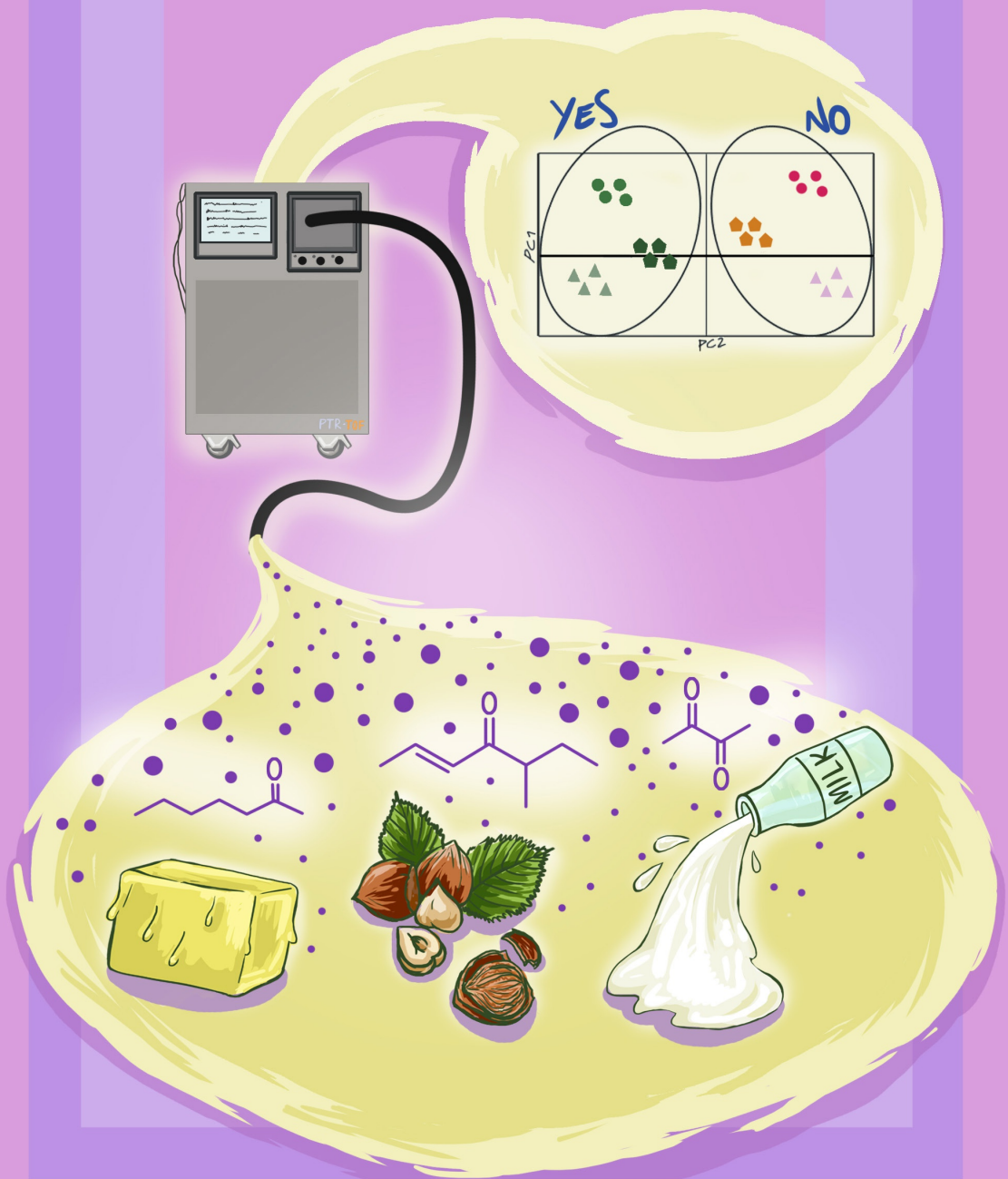
# Show me its volatile profile and I will tell you its quality.

## Rapid fingerprinting of food volatilome at the boundary between food industry and sensory perception.




Michele Pedrotti

Propositions

1) Volatile fingerprinting and *in vivo* monitoring of aroma release help understanding the factors shaping complex concepts as food quality and flavour perception. (this thesis)
2) Predicting the quality of food, established by ambiguous or unprecise sensory evaluation classifications, is like collecting frogs in a bucket. (this thesis)
3) Mapping inter-individual variability is one of the most fascinating challenges for sensory and consumer science and will assist the development of personalized food design.
4) Canned spaghetti is an alarm bell for the limits of food technology that should not be exceeded.
5) The problem is not to feed the planet but the way in which we want to do it.
6) *Techne* and *technik* will eventually fail in stretching planetary boundaries for ensuring the perpetual growth needed by our current global economic system.

Propositions belonging to the thesis, entitled
Show me its volatile profile and I will tell you its quality.
Rapid fingerprinting of food volatilome at the boundary between food industry and sensory perception.
Michele Pedrotti
Wageningen, 1 September 2020

# Show me its volatile profile and I will tell you its quality.

Rapid fingerprinting of food volatilome at the boundary between food industry and sensory perception.

**Michele Pedrotti**

## Thesis committee

### Promotor

Prof. Dr Vincenzo Fogliano
Professor of Food Quality and Design
Wageningen University & Research

### Co-promotor

Dr Franco Biasioli
Food Quality and Nutrition department
Edmund Mach Foundation, San Michele all'Adige, Italy

### Other members

Prof. Dr C. (Kees) de Graaf, Wageningen University & Research
Dr J. L. Le Quéré, Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), Dijon, France
Prof. Dr C.E.I. Cordero, University of Turin, Italy
Prof. Dr M.W.F. Nielen, Wageningen University & Research

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

# Show me its volatile profile and I will tell you its quality.

Rapid fingerprinting of food volatilome at the boundary between food industry and sensory perception.

**Michele Pedrotti**

**Thesis**

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Tuesday 1 September 2020

at 1:30 p.m. in the Aula.

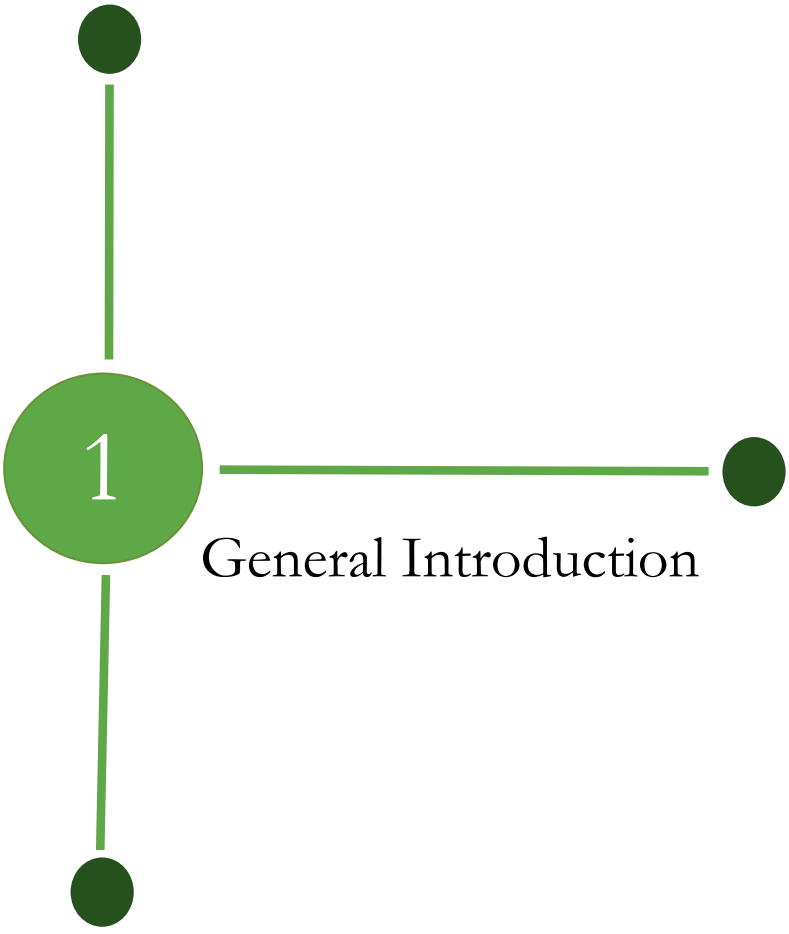
Michele Pedrotti
Show me its volatile profile and I will tell you its quality.
Rapid fingerprinting of food volatilome at the boundary between food industry and sensory perception.
24 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2020)
With references, with summary in English

*ISBN* 978-94-6395-417-4
*DOI* 10.18174/522719

*Que la vida es un carnaval*

# Table of contents:




# General Introduction

## 1.1 Food Quality: a multifaceted issue

Food quality is a main determinant of consumer choices and food intake. Food quality, comprises many different aspects as it represents the sum of all properties and assessable attributes of a food item. In a foodomics[1] approach, food quality includes several factors like physical, compositional and microbial features, modifications induced by technological processes or storage, nutritional value and safety (1). In the sensory science world, quality has been defined as the fitness for use (2). This definition recognizes that quality exists in a context or frame of reference for the consumer (2). In his book, Leitzman (3) tried to give a more complete definition by dividing the food quality (FQ) concept in three categories of value:

- **Suitability** value: including economic value, market value, trade value, utilization value, application value, service value, technological value.
- **Health** value: including nutritional and nutritive value, nutritional quality, safety, food value, biological value (*e.g.* concerning protein quality).
- **Sensory** value: including pleasure/hedonic value, quality linked to the sensory perception.

Moreover, other additional domains of quality like the **psychological** value of food, its **cultural** value and its **political** value are currently gaining significance (3). Due to the current critical global context of climate change and increasing environmental pressure, the **ecological** value of foods which assesses the consequences on the environment due to food production and food processing (3) is also gaining interest and according to the author is should be embedded in the concept of food quality (see dedicated box at page 10). Some of these aspects were taken up and extended in the "Total Food Quality" (TFQ) concept proposed by Giusti and colleagues (2008) which is defined by the factors listed in Table 1.1.

**Table 1.1:** Basic factors of the Total Food Quality concept from Giusti et *al.* (2008)

| Factor | Composed by: |
|---|---|
| Sensory | Colour, appearance, texture, juiciness, taste, astringency and aroma |
| Safety | Presence of toxic compounds normally contained in foods, contaminants, mycotoxins, pathogen, and toxigenic microorganisms. |
| Nutritional value | Calories content and macronutrients composition, as well as non-nutrients with high biological activity, compounds from technological processes, digestibility, and bioavailability |
| Functional properties | Ease of use of several ingredients used for processing and transformation |
| Service and stability | Resistance to rapid deterioration (processing, storage, transportation, and shelf-life conditions) |
| Healthiness | Capacity of some food components to exert beneficial effects on consumers' health (*e.g.* probiotics, vitamins) |
| Psychological | Convenience, price, ease of use, novelty, psycho-active effects of food |

[1] Foodomics is defined as "a discipline that studies the Food and Nutrition domains through the application and integration of advanced -omics technologies to improve consumer's well-being, health, and knowledge" (158). Foodomics requires the combination of different disciplines like food chemistry, biological sciences, and data analysis.

In both the concepts (FQ and TFQ) there are properties strictly related to human interpretation of sensory perception (subjective) and others linked to raw materials, technological processing and gastronomic preparations (objective) (4). The distinction between subjective and objective properties, has been formulated by Van Trijp and Steenkamp (5) as the intrinsic and extrinsic quality determinants of a food product. They state that the intrinsic quality determinants refer to physical characteristics such as taste, texture and shelf life and that the extrinsic factors relate to the way in which the food was produced (6). The latter usually has no direct influence on product characteristics, but they can be of overriding importance in the purchasing policy of some consumers like for example the use of pesticides or the use of genetically modified organisms during the production of raw ingredients (6). This multidimensional nature of quality and the importance of consumer's input for food quality should be considered to ensure the success of a commercial food product (7).

Today, where most of Western consumers have an ever-increasing range of foods, a great effort is concentrated on the goal of improving and maintain quality. If with the innovations brought by Pasteur a lot of focus has been put on the microbiological safety aspect of food, nowadays for monitoring and improving food quality in agroindustry is no longer enough to consider only legal and health related issues. The multidimensional aspects of quality should be taken into account. With the affirmation of sensory since in the 90's, growing attention has been put on the added values related to product intrinsic properties and, in particular to sensory and perceived quality and how it changes across product shelf life. Establishing and maintaining product's inherent sensory quality during the whole product's shelf life is a very critical task and technical product phase since it is the basis for future success of a market or consumer product (7). For these reasons, understanding and assessing the factors contributing to sensory quality across the whole food chain, from raw materials to consumer perception, has become essential in agroindustry to guarantee competitiveness.

This thesis focuses on the aspects of food quality related to the sensory part of aroma and flavour.

## Embedding sustainability into food quality concept: a necessary step

Food systems are currently facing unprecedented challenges in terms of nutrition [1] and environment[2]. The transformation towards sustainable and healthy food systems has been recognized as essential for reaching many of the Sustainable Development Goals (SDGs) of the Agenda 2030[3]. For a food system to be sustainable, it needs to provide adequate nutrition and food security while ensuring that all the conditions for these are not compromised for future generations[4]. All the stakeholders of the food supply chain should take action to facilitate this transition. Food scientists, together with food industries, should do their part too, since food science and technology (FS&T) are fundamental for the conversion of agricultural raw materials into safe and healthy food products of high quality. At the last EFFOST conference (33rd edition: Sustainable Food Systems – Performing by Connecting), the Global Challenges and the Critical Needs of Food Science and Technology report was launched, highlighting the FS&T essential role to adapt and find solutions to global challenges in relation to food and nutrition security. Seven missions were identified by the authors to locate the critical needs for future FS&T and highlighted the need for collaborative multidisciplinary research. For example, according to the report, there will be a greater need to promote cohesion between FS&T and nutrition to ensure not only low cost, convenience and palatability, but also the requirements for nutritional balance in diets. Moreover, food technology needs to be increasingly aware of how consumption is linked to economic, demographical and cultural change, and individual health requirements. This is not enough. Including in a more holistic way the sustainability dimension inside food quality concept is an essential step to facilitate food system transition. As observed, sustainability has been included in food quality as ecological value[5] or as extrinsic value in relation to the consumer[6]. However, the concept of food quality cannot be related only to food and to consumers. We should start to relate it to the whole food system. Time has come also for FS&T and food industries to consider more carefully the "externalities" of our food system[7] when developing a new food product. We should not forget that in current food system, for every US$1 spent of food, US$2 is incurred in economic, societal and environmental costs due to both food production and to consumption consequences[8].

The introduction of sustainability aspects into food quality will give food scientists a new framework to evaluate new products and technologies. This will guarantee a new tool for pushing food industry leaders to undertake a series of actions to better align corporate practices in the food sector with the SDGs like the development of products that contribute to healthy and sustainable dietary patterns, and the improvement of sustainable production practices and of their global supply chains[9].

**References:**

[1] Development Initiatives, 2017. Global Nutrition Report 2017: Nourishing the SDGs. Bristol, UK: Development Initiatives.
[2] Foley et al. (2011). Solutions for a cultivated planet. *Nature,* 478, 337-342.
[3] Valentini et al. (2019) Achieving the Sustainable Development Goals through Sustainabl Food Systems. Springer.
[4] Allievi F. (2017) Meat consumption as a wicked problem : evidence from data and policies meat consumption as a wicked problem. PhD thesis.
[5] Leitzmann C. (1993) Food Quality—Definition and a Holistic View. Safeguarding Food Quality.
[6] Linnemann et al. (2006) Consumer-driven food product development. Trends in Food Science and Technology.
[7] Carolan M. (2018). The real cost of cheap food. Routledge
[8] Ellen MacArthur Foundation (2019). Cities and circular economy for food.
[9] BCFN & SDSN (2019). Fixing the business of food.

## 1.2 Monitoring food quality: integrating sensory and instrumental testing

The reliability of a product sensory experience has been recognized as an important feature of product quality: maintaining the constancy of the sensory experience helps to build consumer confidence for a certain brand or product (8). For this reasons, sensory techniques should be an integral part of industrial programs to monitor food quality and are currently applied for product quality control (QC), for product development and for assessing changes during shelf life (9).

However, there are several challenges and problems that sensory evaluation programs in agroindustry face due to intrinsic sensory methods limitations. Sensory traditional methods (*e.g.* quantitative descriptive analysis) are time consuming and expensive since they require the recruitment, training and tuning of a sensory panel which is affected by environment, physiological and psychological factors during evaluation (7). Even when sensory panels are correctly applied, sensory evaluation is not always compatible with the rapid decision time for online testing required in some steps of manufacturing processes (7). Moreover, considering the elevated number of samples daily received or produced by large food industries that, by dealing with global large supply chains may receive tons of raw material per day, is hard to think about an accurate sensory methodology able to test a representative number of samples, especially for QC applications. Finally, traditional sensory methods for QC have to deal with two sources of variability: (i) the measuring instrument (the panellist) which over long time periods could lead to results instability (10) and (ii) the product to be evaluated (8). Therefore, a flexible and comprehensive system may be desired, one that is applicable to different industrial tasks like testing raw materials, finished products, product reformulation, packaging materials, and shelf life tests (11). In this perspective, new technological approaches in food industry are needed to support sensory methods and to investigate perceived quality.

Instrumental tests have a variety of advantages such as precision, accuracy, reproducibility, the possibility to perform continued operation without a restriction on number of samples tested and their convenience in terms of time and economical resources (7). In addition, instrumental measurements comprise measurement of discrete, well-defined physicochemical properties, whereas for sensory evaluation different stimuli interact at both physiological and psychological levels (10). The ideal situation would be to establish correlations between sensory response and instrumental measures or to predict sensory attributes based on instrumental measures (12). In this way, instrumental measures can support sensory testing, especially when a rapid turnaround is needed or in the case of products which are fatiguing to the senses, repetitive or involve health risk in repeated evaluations (8). One of the most intriguing aspects of sensory evaluation and instrumental analysis combination in agroindustry processes and in food science in general, is represented by the investigation of volatile organic compounds (VOCs) since these are at the origin of both aroma and flavour of food products.

## 1.3 Flavour and aroma perception

Sensory properties are fundamental in the relation between humans and food: personal dietary choices, conviviality and emotions are strictly related to what we perceive (13). In particular, flavour plays a key role in determining consumer acceptance and preferences of food products by determining their main organoleptic characteristics (14). Flavour perception is multidimensional and multimodal. It can be described, according to the sensory dictionary ISO 5492:2008 as a "complex combination of the olfactory, gustatory and trigeminal sensations perceived during tasting".

**Tastes** are involatile chemical stimuli that are detected by receptors on the tongue and other oral surfaces. They are divided in the five basic tastes: salt, sweet, sour, bitter and umami (10) at which, more recently have been added the taste of fat (15).

The **trigeminal response** or chemesthesis is the sensation which result from the stimulation of receptors usually associated with thermal perception (thermoreceptors), pain (nociceptors) and touch (mechanoreceptors) which are primarily located in the oral, nasal and ocular mucosae (16). Among the other human senses, the olfactory system, has been neglected for many years but recently, thanks to the understanding of odour mechanisms which led to a Nobel Prize in 2004 (17) and to the recognition of its importance for flavour perception, it earned the right scientific attention (18). VOCs are responsible for the **aroma** and smells perceived by the sense of olfaction. When VOCs are released from food they can reach the olfactory epithelium by two different pathways: directly via the nostrils during direct sniffing (orthonasal olfaction) and via the mouth during consumption (retronasal olfaction) (19), when VOCs are released from the food matrix into saliva and transferred to the air phase in the oral cavity. As shown in Figure 1.1, VOCs are then transported in the nasal cavity via the respiratory flow, especially after swallowing, reaching the olfactory receptors on the olfactory bulb leading to the in-mouth aroma perception (20).

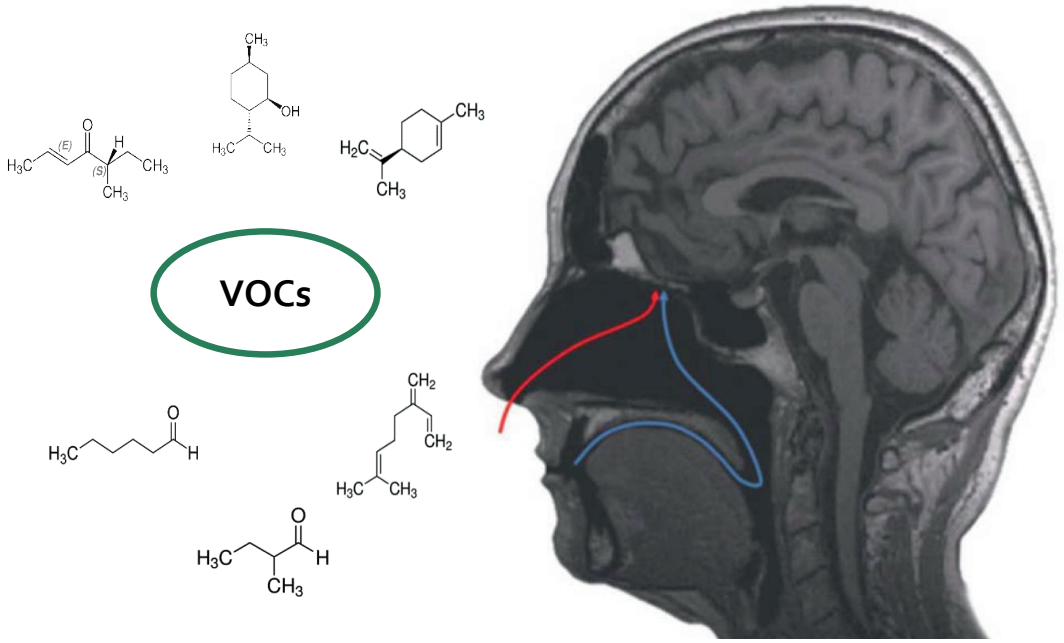



***Figure 1.1:*** *The two different olfaction pathways for volatile organic compounds to reach the olfactory epithelium: The orthonasal route (red arrow) is used during sniffing while the retronasal route (blue arrow) allows for food flavours to be perceived (Source: adapted from Hummel & Seo, 2016). In the figure, examples of key VOCs of the food matrixes analyzed in this thesis are presented.*

## 1.4 VOCs, a promising tool for quality control in food industry

VOCs play a fundamental role in foods science and technology and their analysis seems a promising tool for monitoring industrial processes and support sensory QC in agroindustry. VOCs are constantly released by food products and they are key drivers of food perceived quality both before (odour), during (flavour and aroma) and after (aftertaste, after-flavour) food consumption. Moreover, they are produced and released in most stages of the food-production chain "from farm to fork" making them a crucial subject when dealing with traceability along the supply chain. VOCs also have a huge impact on the consumer sensory experience and they can be detected in a non-invasive way (13,21). Finally, measuring VOCs released during consumption in the nose is the most direct way to investigate the mechanisms underlying flavour perception (22).

However, identifying aroma compounds in a food matrix is one of the most formidable tasks faced by an analytical chemist (16). First of all, more than 12 000 flavour compounds have been reported in various food products (23) but it is estimated that only 5%–10% of them play a significant role in the formation of specific aromas of food products (24). Secondly, most of the analytical methods available are usually not as sensitive

as the human nose. Thirdly, VOCs have very different concentrations in food even at extremely low concentrations (mg/kg or even ng/kg) distributed throughout the food matrix. VOCs usually have a high vapour pressure and they go into gas phase by vaporizing at 0.01 kPa at room temperature with boiling points between the ranges from 50°C-100°C to 240°C -260°C (25). The majority of VOCs are also relatively nonpolar (hydrophobic), relatively small molecules (molecular mass <400 Da) and consist of highly diverse chemical classes especially aldehydes, alcohols, ketones, esters, organic acids, sulphur-containing compounds and heterocyclic compounds like furan-derivatives and pyrazines (26).

The challenge of connecting VOCs composition to perception is also hampered by food matrix complexity (27). The release of aroma compounds from foods is determined by the partition coefficient between air phase, saliva and food matrix. The nature of these interactions depends on the physicochemical properties of flavour compounds but as well on the food components (i.e. proteins, lipids, carbohydrates) and on food structure (28,29). For instance, fat composition, concentrations, emulsion characteristics, texture properties and temperature, can significantly modify interactions between lipids and the small aroma molecules (30,31). Moreover, these could affect consumers oral processing behaviour during consumption which is known to affect flavour release and perception (32,33). Finally, as mentioned, flavour is a multimodal perception and the effect of different sensory dimensions contributes to complicate its understanding and implicates concomitance of different instrumental techniques to cover all the aspects. Therefore, measuring VOCs presence and concentrations in a food product may tell just one piece of the puzzle. Another significant portion of the puzzle, able to help unravelling the complex interaction mechanisms between aroma release and flavour perception during food consumption, may come from the online analysis of VOCs released through the retronasal pathway.

## 1.5 Analytical methods for VOC analysis

Several analytical methods, each one with its strengths and weaknesses, have been developed for the detection of aroma compounds (34,35) and to untangle the extreme complexity and large variation of VOC concentrations in food samples (36). Especially mass spectrometry (MS) developments and their applications in metabolomics had a significant impact for VOC analysis (37) and were used for tackling various food safety and quality issues.

In metabolomics, the major distinction is made between target or untargeted approaches (38) based on the methodology applied and on the aim of the analysis. In untargeted analysis, the aim is to detect as many components as possible in an unbiased manner usually by metabolic fingerprinting or profiling approaches (39). On the other hand, targeted analyses rely on a priori knowledge of the class of metabolites that are expected to contribute to the (sensory) properties of interest (40). For detection of biomarkers often, a combination of untargeted analysis followed by one or several targeted analyses to capture all the information is needed (60). Analysis of food VOCs are performed due to several purposes like obtaining a

complete aroma profile (volatile fingerprint) of a sample, identifying key aroma components responsible for a characteristic food aroma, monitoring the volatiles release or production over time or during processing, detecting off-notes in a food product for quality evaluation or for food spoilage detection, finding links between sensory attributes and perceived aroma and checking the authenticity of aroma compounds by detecting if they come from synthetic or natural sources (34,41,42). Besides that, in the last years, different strategies have been developed to monitor aroma release *in vivo* during food consumption with the aim of better correlating aroma profiles with perceived flavour and investigating the effect of eating and mastication on aroma release (43). Usage proliferation of both targeted and untargeted approaches to investigate volatiles has resulted in the creation of the term 'volatome' or 'volatilome', which is defined as the comprehensive analysis of volatile compounds in any type of sample (44,45).

Traditionally, mass spectrometry methods are applied after VOCs have been separated, usually through gas chromatography. However, new methods that bypass the separation stage by directly applying mass spectrometry have been developed to increase methods time resolution and sensitivity.

### 1.5.1 | Gas Chromatography - Mass Spectrometry

Among instrumental approaches gas chromatography-mass spectrometry (GC-MS) is the reference method for the analysis of food VOCs (46,47). GC-MS systems have high precision, excellent separation capability including distinction of both isobaric and isomeric compounds, excellent selectivity and sensitivity with a reachable detection limits as low as 0.1 pptv (47). However, the method suffers from a relatively low time resolution: the chromatographic separation on the capillary column usually ranges from minutes to tens of minutes. Sample preparation steps to isolate and concentrate aroma constituents are the most time-consuming, tedious and error-prone steps of the total analytical procedure (48) which can highly affect the final results (49). Once VOCs have been isolated and concentrated, they are injected into a column and after the chromatographic separation, the molecules elute and reach a MS detector or a flame ionization detector (FID).

In the last years, different developments were applied to improve different aspects of the standard GC technique such as shortening of analysis time by fastGC and improving the resolving power and increasing peak capacity by multidimensional GC (MDGC) such as comprehensive two-dimensional gas chromatography GCxGC. GCxGC-MS combined with a time-of flight mass spectrometer (TOF-MS) (35) found its application in food, fragrances, biological, environmental, and other scientific fields (50,51).

Another development of the standard GC technique worthy to mention are the gas chromatography-olfactometry-mass spectrometry (GC-O-MS) methods which are a combination of GC-O and GC-MS. GC-O combines an olfactometer – a device in which human nose is applied to detect the odour intensity of analytes (52) – with a GC where the analyte is separate into two equal parts: one part is sent to a detector and another one is sniffed by a trained person or by a panel for recognition of odour and its intensity. From its early invention in 1964 (53), it underwent many developments including the coupling with a mass spectrometer.

The GC-O-MS has many application in the food industry such as identification of key aroma-active compounds, cluster analysis based on the aroma-active compounds, quick mapping of VOCs, relationship between odorants and sensory properties, and clarification of formation mechanism of important odorants (54).

### 1.5.2 | Direct injection methods

GC-MS techniques, despite being the benchmark analytical methods for VOCs identification and quantification, are not designed to examine temporal changes of VOCs in fast processes and, even when using high-speed GC (55,56), the time resolution of GC-based methods is at best in the minutes range. Moreover, often, a sampling and pre-treatment phase introduces time averages of the concentration of the measured mixture (46). Because of these drawbacks, direct injection mass spectrometry (DIMS) methods have been developed which introduces different advantages than GC-MS due to the possibility to perform rapid, non-invasive, direct analysis without any or little need in terms of sample preparation and pre-concentration. In the absence of sample preparation and separation steps, the level of the identification depends on the selectivity of ion chemistry (57). The very high sensitivity achieved by the state-of-the-art DIMS technologies, united with the ability to monitor large dynamic range of compounds and their high time-resolution (even sub-seconds), makes these techniques suitable for a large number of applications in different fields, from atmospheric and environmental chemistry to medical sciences, food and flavour science and industrial process monitoring (58).

DIMS techniques differ in sample preparation, sampling, inlet, ionization and detection (58). The most used DIMS methods for monitoring VOCs in food samples are atmospheric-pressure chemical ionization (APCI), selected ion-flow-tube mass spectrometry (SIFT-MS), proton transfer reaction mass spectrometry (PTR-MS), electronic noses (e-nose) and electronic noses based on mass spectrometry (MS-e-noses).

APCI was one of the first method used to monitor VOCs by DIMS and is commonly used as an ionization source for different MS techniques. The gentle ionization relies on gas phase ion–molecule reactions that take place at or near atmospheric pressure allowing for the formation of intact ions like the protonated molecules. Generally, multiple ion species are generated from air by using a corona discharge that react with the analytes and as well with the water vapor present in the sample to produce [$MH^+$] ions from the neutral molecules [M] (59). APCI presents some issues like the rather complex ionization due to the presence of many possible ionization agents that hampers the unequivocal identification of compounds solely on the basis of their mass to charge ratio (m/z) values, the suppression of ionization in the source leading to non-quantitative results and the relatively low ionization efficiency (58). Successful applications of APCI-MS for monitoring flavour release of food, for *in vivo* studies (22) and in non-targeted foodomics (60) are available in literature.

SIFT-MS and PTR-MS are similar techniques that are comprised of the same three components: an ion generation zone, a reaction zone and a detection zone. They both rely on the principle of chemical ionization

(CI) of the analyte during a defined reaction time: using a known kinetic rate coefficient, *k* and the number of detected product ions to determine the initial VOCs concentration. The reaction that is considered in this case is a ionization reaction of the analyte A with the reagent ion $R^+$ and is mostly a proton transfer from protonated water (58):

$$A + R^+ \xrightarrow{k} A^+ + R \qquad \text{Eq. 1}$$

$$\frac{d[R]}{dt} = k \cdot [A] \cdot [R^+] \qquad \text{Eq. 2}$$

There are two main differences between the two instruments: they differ in the way reagent ions are generated and the portion where the analyte reacts with the ions, namely the drift tube for PTR-MS and the flow-tube for SIFT-MS (61). For the ion source a hollow cathode discharge on water vapor generates $H_3O^+$ ions in PTR-MS (62) while a microwave or electron-impact ion source is utilized on wet air plasma in SIFT-MS (63), generating different ionization agents (i.e. $H_3O^+$, $O_2^+$ and $NO^+$ for positive mode and $O^-$, $O_2^-$, $OH^-$ and $NO_2^-$ for negative mode) which can then be pre-selected through a quadrupole mass filter. Since the three reagent ions react differently with the analyte and may form different association and fragmentation products, more structural information can be obtained but the efficiency of creating the reagent ions is lower than for PTR-MS, leading generally to higher limits of detection (LOD) for SIFT-MS (61). Moreover, due to the usage of a quadrupole as mass analyser, the SIFT-MS is usually less rapid than PTR-MS which has been coupled to a time of flight (ToF) detector (64) and with an additional quadrupole ion guide (65). Finally, no electric filed is employed in SIFT-MS which makes possible to carry out ion-molecule reactions under thermal and controlled conditions (63) to have more precise determination of the ion-molecule reaction rate coefficients for quantitative analysis. More detailed comparison of the two techniques can be found in different reviews (66,67).

E-noses consist of an array of electronic chemical sensors with an integrated pattern recognition system (68) which are designed to mimic the human olfactory system. The output of unspecific sensors can be used to build models to classify products or processes, by means of chemometrics or data mining (69). In MS-e-noses, VOCs are introduced into the ionization chamber of a MS instrument (usually a quadrupole mass spectrometer) without prior chromatographic separation and each fragment ion (m/z ratio) of the mass spectrum obtained acts as a "sensor" and its abundance is equivalent to the sensor signal (70). The output of MS-e-nose is a fingerprint of the sample, meaning that the technique cannot identify specific VOCs. With the existing pattern recognition system it is possible to recognize simple or complex odours (59) but the degree of fragmentation has to be taken into account for data interpretation. The application of MS-e-noses for monitoring food quality has been explored in many different food matrixes (70,71).

Other DIMS techniques used for food analysis are the ion-mobility spectrometry-mass spectrometry (IMS-MS) and the many ambient mass spectrometry (AMS) methods that have been recently implemented

(72,73) like the direct analysis in real time mass spectrometry (DART-MS) (74). These methods will not be further discussed in this thesis, which will explore application of PTR-MS for the rapid, direct and high throughput analysis of volatile compounds in the headspace of different food products and for monitoring *in vivo* flavour release. The following sections will present a detailed description about the utilized methodology and its applications for both food quality and *in vivo* flavour release by nose-space analysis.

## 1.6 Proton Transfer Reaction Mass Spectrometry

PTR-MS was developed in 1995 by W. Lindinger and his team at the University of Innsbruck for the analysis of volatile organic traces in ambient air (62,75,76). PTR-MS became successful due to its soft ionization which allows to reduce fragmentation and to observe the molecular ion of a compound of interest. The first PTR-MS instrument was commercialized in 1998 by Ionicon Analytik GmbH which is in Innsbruck, Austria. Other PTR-MS producing companies are KORE Technology Ltd. located in Ely, UK and TOFWERK AG which recently started to produce PTR-MS like instrument (Vocus) in Thun, Switzerland.
As the other DIMS methods, PTR-MS allows the analysis of volatiles without any pre-treatment in real-time and with high sensitivity. The technique is based on ionization of VOCs by means of protonated water, hydronium ions ($H_3O^+$). The proton transfer from the hydronium ions is exothermic when the proton affinities of the targeted VOCs are higher than the one of water. This is very advantageous since common constituents of air, such as $N_2$, $O_2$, $CO_2$ and inorganic species have low proton affinities and do not undergo proton transfer reaction. On the other hand, most of VOCs responsible for aroma in food have higher proton affinities than that of protonated water molecules and undergo very selective non-dissociative proton transfer reaction that proceed at a collision-limited rate (77).
Usually PTR-MS consists of mainly three parts: an ion source, a reaction region and a mass analyser as it is shown in Figure 1.2. Briefly, VOCs under investigation are injected in the drift tube by a continuous flow of air where are ionized by proton transfer from $H_3O^+$ ions produced by the ion source. VOCs are then separated and detected by a suitable mass analyser.

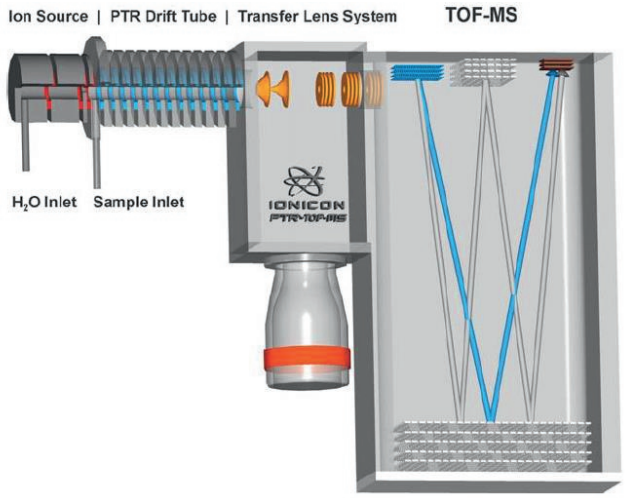



***Figure 1.2**: Schematic drawing of the Ionicon PTR-ToF-MS instrument from Jordan et al. (2009)*

### 1.6.1 | Ion source: $H_3O^+$ and the Selective Reagent Ionization system

In most PTR-MS apparatus, the first part is constituted by a hollow-cathode ion source that usually produces a $H_3O^+$ primary-ion beam by discharging on water vapor which is injected into the ion source region (75). The discharge is very fast and most of the ions produced react in cascades of reactions which brings to the formation of high purity $H_3O^+$ primary ions (> 99.5%) that can then be injected directly into the reaction chamber without prior mass selection (62). This simplifies system realization and allows for better sensitivity.

Other primary parent ions can also be used. Some years ago, the selective reagent ionization (SRI) system was released, which allows for rapid switching of the primary parent ions like $NO^+$, $O_2^+$ $Kr^+$ and $Xe^+$ (64). In this case, the reagent ions are produced by using $O_2$, charcoal filtered air (or, alternatively, pure $N_2$ and $O_2$ flows) and Kr and $Xe^+$ as reagent gases in the ion source, respectively. This system allows to measure compounds with a proton affinity higher than water (i.e. some alkanes) and in general leads to a better compound identification by separating isobaric compounds such as isobaric aldehyde and ketone pairs (58,77,78).

### 1.6.2 | Drift tube and the proton transfer reactions

The second part of a PTR-MS apparatus is the drift region, where parent ions and analytes are mixed and proton transfer reactions can occur via collision. The charged compounds are driven by an electric field and are carried by a buffer gas, such as air. Proton transfer reactions are controlled by the reduced electric filed, *E/N* value, where E is the electric field across the drift tube and N is the gas number density (number of gas particles per unit volume). This value should be chosen carefully to balance between giving sufficient energy to the collisions between ions and neutral molecules to break up any ion clusters that might be

formed and avoiding excessive fragmentation of analyte molecular ions (77) that happens at high *E/N* rations due to the more energetic collision. This, is normally achieved by employing an *E/N* between 100 and 140 Td (Townsend, 1 Td =$10^{-17}$ V cm$^2$) since excessive production of $H_3O^+$ $(H_2O)n$ clusters may affect the quantification: these clusters have different interactions with certain VOCs when compared with $H_3O^+$ interaction chemistry (79). As show in equation 3, drift pressure, temperature and length (usually around 10 cm) are fundamental parameters that should be controlled to provide a fixed reaction time for the ions as they pass along the tube: this time can be measured, or it can be calculated from ion transport properties.

$$E/N = \frac{U_{drift}}{L_{drift}} \times \frac{R \times T_{drift}}{N_A \times P_{drift}} \qquad \text{Eq. 3}$$

Where R is the gas constant and $N_A$ the Avogadro constant. A controlled high temperature was shown to improve *response time* of the instrument by also reducing the so-called *memory effect* where molecules are adsorbed and slowly released by the surfaces of the gas inlet line and of the drift tube (77,80).

Taking the drift tube as a chemical reactor, VOCs perform many non-reactive collisions with buffer gas atoms and molecules, but only when they collide with the reactants, the proton transfer reaction can occur (if energetically allowed) through a dissociative (fragmentation) or non-dissociative way. In most of the cases, VOCs undergo a fast (collisional rate) non-dissociative reaction with the $H_3O^+$ donating the proton as described in Eq. 1. However, some molecules like all $C_3$ and higher alcohols, some aldehydes and esters possess a tendency to undergo a dehydration or protonation reaction (77). If the analyte molecules are present in trace amounts, we can assume that the proton donor concentration is largely unchanged by the addition of the analyte sample ($[VOCH^+] << [H_3O^+]$). Therefore, it is possible to calculate the concentration of reagents from the ions signal ratio according to the principles of chemical kinetics (62):

$$C\ (ppbv) = \frac{1}{kt} \cdot \frac{[VOCH^+]}{[H_3O^+]} \cdot \frac{1}{N} \cdot 10^9 \qquad \text{Eq. 4}$$

where *C* is the concentration reported in ppbv, *k* is the reaction rate constant for a given molecule ($s^{-1}cm^3$), *t* is the ion travel time in the drift tube (~100 µs) which can be calculated from the drift tube parameters, $[VOCH^+]$ and $[H_3O^+]$ indicate the measured counts per second (cps) and N is the gas density in the drift tube (molecules/cm$^3$). To obtain the precise VOC quantification without any additional calibration, the reactions should occur under well-defined and controlled conditions since the equation parameters, together with the previously drift tube parameters mentioned (i.e. *E/N*, temperature, pressure) play an important role in chemical reactions. Usually, in food applications, an experimental *k* value is not available for every compound and a constant reaction rate coefficient ($kR$=2x$10^{-9}$ cm$^3$/s) is used for calculations. This can introduce a systematic error for the absolute concentration of each compound which is below 30% in most of the cases and can be accounted for when the actual reaction rate constant is not available (81).

When other primary ions then $H_3O^+$ are used as ionization agents, different chemical ionization processes take place. In this thesis we will consider the case of $O_2^+$ and $NO^+$. When $O_2^+$ is used the reactions occur as given in Eq. 5-6 via charge transfer:

*Non-dissociative:* $$O_2^+ + A \rightarrow A^+ + O_2$$ Eq. 5

*Dissociative:* $$O_2^+ + A \rightarrow B^+ + F + O_2$$ Eq. 6



Where B and F represent two different fragments of the initial molecule A. Due to the amount of excess energy on charge transfer, there is a strong tendency for dissociative reactions and therefore, $O_2^+$ has a limited application in food quality control such as monitoring of hydrocarbons contaminants. The case of $NO^+$ as primary ion for food application, may be more interesting due to the possibility to separate isomers like aldehydes and ketones. VOCs may undergo different reactions with $NO^+$ such as charge transfer producing $M^+$ ions, hydride ($H^-$) or hydroxide ($OH^-$) ion transfer producing $(M - H)^+$ and $(M - OH)^+$ ions and ion-molecule association as shown by many studies conducted by using SIFT-MS methods (82,83). Aldehydes usually react by hydride abstraction, forming mass (*m*-1) ions while ketones cluster with $NO^+$ forming mass (*m* +30) ions as shown in Eq. 7-8:

*Aldehyde reaction:* $$NO^+ + A \rightarrow [A - X]^+ + XNO$$ Eq. 7

*Ketone reaction:* $$NO^+ + A \rightarrow ANO^+$$ Eq. 8

Where, X represents the abstracted ion (H- or OH-). Once VOCs react with the primary ion and they acquire a charge, their generated fragments are transported to the last portion of PTR-MS instrument, the mass analyser.

### 1.6.3 | The mass analyser and the injection region

The third part of a PTR-MS instrument is an ion detection system or a mass analyser as show in Figure 1.2. Its main aim is to separate ions according to their *m/z* which is the mass number of an ion m, divided by its charge number, z which in PTR-MS is always equal to one. Different type of mass analysers have been used in combination with PTR-MS, each one with its own mass resolution, sensitivity, response time, transmission and dynamic range. The traditional mass analyser was a quadrupole but the ones used for the studies in this thesis are the time-of-flight (ToF) and the ToF in combination with a quadrupole interface (Qi).

The essential principle of a ToF mass spectrometer is that ions moving in the same direction and having a distribution of masses but a (more-or-less) constant kinetic energy, will have a corresponding distribution of velocities in which velocity is inversely proportional to the square root of m/z (84,85). Ions are extracted from the drift tube in pulses and they are separated according to their flight times under the influence of an electric field. Ions of different mass will arrive at the detector sequentially and in principle it is possible to

detect all of them. Compared to the quadrupole version, the ToF analyser eliminated the necessity to select a subset of ions to be monitored by allowing to obtain a whole mass spectrum of complex trace gas mixtures in a short time (a second or even less) and, thanks to its improved mass resolution ($10^3$ – $10^5$), allows the separation of most nominally isobaric ions (64). As well, the PTR-ToF-MS instrument has a very low detection limit (1 ppbv for 1 min integration time) and high sensitivity.

Another component that should be considered is the lens system that connects the drift tube to the mass analyser. One factor that critically limits the detection sensitivity, derives from the fact that most ions traversing the drift tube do not pass through the small exit aperture at the end of the drift tube and so, a large quantity of ion signal is wasted. This loss of ions was partially mitigated by the introduction of a radio frequency ion funnel (RF) at the end of the drift tube portion, which, by focusing radially the ion beam was shown to increase detection sensitivity for VOCs between 1 and 2 orders of magnitudes (86,87). More recently, the introduction of a "Quadrupole interface" (Qi), in combination with a ToF analyser was presented by Ionicon (65). This new interface in the injection region, in combination with a higher drift tube pressure in the instrument, greatly improved the ToF mass resolution by approximately 30% through a more effective transfer of ions from the drift tube to the ToF analyser.

### 1.6.4 | Data managing

PTR-ToF-MS data are very complex datasets. The high mass resolution provided by the technique, united with the spectra fast acquisition rate (approx. 1 spectrum per second), results in production of many complex mass spectrum that preserve analytical information of hundreds of mass peaks varying in intensities (88). To manage all these information, for improving mass accuracy and for quantification of mass peaks concentrations advanced methodologies are required (89,90). A three-step approach was described by Cappellin *et al.* (2011) and summarized in Figure 1.3 to properly extract and analyse PTR-MS data: spectra analysis, multivariate analysis and data mining, and analytical information.

For the spectra analysis pillar, different specific software have been developed. In this thesis, the TOFOffice software (Foundation Edmund Mach, San Michele all' Adige, Italy) was employed to elaborate the data obtained from both PTR-ToF-MS and PTR-QiToF-MS for the steps from external calibration till peak extraction. While internal mass-scale calibration during the measurement is performed to facilitate online monitoring, external mass-scale calibration is made for spectra alignment. Both are based on the usage of compounds which are constantly present during the measurement such as primary ions, other peaks belonging to impurities of the machine ($H_3O^+$, $O_2^+$, $NO^+$, acetone) and as well 1,3-Diiodobenzene ($C_6H_4I_2$) and its fragments (typically as *m/z* 330.848 and *m/z* 203.946 for its fragment in $H_3O^+$ mode and as *m/z* 329.840 in both $O_2^+$ and $NO^+$). This compound is continually injected into the drift tube through the PerMaSCAl device (Ionicon Analytik GmbH, Innsbruck, Austria). After this step, dead time correction, noise reduction and baseline removal are applied by the software to improve data quality.

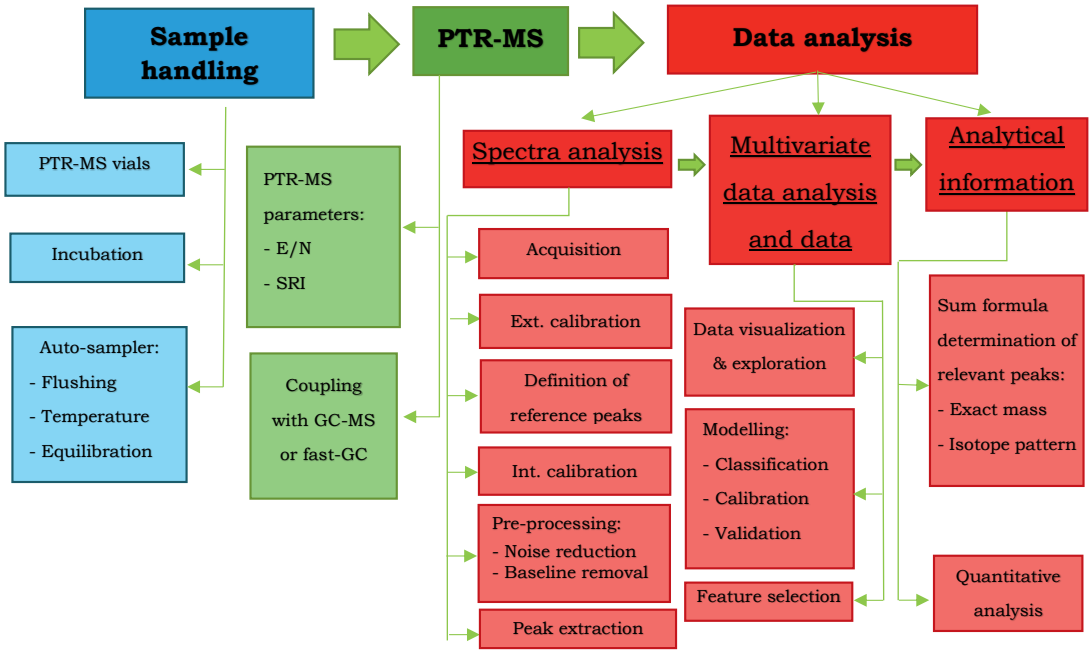



***Figure 1.3**: Example of experimental flow of a PTR-MS experiment adapted from Cappellin et al. (2011)*

Peaks are then identified in a semi-automated way by considering peak shapes and width and by employing Gaussian functions to fit the mass spectrometric peaks (90) and to extract data in counts per second (cps). For quantitative analyses, by using Equation 3 the so-called volume mixing ratio (VMR), expressed in parts per billion by volume, can be determined. VMR is the same inside of the drift tube as it is outside and is temperature independent. However, it is common to refer to VMR as a concentration and so in this work we will refer to the VMR as concentration (77).

Once data are extracted they can be analysed through data mining and a variety of uni or multivariate data analysis methods based on the experiment purpose. Different examples have been reported for supporting the utility and effectiveness of coupling PTR-ToF-MS with multivariate and data mining methods in fruit metabolomics (91) and for a reliable and fast characterization of agroindustry products (92).

## 1.7 PTR-MS applications for monitoring and understanding factors shaping food quality

PTR-MS technology was described 'as an accurate, highly sensitive, DIMS technique that allow for the rapid characterization of food products and for the monitoring of processes in food science and technology and agroindustry, without any pre-treatment' (21) that briefly summarize the technique benefits

for investigating VOCs in food and drinks. However, the difficulties to exactly associate a *m/z* signal to a molecule should be kept in mind. Despite the potential of using fragmentation ion patterns (93,94) and $NO^+$ as reagent ion to discriminate some isobaric and isomeric compounds, the real advantage of PTR-MS in food science is not VOCs identification but its real-time and non-invasive monitoring and fingerprinting potentialities. Two main distinctions should be made when referring to its application: headspace (HS) analysis and on-line analysis summarized in Figure 1.4.

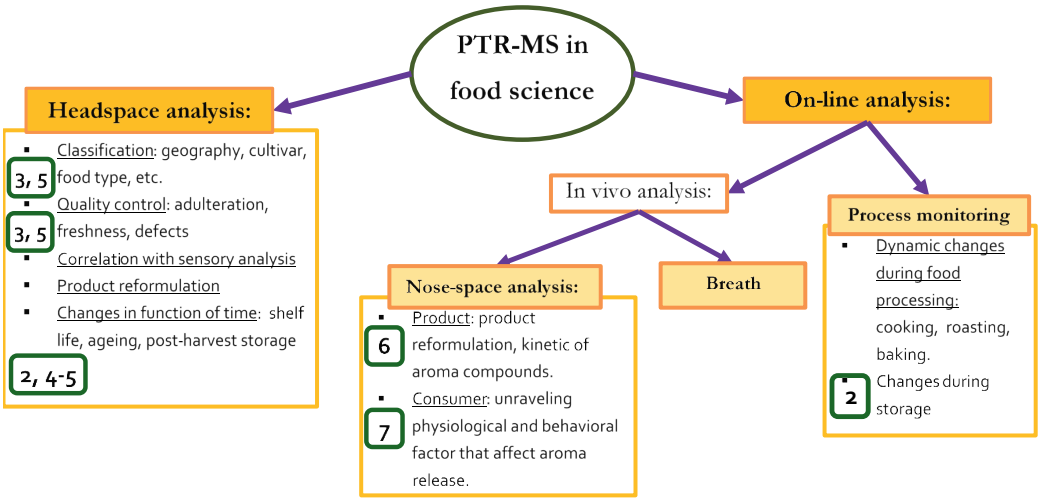



***Figure 1.4**: Applications of PTR-MS in food science. The numbers indicate the thesis chapter that explore the relative application.*

### 1.7.1 | Headspace analysis

HS analysis are usually associated to the fingerprint concept of MS e-noses where an informative full scan of the whole mass spectra is generated (79) and, with the appropriate statistical analysis, is possible to classify food samples based on quality standards, origin, genetics or others (21). HS PTR-MS fingerprints were applied to monitor food VOCs evolution as a function of time (*e.g.* shelf life, ageing, post-harvest storage, ripening and fermentation), as a function of ingredients reformulations (*e.g.* change of ingredient, change of concentrations), for classification challenges (*e.g.* geographical origin, cultivar, food type) and for quality control (*e.g.* freshness, adulteration, quality classification). Some of the most recent PTR-MS applications are in saffron quality control (95), for evaluating shelf life of poultry meat (96), for evaluating botanical and geographical origins of both cocoa (97) and coffee beans (98,99), for assessing VOCs produced by three commercial bakery starter cultures (100) and to quantify phenolic compounds in a selection of Islay whiskies in comparison to non-peaty whiskies (101).

Different works also tried to correlate PTR-MS fingerprints with other type of data, in particular with sensory data. As aforementioned, the possibility to correlate instrumental and sensory analysis has huge potentials in agroindustry quality control programs. The first pioneer work compared the classifications of seven varieties of Italian mozzarella cheese made with classical sensory analysis with VOC headspace analysis by a quadrupole PTR-MS (102). The two methods achieved comparable sample description (102). The same group investigated changes in red orange juice after different types of stabilizing treatments and, in this case, PTR-MS had a greater accuracy in discriminating samples than the sensory panel (103). More recent works assessed PTR-ToF-MS potentiality to predict aroma-based sensory categories of 206 dark chocolates classified into four sensory poles by a quantitative descriptive analysis from a trained panel (104), to investigate aroma and sensory profiles of 43 commercial cultivars of peaches (*Prunus persica* L. Batsch.) of two consecutive harvest years (105), to compare VOCs and sensory analysis of three pears cultivars by also identifying cultivar specific volatile markers with a influence on consumers liking levels (106) and to investigate the relationship between sensory characteristics and PTR-ToF-MS spectra of 24 virgin olive oils with different cultivar, geographical location and harvesting time (107).

### 1.7.2 | On-line analysis

This is the application that takes most advantage of the high time resolution and sensitivity of the technique, which allows to follow in real time VOCs release. As highlighted by Figure 1.4, when it comes to on-line analysis a distinction should be made between process monitoring and *in vivo* analysis (21), that can be further divided in breath and nose-space analysis.

#### 1.7.2.1 Process monitoring

PTR-MS was successfully applied to monitor different processes related to food quality and interesting to food industry. The first works in this direction followed VOCs released during coffee preparation from beans roasting to the cup of coffee (108,109) and PTR-ToF-MS is still applied in coffee aroma researches (110). Changes in metabolic and catabolic pathways during storage were also monitored through PTR-MS in meat to detect spoilage (111,112), in fruits for following ripening and spoilage (113,114) and in dairy products for assessing VOCs production upon exposure to light (115). PTR-MS was also exploited to follow the formation of different compounds during food processing as Maillard reactions (116), formation of acrylamide (117) and furan (118) and to monitoring lactic acid fermentation of milk (119).

#### 1.7.2.2 *In vivo* aroma release: combining instrumental and sensory measurements

Aroma perception is a dynamic process where the intensity and profile of VOCs evolve with time. Measuring aroma release in the nose, combined with other approaches, is the most direct way to investigate the mechanisms underlying flavour perception (43). However, monitoring aroma release *in vivo* presents

many issues, brilliantly summarized by Taylor & Linforth (2010b). They argue that a suitable technique needs to be able to:

- have an extremely rapid sampling time of about 20-100 milliseconds per data point to follow human breath;
- analyse a wide variety of compounds at low concentrations by at the same time managing interfering factors introduced by human breath as air gases and water vapor;
- have a wide high sensitivity range (across 6 orders of magnitude).

PTR-ToF-MS possess these characteristics with a sensitivity that is often in the range of the one of human nose. This makes the technique suitable for *in vivo*, breath-by-breath monitoring of flavour compounds during food consumption. *In vivo* aroma release is a complex process, influenced by many different factors like food and aroma compound properties, chemical interactions that occur within food, between aroma compounds, between food and saliva and the transmission through the retronasal pathway. As well, the eating process (*e.g.* mastication, swallowing) and individual characteristics (*e.g.* biological and psychological) of the human subject can greatly affect aroma release and perception. The PTR-MS technique can be used to collect information on individuals and on the factors affecting flavour release and food metabolism (21), all key elements that can help agroindustry to formulate successful food products.

The first reported cases of PTR-MS application for *in vivo* analysis were *breath analysis* by the group of Lindinger. Methanol and ethanol in human breath were followed after consumption of alcoholic drinks or fruits (120,121) while few years later, acetone and volatile sulphur compounds in human breath were monitored for 32 hours after the ingestion of raw garlic (122). These first pioneering works opened the doors at PTR-MS breath analysis for both medical researches and characterization of human and food metabolism. PTR-MS has been employed to investigate exhaled concentrations of isoprene, methanol, volatile anaesthetics and potential breath biomarkers for a range of diseases including respiratory inflammatory conditions and tumours (123–125). Moreover, the technique was used to assess demographic and physiological effects on endogenous breath compounds like the effect of age, gender and BMI on exhaled acetone and isoprene (126,127). The proliferation of international researches in breath analysis has resulted in a range of different breath sampling and analytical techniques which made comparison and assimilation of research difficult, and likely contributes to the current lack of replication of research findings (128). For this reason an agenda for methods standardization in breath research has been recently set up (129).

The other approach to *in vivo* analysis, is the so-called *nose-space analysis* which consists of sampling exhaled breath through the nose via a non-invasive nosepiece of different materials from glass to Teflon which allows panellists to consume food and beverages (130). The impact of the aforementioned factors on aroma release and perception have been investigated by applying PTR-MS nose-space analysis as

highlighted in Figure 1.4. A main distinction is made when the focus is on characterizing the product or on the human subjects.

For **product characterization** a wide variety of food systems have been analysed by nose-space PTR-MS. These experiments greatly contributed to our knowledge on how food properties, like food texture, structure, viscosity and fat content affect aroma release (131–133). Moreover, Heenan *et al* (2012) described the impact of sugar concentration on aroma release of different VOCs of flavoured cereal bars, while Ting *et al.* (2012) investigated the relation between *in vivo* aroma release and textural and physicochemical parameters of apples. For a profound understanding of food flavour, the dynamic process of aroma release must be matched by the dynamic process of flavour perception (136). For this reason, in the last years, PTR-MS has been extensively combined with sensory dynamic methods which are likely to produce more valid results than static methods (137). The first dynamic sensory method applied in combination with nose-space measurements was time intensity (TI). The method is based on measuring the intensity of a sensation in relation to the time of its perception (138) through which is possible to obtain detailed information on dynamic evolution of the sensory attribute under investigation (8). These information is especially important when studying products with a distinctive time profile like chewing gums. The TI methodology has been applied extensively on this matrix, as testified by the high number of researches available in literature (139–142). Different variations of TI method were also proposed like the dual-attribute TI, where two attributes are monitored at the same time in slowly changing products (143,144) and Discrete TI where panellists are asked for repeated rating of single or few attributes at discrete time points (8,145,146). TI coupled to PTR-MS has also helped to understand the effect of fat reduction on aroma flavour and perception (147) and the influence of $CO_2$ and sugar concentrations in flavoured carbonated drinks (148).

Recently, another dynamic sensory method was coupled to nose-space analysis, the temporal dominance of sensation (TDS). TDS describes the temporal evolution of different sensations developed during consumption where trained panellists are asked to choose the attribute which dominates their perception (the attribute that catches consumer attention) among a pre-defined list of attributes (maximum 10) (149).

While real products are tested when the focus is in understanding food properties effect on flavour perception and release, model food systems are usually employed when the aim is to **characterize consumer** and the factors affecting flavour release. Early *in vivo* nose-space experiments demonstrated a great inter-individual variability (150–152). The causes of this variability are manifold and have not been fully understood due to the complexity of in-mouth mechanisms (153). Peripheral factors like salivary flow, saliva composition in terms of proteins, peptides and microbiota (154), breathing rates, absorption of aroma compounds by the pharyngeal mucosa and oral processing including chewing, swallowing, eating rates and tongue movements play an important role in retronasal flavour release and perception (29,153,155). Some of these physiological factors are also correlated to demographic elements like gender and age (156,157).

PTR-MS nose-space measurements can then help understanding the mechanisms and the influence of these peripheral factors in flavour release.

## 1.8 Rational and thesis outline

The challenge of delivering to consumers food products that are safe, cheap and with excellent sensory qualities has shaped Western (largely unsustainable) food systems. Despite PTR-MS technique has been widely used in food science (Figure 1.4), only a very limited number of food companies implemented this technology in their plants and use it mostly for R&D purposes. This thesis therefore, focuses on the aspects of food quality related to the flavour and aroma sensory part by exploring different applications of PTR-MS along the food chain and throughout consumption (Figure 1.5).

In the first chapters (**Chapter 2-5),** we explored the suitability of headspace PTR-MS measurement to monitor raw materials quality life in a real industrial scenario in terms of sample numerosity and variability through headspace analysis. PTR-ToF-MS technique suitability, together with some recent implementations like SRI and RF, were investigated for different applications in agroindustry. The experiments in **Chapter 2 and 3** were performed on anhydrous milk fat, a dairy product widely used in confectionary industry, **Chapter 4** investigated ultrahigh-temperature lactose-free milk, and **Chapter 5** investigated raw hazelnuts. In these first chapters, the quality of these ingredients was screened by obtaining their VOCs fingerprints, after which they were correlated with industrial quality control sensory data. More specifically, **Chapter 2** assess the impact of two type of packaging on aroma changes during shelf life at both refrigerated temperature (up to 240 days) and thermal treatment (50°C up to 11 days). **Chapter 3** introduces the sensory aspect. Three quality categories of anhydrous milk fat obtained from industrial sensory analysis were predicted based on data mining of PTR/SRI-ToF-MS fingerprints. In **Chapter 4,** PTR-ToF-MS and GS-MS were used to get insights on the phenomena occurring during shelf life at 20°C of ultrahigh-temperature lactose-free milk. Changes in VOCs profile over a 150 days period, were evaluated by considering milk batch-to-batch variability and different commercial lactase preparations. **Chapter 5** continues the investigation into quality control of raw materials by focusing on raw hazelnuts. In this case three different experiments tested the method ability to classify samples with different sensory values and to detect quality biomarkers in hazelnuts with visual defects and with sensory defects. Technique sensitivity in detecting sample defects when simulating industrial lots variability was also explored.

In case of food quality throughout consumption, **Chapter 6 and 7** explore *in-vivo* PTR-QiToF-MS appropriateness when being simultaneously coupled with dynamic sensory methods. The experiment in **Chapter 6** was designed to unravel the mechanisms by which single foods affect the sensory properties of composite foods. In-nose aroma release and dynamic aroma intensity perception were assessed simultaneously for mayonnaises without and with different carrier foods (bread, cooked potato) varying in hardness (soft, hard). **Chapter 7** assesses the influence of consumer characteristics (gender, ethnicity and

physiological parameters) on flavour release and perception of a model food like mint chewing gum. Collecting information on how consumers and products characteristics affect aroma perception and release can play an important role for product reformulation and for the new emerging field of personalized product design and nutrition.

Finally, **Chapter 8**, provides a general, discussion of all studies and reflects on potentials and pitfalls related to PTR-MS application in agroindustry. Methodological considerations for both HS and nose-space analysis, suggestions for future research and main conclusions are also provided.

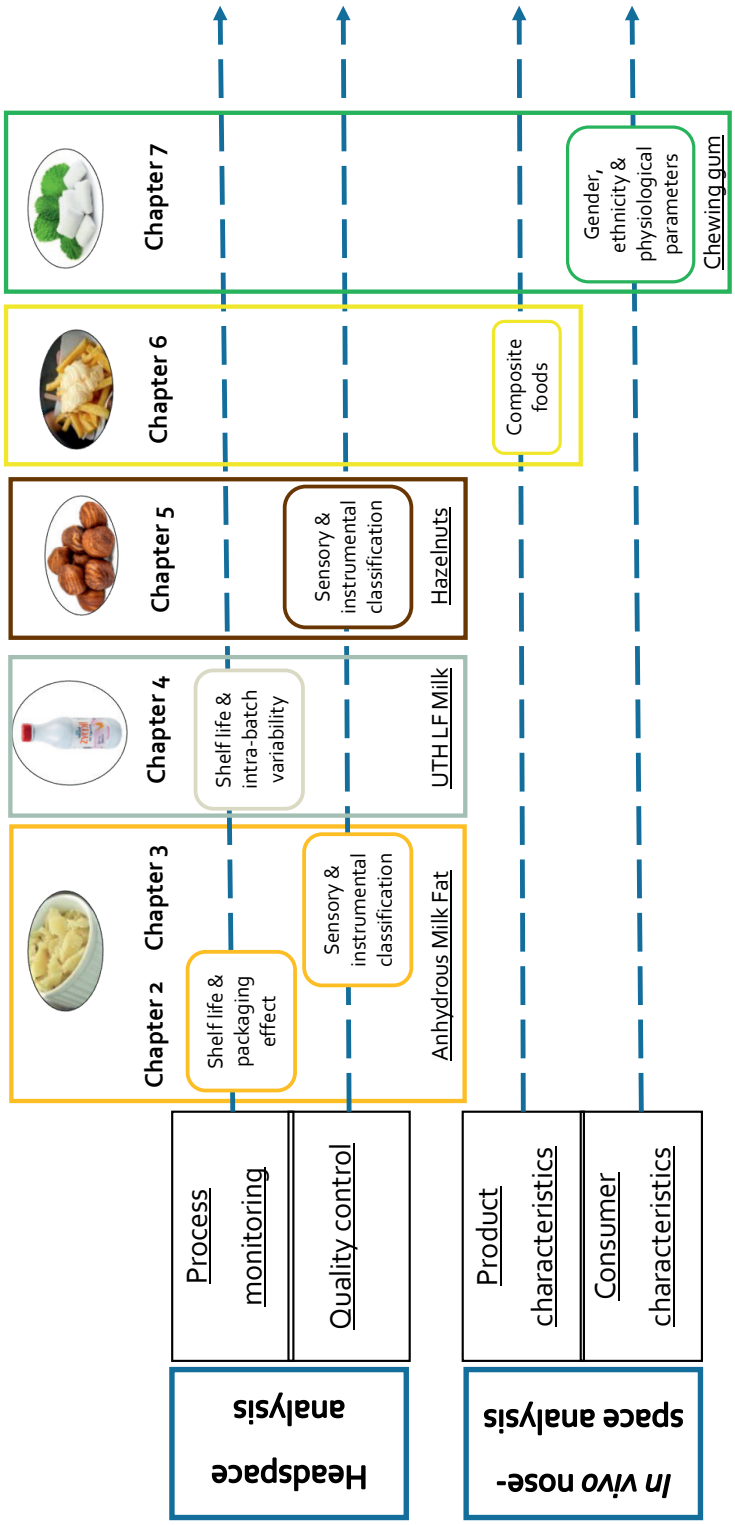



***Figure 1.5:*** *Schematic overview of thesis chapters. While the first chapters focused on headspace (HS) PTR-ToF-MS applications for quality control and monitoring shelf life, the last chapters explored PTR-QiToF-MS for in-vivo nose-space applications to explore factors affecting flavour release and perception during consumption*

## 1.9 References


1. Trimigno A, Marincola FC, Dellarosa N, Picone G, Laghi L. Definition of food quality by NMR-based foodomics. Current Opinion in Food Science. 2015.
2. Lawless H. Dimensions of sensory quality: A critique. Food Qual Prefer. 1995;
3. Leitzmann C. Food Quality—Definition and a Holistic View. In: Safeguarding Food Quality. 1993.
4. Giusti AM, Bignetti E, Cannella C. Exploring new frontiers in total food quality definition and assessment: From chemical to neurochemical properties. Food and Bioprocess Technology. 2008.
5. Van Trijp JCM, Steenkamp JBEM. Consumer-oriented new product development: Principles and practice. In: Innovation in Agri-Food Systems: Product Quality and Consumer. 2005.
6. Linnemann AR, Benner M, Verkerk R, Van Boekel MAJS. Consumer-driven food product development. Trends in Food Science and Technology. 2006.
7. Munoz AM. Sensory evaluation in quality control: an overview, new developments and future opportunities. Food Qual Prefer. 2002;13(6):329–39.
8. Lawless HT, Heymann H. Sensory evaluation of food : principles and practices [Internet]. 2nd ed. Food science text seriesFood Sensory evaluationChimieScience des matériaux. New York: Springer; 2010. 1 online resource (xxiii, 596 pages). Available from: http://link.springer.com/10.1007/978-1-4419-6488-5
9. Cayot N. Sensory quality of traditional foods. Food Chem. 2007;101(1):154–62.
10. Kilcast D. Measurement of the sensory quality of food: an introduction. In: Instrumental Assessment of Food Sensory Quality. 2013.
11. Reece RN. Quality Assurance Perspective of Sensory Evaluation. Food Technol. 1979;33(9):37.
12. Corollaro ML, Aprea E, Endrizzi I, Betta E, Dematte ML, Charles M, et al. A combined sensory-instrumental tool for apple quality evaluation. Postharvest Biol Technol. 2014;96:135–44.
13. Yeretzian C, Pollien P, Lindinger C, Ali S. Individualization of Flavor Preferences: Toward a Consumer-centric and Individualized Aroma Science. Compr Rev Food Sci Food Saf. 2004;
14. Taylor AJ. Release and transport of flavors in vivo: Physicochemical, physiological, and perceptual considerations. Compr Rev Food Sci Food Saf. 2002;
15. Besnard P, Passilly-Degrace P, Khan NA. Taste of fat: A sixth taste modality? Physiological Reviews. 2015.
16. Reineccius G, Peterson D. Principles of food flavor analysis. In: Instrumental Assessment of Food Sensory Quality. 2013.
17. Buck L, Axel R. A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. Cell. 1991;
18. Shepherd GM. "Neurogastronomy. How the Brain Creates Flavor and Why it Matters", Gordon M. Shepherd. 2013;
19. Shepherd GM. Perspectives on olfactory processing, conscious perception, and orbitofrontal cortex. In: Annals of the New York Academy of Sciences. 2007.
20. Guichard E, Salles C. Retention and release of taste and aroma compounds from the food matrix during mastication and ingestion. Flavor: From Food to Behaviors, Wellbeing and Health. Elsevier Ltd; 2016. 3–22 p.
21. Biasioli F, Yeretzian C, Gasperi F, Mark TD. PTR-MS monitoring of VOCs and BVOCs in food science and technology. Trac-Trends Anal Chem. 2011;30(7):968–77.
22. Taylor AJ, Linforth RST. Food Flavour Technology: Second Edition. Food Flavour Technology: Second Edition. 2010.
23. Schieberle P, Hofmann T. Mapping the combinatorial code of food flavors by means of molecular sensory science approach. In: Food Flavors: Chemical, Sensory and Technological Properties. 2011.

24. Jeleń H. Specificity of food odorants. In: Food Flavors: Chemical, Sensory and Technological Properties. 2011.
25. Belitz HD, Grosch W, Schieberle P. Food chemistry. Food Chemistry. 2009.
26. Diez-Simon C, Mumm R, Hall RD. Mass spectrometry-based metabolomics of volatiles as a new tool for understanding aroma and flavour chemistry in processed food products. Vol. 15, Metabolomics. 2019.
27. Dresow JF, Böhm H. The influence of volatile compounds of the flavour of raw, boiled and baked potatoes: Impact of agricultural measures on the volatile components. Landbauforsch Volkenrode. 2009;
28. Jeleń HH, Majcher M, Dziadas M. Microextraction techniques in the analysis of food flavor compounds: A review. Analytica Chimica Acta. 2012.
29. Buettner A, Beauchamp J. Chemical input - Sensory output: Diverse modes of physiology-flavour interaction. Food Qual Prefer. 2010;
30. Guichard E. Interaction of aroma compounds with food matrices. In: Flavour Development, Analysis and Perception in Food and Beverages. 2015.
31. Guichard E. Interactions between flavor compounds and food ingredients and their influence on flavor perception. Food Rev Int. 2002;
32. Poinot P, Arvisenet G, Ledauphin J, Gaillard JL, Prost C. How can aroma-related cross-modal interactions be analysed? A review of current methodologies. Food Quality and Preference. 2013.
33. van Eck A, Hardeman N, Karatza N, Fogliano V, Scholten E, Stieger M. Oral processing behavior and dynamic sensory perception of composite foods: Toppings assist saliva in bolus formation. Food Qual Prefer. 2019;
34. Reineccius G. Choosing the correct analytical technique in aroma analysis. In: Flavour in Food. 2006.
35. Reineccius G. Instrumental Methods of Analysis. In: Food Flavour Technology: Second Edition. 2010.
36. Ibáñez C, García-Cañas V, Valdés A, Simó C. Novel MS-based approaches and applications in food metabolomics. TrAC - Trends in Analytical Chemistry. 2013.
37. Herrero M, Simõ C, García-Cañas V, Ibáñez E, Cifuentes A. Foodomics: MS-based strategies in modern food science and nutrition. Mass Spectrometry Reviews. 2012.
38. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nature Reviews Molecular Cell Biology. 2012.
39. Hall RD. Plant metabolomics: From holistic hope, to hype, to hot topic. New Phytologist. 2006.
40. Scalbert A, Brennan L, Fiehn O, Hankemeier T, Kristal BS, van Ommen B, et al. Mass-spectrometry-based metabolomics: Limitations and recommendations for future progress with particular focus on nutrition research. Metabolomics. 2009.
41. Grosch W. Aroma compounds. In: Handbook of Food Analysis Second Edition: Physical Characterization and Nutrient Analysis. 2004.
42. Heenan SP, van Ruth SM. Emerging flavour analysis methods for food authentication. In: Instrumental Assessment of Food Sensory Quality. 2013.
43. Taylor AJ, Linforth RST. On-Line Monitoring of Flavour Processes. In: Food Flavour Technology: Second Edition. 2010. p. 266–95.
44. Phillips M, Cataneo RN, Chaturvedi A, Kaplan PD, Libardoni M, Mundada M, et al. Detection of an Extended Human Volatome with Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry. PLoS One. 2013;
45. Das MK, Bishwal SC, Das A, Dabral D, Varshney A, Badireddy VK, et al. Investigation of gender-specific exhaled breath volatome in humans by GCxGC-TOF-MS. Anal Chem. 2014;
46. Dewulf J, Van Langenhove H. Analysis of volatile organic compounds using gas chromatography. Trac-Trends Anal Chem. 2002;21(9–10):637–46.
47. Beale DJ, Pinu FR, Kouremenos KA, Poojary MM, Narayana VK, Boughton BA, et al. Review of recent developments in GC–MS approaches to metabolomics-based research. Metabolomics. 2018.

48. De Koning S, Janssen HG, Brinkman UAT. Modern methods of sample preparation for GC analysis. Chromatographia. 2009.
49. Gu Q, David F, Lynen F, Rumpel K, Dugardeyn J, Van Der Straeten D, et al. Evaluation of automated sample preparation, retention time locked gas chromatography-mass spectrometry and data analysis methods for the metabolomic study of Arabidopsis species. J Chromatogr A. 2011;
50. Alves Z, Melo A, Figueiredo AR, Coimbra MA, Gomes AC, Rocha SM. Exploring the saccharomyces cerevisiae volatile metabolome: Indigenous versus commercial strains. PLoS One. 2015;
51. Strączyński G, Ligor T. Comprehensive Gas Chromatography: Food and Metabolomocs Applications. Critical Reviews in Analytical Chemistry. 2018.
52. d'Acampora Zellner B, Dugo P, Dugo G, Mondello L. Gas chromatography-olfactometry in food flavour analysis. Journal of Chromatography A. 2008.
53. Fuller GH, Steltenkamp R, Tisserand GA. THE GAS CHROMATOGRAPH WITH HUMAN SENSOR: PERFUMER MODEL. Ann N Y Acad Sci. 1964;
54. Song H, Liu J. GC-O-MS technique and its applications in food flavor analysis. Food Research International. 2018.
55. Qi M, Ge X, Liang M, Fu R. Flash gas chromatography for analysis of volatile compounds from Houttuynia cordata Thunb. Anal Chim Acta. 2004;
56. Vandendriessche T, Nicolai BM, Hertog MLATM. Optimization of HS SPME Fast GC-MS for High-Throughput Analysis of Strawberry Aroma. Food Anal Methods. 2013;
57. Nozière B, Kalberer M, Claeys M, Allan J, D'Anna B, Decesari S, et al. The Molecular Identification of Organic Compounds in the Atmosphere: State of the Art and Challenges. Chemical Reviews. 2015.
58. Biasioli F, Yeretzian C, Mark TD, Dewulf J, Van Langenhove H. Direct-injection mass spectrometry adds the time dimension to (B)VOC analysis. Trac-Trends Anal Chem. 2011;30(7):1003–17.
59. McEwan MJ. Direct analysis mass spectrometry. In: Ion/Molecule Attachment Reactions: Mass Spectrometry. 2015.
60. Ibáñez C, Simó C, García-Cañas V, Acunha T, Cifuentes A. The role of direct high-resolution mass spectrometry in foodomics. Anal Bioanal Chem. 2015;
61. Lehnert A-S, Behrendt T, Ruecker A, Pohnert G, Trumbore S. Performance of SIFT-MS and PTR-MS in the measurement of volatile organic compounds at different humidities. Atmos Meas Tech Discuss. 2019;
62. Lindinger W, Hansel A, Jordan A. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) - Medical applications, food control and environmental research. Int J Mass Spectrom. 1998;173(3):191–241.
63. Smith D, Španěl P. Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis. Mass Spectrom Rev. 2005;24(5):661–700.
64. Jordan A, Haidacher S, Hanel G, Hartungen E, Herbig J, Märk L, et al. An online ultra-high sensitivity Proton-transfer-reaction mass-spectrometer combined with switchable reagent ion capability (PTR + SRI - MS). Int J Mass Spectrom. 2009;286(1):32–8.
65. Sulzer P, Hartungen E, Hanel G, Feil S, Winkler K, Mutschlechner P, et al. A Proton Transfer Reaction-Quadrupole interface Time-Of-Flight Mass Spectrometer (PTR-QiTOF): High speed due to extreme sensitivity. Int J Mass Spectrom [Internet]. 2014;368:1–5. Available from: http://www.sciencedirect.com/science/article/pii/S1387380614001584
66. Byliński H, Gębicki J, Dymerski T, Namieśnik J. Direct Analysis of Samples of Various Origin and Composition Using Specific Types of Mass Spectrometry. Critical Reviews in Analytical Chemistry. 2017.
67. Smith D, Španěl P. Direct, rapid quantitative analyses of BVOCs using SIFT-MS and PTR-MS obviating sample collection. TrAC - Trends in Analytical Chemistry. 2011.

68. Gardner JW, Bartlett PN. A brief history of electronic noses. Sensors Actuators B Chem. 1994;
69. Montuschi P, Santonico M, Mondino C, Pennazza G, Maritini G, Martinelli E, et al. Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. Chest. 2010;
70. Peris M, Escuder-Gilabert L. A 21st century technique for food control: Electronic noses. Analytica Chimica Acta. 2009.
71. Loutfi A, Coradeschi S, Mani GK, Shankar P, Rayappan JBB. Electronic noses for food quality: A review. Journal of Food Engineering. 2015.
72. Venter A, Nefliu M, Graham Cooks R. Ambient desorption ionization mass spectrometry. TrAC - Trends Anal Chem. 2008;
73. Alberici RM, Simas RC, Sanvido GB, Romão W, Lalli PM, Benassi M, et al. Ambient mass spectrometry: Bringing MS into the "real world." Analytical and Bioanalytical Chemistry. 2010.
74. Cody RB, Laramée JA, Nilles JM, Durst† HD. Direct Analysis in Real Time (DART®) Mass Spetrometry. New Dev Mass Spectrom. 2015;
75. Hansel A, Jordan A, Holzinger R, Prazeller P, Vogel W, Lindinger W. Proton transfer reaction mass spectrometry: on-line trace gas analysis at the ppb level. Int J Mass Spectrom Ion Process. 1995;149–150(C):609–19.
76. Lindinger W, Hansel A. Analysis of trace gases at ppb levels by proton transfer reaction mass spectrometry (PTR-MS). Plasma Sources Sci Technol. 1997;
77. Ellis AM, Mayhew CA. Proton transfer reaction mass spectrometry: Principles and applications. Proton Transfer Reaction Mass Spectrometry: Principles and Applications. 2013.
78. Sulzer P, Edtbauer A, Hartungen E, Jurschik S, Jordan A, Hanel G, et al. From conventional proton-transfer-reaction mass spectrometry (PTR-MS) to universal trace gas analysis. Int J Mass Spectrom. 2012;321:66–70.
79. Blake RS, Monks PS, Ellis AM. Proton-Transfer Reaction Mass Spectrometry. Chem Rev. 2009;109(3):861–96.
80. Mikoviny T, Kaser L, Wisthaler A. Development and characterization of a High-Temperature Proton-Transfer-Reaction Mass Spectrometer (HT-PTR-MS). Atmos Meas Tech. 2010;
81. Cappellin L, Karl T, Probst M, Ismailova O, Winkler PM, Soukoulis C, et al. On Quantitative Determination of Volatile Organic Compound Concentrations Using Proton Transfer Reaction Time-of-Flight Mass Spectrometry. Environ Sci Technol. 2012;46(4):2283–90.
82. Koss AR, Warneke C, Yuan B, Coggon MM, Veres PR, De Gouw JA. Evaluation of NO+ reagent ion chemistry for online measurements of atmospheric volatile organic compounds. Atmos Meas Tech. 2016;
83. Smith D, Španěl P. Ambient analysis of trace compounds in gaseous media by SIFT-MS. Analyst. 2011.
84. Guilhaus M. Principles and Instrumentation in Time-of-flight Mass Spectrometry. J Mass Spectrom. 1995;
85. Blake RS, Whyte C, Hughes CO, Ellis AM, Monks PS. Demonstration of proton-transfer reaction time-of-flight mass spectrometry for real-time analysis of trace volatile organic compounds. Anal Chem. 2004;76(13):3841–5.
86. Barber S, Blake RS, White IR, Monks PS, Reich F, Mullock S, et al. Increased sensitivity in proton transfer reaction mass spectrometry by incorporation of a radio frequency ion funnel. Anal Chem. 2012;
87. Brown PA, Cristescu SM, Mullock SJ, Reich DF, Lamont-Smith CS, Harren FJM. Implementation and characterization of an RF ion funnel ion guide as a proton transfer reaction chamber. Int J Mass Spectrom. 2017;
88. Cappellin L, Loreto F, Aprea E, Romano A, del Pulgar JS, Gasperi F, et al. PTR-MS in Italy: A Multipurpose Sensor with Applications in Environmental, Agri-Food and Health Science. Sensors. 2013;13(9):11923–55.
89. Cappellin L, Biasioli F, Fabris A, Schuhfried E, Soukoulis C, Mark TD, et al. Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS. Int J Mass Spectrom. 2010;290(1):60–3.
90. Cappellin L, Biasioli F, Granitto PM, Schuhfried E, Soukoulis C, Costa F, et al. On data analysis in PTR-TOF-MS: From raw spectra to data mining. Sensors and Actuators B-Chemical. 2011;155(1):183–90.

91. Cappellin L, Soukoulis C, Aprea E, Granitto P, Dallabetta N, Costa F, et al. PTR-ToF-MS and data mining methods: A new tool for fruit metabolomics. Metabolomics. 2012;
92. Granitto PM, Biasioli F, Aprea E, Mott D, Furlanello C, Märk TD, et al. Rapid and non-destructive identification of strawberry cultivars by direct PTR-MS headspace analysis and data mining techniques. Sensors Actuators, B Chem. 2007;121(2):379–85.
93. Aprea E, Biasioli F, Märk TD, Gasperi F. PTR-MS study of esters in water and water/ethanol solutions: Fragmentation patterns and partition coefficients. Int J Mass Spectrom. 2007;
94. Buhr K, van Ruth S, Delahunty C. Analysis of volatile flavour compounds by Proton Transfer Reaction-Mass Spectrometry. Int J Mass Spectrom. 2002;221:1–7.
95. Nenadis N, Heenan S, Tsimidou MZ, Van Ruth S. Applicability of PTR-MS in the quality control of saffron. Food Chem. 2016;
96. Wojnowski W, Majchrzak T, Szweda P, Dymerski T, Gębicki J, Namieśnik J. Rapid Evaluation of Poultry Meat Shelf Life Using PTR-MS. Food Anal Methods. 2018;
97. Acierno V, Yener S, Alewijn M, Biasioli F, Van Ruth S. Factors contributing to the variation in the volatile composition of chocolate: Botanical and geographical origins of the cocoa beans, and brand-related formulation and processing. Food Res Int. 2016;
98. Yener S, Romano A, Cappellin L, Granitto PM, Aprea E, Navarini L, et al. Tracing coffee origin by direct injection headspace analysis with PTR/SRI-MS. Food Res Int. 2015;69:235–43.
99. Yener S, Romano A, Cappellin L, Mark TD, del Pulgar JS, Gasperi F, et al. PTR-ToF-MS characterisation of roasted coffees (C-arabica) from different geographic origins. J Mass Spectrom [Internet]. 2014;49(9):929–35. Available from: http://onlinelibrary.wiley.com/store/10.1002/jms.3455/asset/jms3455.pdf?v=1&t=j488vtqk&s=5e5f068e2b46bbd362bfc98556585ea01039f6a8
100. Capozzi V, Makhoul S, Aprea E, Romano A, Cappellin L, Jimena AS, et al. PTR-MS Characterization of VOCs Associated with Commercial Aromatic Bakery Yeasts of Wine and Beer Origin. Molecules. 2016;
101. Beauchamp J, Biberacher S, Gao S. Rapid Quantitation of Phenolic Compounds in Islay Single Malt Scotch Whiskies by Direct Injection Mass Spectrometry. ACS Symp Ser. 2019;
102. Gasperi F, Gallerani G, Boschetti A, Biasioli F, Monetti A, Boscaini E, et al. The mozzarella cheese flavour profile: A comparison between judge panel analysis and proton transfer reaction mass spectrometry. J Sci Food Agric. 2001;
103. Biasioli F, Gasperi F, Aprea E, Colato L, Boscaini E, Märk TD. Fingerprinting mass spectrometry by PTR-MS: Heat treatment vs. pressure treatment of red orange juice - A case study. Int J Mass Spectrom. 2003;
104. Deuscher Z, Andriot I, Sémon E, Repoux M, Preys S, Roger JM, et al. Volatile compounds profiling by using proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS). The case study of dark chocolates organoleptic differences. Journal of Mass Spectrometry. 2019.
105. Bianchi T, Weesepoel Y, Koot A, Iglesias I, Eduardo I, Gratacós-Cubarsí M, et al. Investigation of the aroma of commercial peach (Prunus persica L. Batsch) types by Proton Transfer Reaction–Mass Spectrometry (PTR-MS) and sensory analysis. Food Res Int. 2017;
106. Taiti C, Marone E, Lanza M, Azzarello E, Masi E, Pandolfi C, et al. Nashi or Williams pear fruits? Use of volatile organic compounds, physicochemical parameters, and sensory evaluation to understand the consumer's preference. Eur Food Res Technol. 2017;
107. Marone E, Masi E, Taiti C, Pandolfi C, Bazihizina N, Azzarello E, et al. Sensory, spectrometric (PTR–ToF–MS) and chemometric analyses to distinguish extra virgin from virgin olive oils. J Food Sci Technol. 2017;
108. Yeretzian C, Jordan A, Lindinger W. Analysing the headspace of coffee by proton-transfer-reaction mass-spectrometry. Int J Mass Spectrom [Internet]. 2003;223(1–3):115–39. Available from: http://ac.els-

cdn.com/S1387380602007856/1-s2.0-S1387380602007856-main.pdf?_tid=2c9b5aa4-6488-11e6-b797-00000aacb360&acdnat=1471444850_d96d29cc47ade186fe654261be16e66e

109. Yeretzian C, Jordan A, Badoud R, Lindinger W. From the green bean to the cup of coffee: Investigating coffee roasting by on-line monitoring of volatiles. Eur Food Res Technol. 2002;
110. Zanin RC, Smrke S, Kurozawa LE, Yamashita F, Yeretzian C. Novel experimental approach to study aroma release upon reconstitution of instant coffee products. Food Chem. 2020;
111. Mayr D, Margesin R, Schinner F, Märk TD. Detection of the spoiling of meat using PTR-MS. Int J Mass Spectrom. 2003;
112. Franke C, Beauchamp J. Real-Time Detection of Volatiles Released During Meat Spoilage: a Case Study of Modified Atmosphere-Packaged Chicken Breast Fillets Inoculated with Br. thermosphacta. Food Anal Methods. 2017;
113. Soukoulis C, Cappellin L, Aprea E, Costa F, Viola R, Mark TD, et al. PTR-ToF-MS, A Novel, Rapid, High Sensitivity and Non-Invasive Tool to Monitor Volatile Compound Release During Fruit Post-Harvest Storage: The Case Study of Apple Ripening. Food Bioprocess Technol. 2013;6(10):2831–43.
114. Boschetti A, Biasioli F, van Opbergen M, Warneke C, Jordan A, Holzinger R, et al. PTR-MS real time monitoring of the emission of volatile organic compounds during postharvest aging of berryfruit. Postharvest Biol Technol. 1999;17(3):143–51.
115. Beauchamp J, Zardin E, Silcock P, Bremer PJ. Monitoring photooxidation-induced dynamic changes in the volatile composition of extended shelf life bovine milk by PTR-MS. J Mass Spectrom [Internet]. 2014;49(9):952–8. Available from: http://onlinelibrary.wiley.com/store/10.1002/jms.3430/asset/jms3430.pdf?v=1&t=j5b2bzy9&s=d893aef031c4b635db87264be6644d5634fd39b2
116. Pollien P, Lindinger C, Yeretzian C, Blank I. Proton transfer reaction mass spectrometry, a tool for on-line monitoring of acrylamide formation in the headspace of Maillard reaction systems and processed food. Anal Chem. 2003;75(20):5488–94.
117. Robert F, Vuataz G, Pollien P, Saucy F, Alonso MI, Bauwens I, et al. Acrylamide formation from asparagine under low-moisture Maillard reaction conditions. 1. Physical and chemical aspects in crystalline model systems. J Agric Food Chem. 2004;
118. Märk J, Pollien P, Lindinger C, Blank I, Märk T. Quantitation of furan and methylfuran formed in different precursor systems by proton transfer reaction mass spectrometry. J Agric Food Chem. 2006;
119. Soukoulis C, Aprea E, Biasioli F, Cappellin L, Schuhfried E, Märk TD, et al. Proton transfer reaction time-of-flight mass spectrometry monitoring of the evolution of volatile compounds during lactic acid fermentation of milk. Rapid Commun Mass Spectrom. 2010;
120. Taucher J, Lagg A, Hansel A, Vogel W, Lindinger W. Methanol in Human Breath. Alcohol Clin Exp Res. 1995;
121. Lindinger W, Taucher J, Jordan A, Hansel A, Vogel W. Endogenous production of methanol after the consumption of fruit. Alcohol Clin Exp Res. 1997;
122. Taucher J, Hansel A, Jordan A, Lindinger W. Analysis of Compounds in Human Breath after Ingestion of Garlic Using Proton-Transfer-Reaction Mass Spectrometry. J Agric Food Chem. 1996;
123. Lirk P, Bodrogi F, Rieder J. Medical applications of proton transfer reaction-mass spectrometry: Ambient air monitoring and breath analysis. Int J Mass Spectrom. 2004;
124. Amann A, Costello BDL, Miekisch W, Schubert J, Buszewski B, Pleil J, et al. The human volatilome: Volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva. Journal of Breath Research. 2014.
125. Filipiak W, Sponring A, Filipiak A, Baur M, Troppmair J, Amann A. Volatile Biomarkers: Non-Invasive Diagnosis in Physiology and Medicine. Volatile Biomarkers. 2013.

126. Schwarz K, Pizzini A, Arendacká B, Zerlauth K, Filipiak W, Schmid A, et al. Breath acetone - Aspects of normal physiology related to age and gender as determined in a PTR-MS study. J Breath Res. 2009;
127. Kushch I, Arendacká B, Štolc S, Mochalski P, Filipiak W, Schwarz K, et al. Breath isoprene - Aspects of normal physiology related to age, gender and cholesterol profile as determined in a proton transfer reaction mass spectrometry study. Clin Chem Lab Med. 2008;
128. Fowler SJ. Breath analysis for label-free characterisation of airways disease. European Respiratory Journal. 2018.
129. Herbig J, Beauchamp J. Towards standardization in the analysis of breath gas volatiles. J Breath Res. 2014;
130. Beauchamp J, Herbig J. Proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOFMS) for aroma compound detection in real-time: Technology, developments, and applications. In: ACS Symposium Series. 2015.
131. Frasnelli J, van Ruth S, Kriukova I, Hummel T. Intranasal concentrations of orally administered flavors. Chem Senses. 2005;
132. van Ruth SM, Frasnelli J, Carbonell L. Volatile flavour retention in food technology and during consumption: Juice and custard examples. Food Chem. 2008;
133. Boland AB, Delahunty CM, Van Ruth SM. Influence of the texture of gelatin gels and pectin gels on strawberry flavour release and perception. Food Chem. 2006;96(3):452–60.
134. Heenan S, Soukoulis C, Silcock P, Fabris A, Aprea E, Cappellin L, et al. PTR-TOF-MS monitoring of in vitro and in vivo flavour release in cereal bars with varying sugar composition. Food Chem. 2012;131(2):477–84.
135. J.L. Ting V, Soukoulis C, Silcock P, Cappellin L, Romano A, Aprea E, et al. In Vitro and In Vivo Flavor Release from Intact and Fresh-Cut Apple in Relation with Genetic, Textural, and Physicochemical Parameters. J Food Sci. 2012;
136. Ross CF. Sensory science at the human-machine interface. Trends Food Sci Technol [Internet]. 2009;20(2):63–72. Available from: http://ac.els-cdn.com/S0924224408003178/1-s2.0-S0924224408003178-main.pdf?_tid=abffc7e8-6a06-11e6-afed-00000aab0f26&acdnat=1472048937_4e8f3be2b336312cbfcf8d805222be9a
137. Hort J. Application of Time-Dependent Measures to Understand Sensory Perception. In: Time-Dependent Measures of Perception in Sensory Evaluation. 2017.
138. Cliff M, Heymann H. Development and use of time-intensity methodology for sensory evaluation: A review. Food Research International. 1993.
139. Galmarini M V., Symoneaux R, Visalli M, Zamora MC, Schlich P. Could Time-Intensity by a trained panel be replaced with a progressive profile done by consumers? A case on chewing-gum. Food Qual Prefer. 2016;48:274–82.
140. McGowan BA, Lee SY. Comparison of methods to analyze time-intensity curves in a corn zein chewing gum study. Food Qual Prefer. 2006;17(3–4):296–306.
141. Potineni R V., Peterson DG. Mechanisms of flavor release in chewing gum: Cinnamaldehyde. J Agric Food Chem. 2008;56(9):3260–7.
142. Davidson JM, Linforth RST, Hollowood TA, Taylor AJ. Effect of sucrose on the perceived flavor intensity of chewing gum. J Agric Food Chem. 1999;47(10):4336–40.
143. Duizer LM, Bloom K, Findlay CJ. Dual-attribute time-intensity sensory evaluation: A new method for temporal measurement of sensory perceptions. Food Qual Prefer. 1997;8(4):261–9.
144. Duizer LM, Bloom K, Findlay CJ. Dual-attribute Time-intensity Measurement of Sweetness and Peppermint Perception of Chewing Gum. Inst Food Technol. 1996;61(3):636–8.
145. Delarue J, Loescher E. Dynamics of food preferences: A case study with chewing gums. Food Qual Prefer. 2004;15(7-8 SPEC.ISS.):771–9.

146. Kuesten C. Time – Intensity Using Discrete Time Points. 2017;182–236.
147. Frank D, Appelqvist I, Piyasiri U, Wooster TJ, Delahunty C. Proton transfer reaction mass spectrometry and time intensity perceptual measurement of flavor release from lipid emulsions using trained human subjects. J Agric Food Chem. 2011;
148. Saint-Eve A, Déléris I, Aubin E, Semon E, Feron G, Rabillier JM, et al. Influence of composition ($CO_2$ and sugar) on aroma release and perception of mint-flavored carbonated beverages. J Agric Food Chem. 2009;
149. Pineau N, Schlich P, Cordelle S, Mathonnière C, Issanchou S, Imbert A, et al. Temporal Dominance of Sensations: Construction of the TDS curves and comparison with time-intensity. Food Qual Prefer. 2009;
150. Gierczynski I, Laboure H, Guichard E. In vivo aroma release of milk gels of different hardnesses : Inter-individual differences and their consequences on aroma perception. J Agric Food Chem. 2008;56(5):1697–703.
151. Mestres M, Kieffer R, Buettner A. Release and perception of ethyl butanoate during and after consumption of whey protein gels: Relation between textural and physiological parameters. J Agric Food Chem. 2006;54(5):1814–21.
152. Labouré H, Repoux M, Courcoux P, Feron G, Guichard E. Inter-individual retronasal aroma release variability during cheese consumption: Role of food oral processing. Food Res Int. 2014;
153. Salles C, Chagnon MC, Feron G, Guichard E, Laboure H, Morzel M, et al. In-mouth mechanisms leading to flavor release and perception. Crit Rev Food Sci Nutr. 2011;51(1):67–90.
154. Piombino P, Genovese A, Esposito S, Moio L, Cutolo PP, Chambery A, et al. Saliva from obese individuals suppresses the release of aroma compounds from wine. PLoS One. 2014;
155. Canon F, Neiers F, Guichard E. Saliva and flavour perception : perspectives. 2018;
156. Doyennette M, Déléris I, Féron G, Guichard E, Souchon I, Trelea IC. Main individual and product characteristics influencing in-mouth flavour release during eating masticated food products with different textures: Mechanistic modelling and experimental validation. J Theor Biol. 2014;340:209–21.
157. Repoux M, Sémon E, Feron G, Guichard E, Labouré H. Inter-individual variability in aroma release during sweet mint consumption. Flavour Fragr J. 2012;27(1):40–6.
158. Cifuentes A. Food analysis and foodomics. Journal of Chromatography A. 2009.

2

# Rapid and non-invasive quality control of anhydrous milk fat by PTR-MS: the effect of storage time and packaging

Pedrotti M., Khomenko I., Cappellin L., Fontana M., Somenzi M., Falchero L., Arveda M., Fogliano V., Biasioli F.

*Journal of Mass Spectrometry,* 2018 53(9)

DOI: 10.1002/jms.4204

## Abstract

In this study, proton transfer reaction mass spectrometry (PTR-MS), coupled with a time-of-flight mass analyser and a multipurpose automatic sampler, was evaluated as a rapid and non-destructive tool for the quality control (QC) of anhydrous milk fat (AMF). AMFs packed in cardboard and bag-in-box were compared during refrigerated shelf-life at 4°C for 9 months. AMF samples were taken at 120, 180 and 240 days and measured by PTR-MS during storage at 50°C for 11 days. Univariate and multivariate data analysis were performed to classify samples according to the packaging type and to compare aromatic profiles. Markers related to both packaging and storage duration were identified and all stored samples were clearly distinguishable from reference fresh samples. Significant differences in some key butter aroma compounds such as 2-pentanone, 2-heptanone, 2/3-methylbutanal, acetoin, and butanoic acid were observed between different types of packaging. During the refrigerated storage, differences related to packaging are more evident, while during the storage at 50°C, the fat oxidation induced by the high temperature becomes the most relevant phenomenon independently of the packaging type. These results indicate the importance of avoiding AMF storage at 50°C for long times during industrial production processes. All together the data demonstrated the viability of PTR-MS as a rapid and high sensitivity tool in agroindustry quality control program.

**Keywords:** PTR-MS, VOCs, anhydrous milk fat, shelf life, industrial quality control, packaging

## 2.1 Introduction

Anhydrous milk fat (AMF) is an important industrial raw material composed by a complex mixture of triacylglycerols that should contain at least 99.8% dairy fat obtained by eliminating water and non-fat dry matter. Water elimination makes this product easier to transport, more stable and with a longer shelf life when compared to butter. Therefore, AMF is an ingredient of choice for pastry, confectionery, and ice-cream industries. As for all milk fats, oxidative rancidity is one of the major factors limiting AMF shelf-life (1, 2) by causing major changes in quality parameters (3, 4). Peroxidation of the lipid fraction leads to the formation of secondary oxidation products, especially aldehydes, which affects consumer acceptability and reduces product shelf-life (5, 6).



Food industries are constantly looking for flexible, fast and reliable methodologies to monitor the quality of their product by focusing on flavour and aroma (7). A quality control based on volatile organic compounds (VOCs) monitoring has the advantages of being non-invasive and relevant for consumer acceptance. In fact, VOCs profile is directly linked with perceived food quality at every stage of consumption (8). Extensive research has been focused on detecting, quantifying and characterizing the different flavours and off-flavours originating from dairy fat ingredients: the aroma constituent of butter and AMF have been under investigation for more than seven decades leading to the identification of more than 230 VOCs (9).

In the last years, different direct injection mass spectrometry (MS) technologies have been used to investigate VOCs. Among these technologies, proton transfer reaction mass spectrometry (PTR-MS) is an accurate, highly sensitive and non-destructive technique suitable for a rapid characterization of food products and for monitoring processes in agroindustry without any pre-treatment (8). It is based on an efficient implementation of chemical ionization by proton transfer (10). The gas mixture under investigation is continuously injected in the reaction region – the drift tube – where the VOCs are ionized by proton transfer from $H_3O^+$ ions produced by the hollow cathode ion source. The generated ions are then analysed according to their mass/charge ratio (m/z) using a time-of-flight (ToF) mass analyser which provides high mass resolution (up to 5000 m/Δm) and a broad mass range (11). The outcome is a rapid mass-resolved fingerprint of the total "volatilome" of the samples with ultra-high sensitivity (ppb) which can be of great interest for different applications supporting industrial quality control programs.

PTR-MS analysis has been used by Van Ruth *et al.* (12, 13) to classify butter oils and to verify the geographical origin of European butters in terms of headspace volatile composition. The method was deemed as a promising approach for control of regulations and quality. These pioneering researches were based on the first version of PTR-MS, which was equipped with a quadrupole mass analyser providing only the nominal mass-to-charge ratio of the ions. The introduction of a ToF mass analyser performed by Jordan *et al.* in 2009 (11) improved the mass resolution leading to a better separation and identification of butter key aroma compounds. Makhoul *et al.* (14) explored the potential of PTR-ToF-MS analysis for the classification of three

different AMFs samples based on VOC samples' profiles and sensory evaluation. However, this proof of concept on a reduced dataset did not allow reaching a clear indication on PTR-MS applicability for industrial quality control.

Other different approaches have been previously applied to investigate both butter and AMF VOCs upon storage and oxidation. Krause *et al.* (15) characterized the effect of refrigerated (5°C) and frozen (-20°C) storage over a year on the physical and sensory properties of butter. Optimum quality for butter stored as bulk at refrigerated storage was found to be stable up to 9 months, while when frozen at –20°C, it could be stored for up to 18 months. In a similar study, *Lozano et al.* (16) by combining GC-olfactometry and GC-MS, studied the effect of long-term storage (0, 6, 12 months) on VOCs and sensory properties on salted butter under refrigerated (4 °C) and frozen (-20 °C) storage. The effect of wrapping materials on butter shelf life was also compared: foil packaging significantly improved refrigerated and frozen flavour stability of salted butter compared with parchment paper (16). The effect of packaging on milk fat storage stability and aroma influence was also investigated by Tomlinson and Dixon (17) that confirmed polyethylene films as the best protection against surface oxidation, providing the best freeze-thaw stability. Widder *et al.* (18), used the aroma extraction dilution analysis approach and the dilution factor calculation for investigating the key VOC profile of butter oil during a storage of 42 days at room temperature. The study detected 19 odour compounds as the constituents of the volatile fraction from fresh butter oil (diacetyl, butyric acid, skatole and δ-decalactone as examples of the most relevant ones).

Nowadays, in industry, AMF is usually refrigerated and stored after wrapping in plastic films and putting in cardboard packages (CT) of about 25 kg or inserted in the so-called bag-in-box (BIB) containers. Different layers of polyethylene and metallized polyester (Met. PET) compose bag-in-box material that is hermetically sealed and is significant more expensive than CT packages. As mentioned, in food industry AMF is mainly used for recombination of various dairy and confectionary products. For this purpose AMF is usually melted in tanks at 50°C and used as liquid form because is easy to mix with other products (4). Before being used a variable amount of time (days) can pass where AMF is kept at this temperature. Investigating AMF VOCs changes at this temperature can be of primary interest both to highlight the development of sensorial defects in the matrix and to get insights into oxidative rancidity which is heavily affected by temperature (19).

To our knowledge, despite the relevant effect of AMF packaging and storage conditions on product quality, no previous studies comparing the effects of BIB and CT packaging on AMF quality have been published. Moreover, even if PTR-MS has already been used to classify different AMF samples, the technique has not been applied to monitor how packaging and storage time in practical industrial conditions can affect AMF VOCs release.

In this work, we evaluate a rapid and innovative approach by using PTR-ToF-MS coupled to a multipurpose automatic sampler as tool for AMF industrial quality control and study the effect of storage time and temperature on AMF volatile compound profile.

## 2.2 Material and Methods

### 2.2.1 | Anhydrous milk fat samples and packaging

Three AMF production batches (indicated as 321, 322 and 323) from the same production facility were sampled in different days to evaluate production variability. All batches were produced from pasteurized sweet cream with 35-40% fat content. AMF was produced by applying the standard method in continuous flow directly from the cream according to a procedure described elsewhere (4). Immediately after production, each batch was split in the two different types of packaging (BIB and CT) and stored in a temperature-controlled room with a mean temperature of 4°C ± 1.0 °C under control humidity conditions (65%) for the entire shelf-life period.

The BIB packaging is composed of an outer cover and an inner lining that is in direct contact with the food matrix. The outer cover is made by a metal foil of polyethylene terephthalate 12 µm thick between two layers of polyethylene (PE) 45 µm each. The inner lining is composed by a PE layer with a thickness of 65 µm. In the CT packaging, the AMF block is stored inside a cardboard and wrapped in a layer of high density PE. Information about the different samples used are summarized in Table 2.1. As reference, fresh samples (FREs) were obtained for each time point from the same manufacturer and stored in glass jars to compare fresh AMF to the one stored.

**Table 2.1:** List of AMF samples used during the experiment.

| Code | Type of packaging | Batch | Storage time for 120 days [days] | Storage time for 180 days [days] | Storage time for 240 days [days] |
|---|---|---|---|---|---|
| 321CT | CT | 1 | 142 | 204 | 296 |
| 322CT | CT | 2 | 141 | 203 | 295 |
| 323CT | CT | 3 | 140 | 202 | 294 |
| 321BIB | BIB | 1 | 142 | 204 | 296 |
| 322BIB | BIB | 2 | 141 | 203 | 295 |
| 323BIB | BIB | 3 | 140 | 202 | 294 |
| 711FRE | FRE | - | 30 | - | - |
| FRE829 | FRE | - | - | 34 | - |
| FRE830 | FRE | - | - | 35 | - |
| FRE831 | FRE | - | - | 36 | - |
| FRE101 | FRE | - | - | - | 74 |
| FRE201 | FRE | - | - | - | 77 |
| FRE801 | FRE | - | - | - | 72 |

### 2.2.2 | Sample preparation

After 120, 180 and 240 days of refrigerated storage, samples were packed in plastic bags for shipment and transported on ice packs from the industrial partner to the analytical laboratory at Fondazione Edmund Mach (San Michele all'Adige, Italy). Upon receipt, products were removed from shipping containers, examined for damage and then assigned to frozen storage (-20 ± 1.0 °C) until the day of measurement. On the first day of measurement (day 0) samples were melted in a thermal bath (50°C) and prepared for each day of measurement (day 0, 2, 4, 7, 9 and 11) following a factorial design for each storage point (120, 180 and 240 days) as shown in Figure 2.1. For each sample, five 2.5 mL aliquots of AMF were transferred into sampling vials, which were previously conditioned for two days at 65°C. Vials were then closed, labelled and stored at 50°C until measurement to simulate aging. Empty vials were used as blanks. The procedure was the same for all time points.

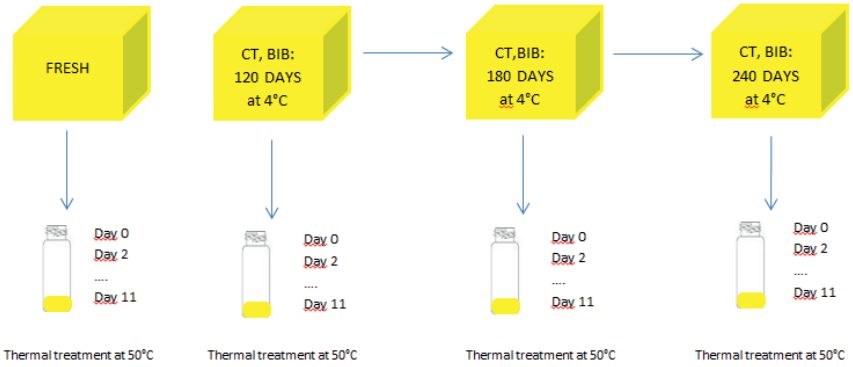



***Figure 2.1:*** *Schematic drawing of the experimental design. Bag-In-Box (BIB) and Cardboard (CT) samples after production were assigned to refrigerated storage (4°C) for the whole experiment. After reaching a specific time point (120, 180, 240 days) each AMF sample was melted and held at 50°C. Vials (in five replicates) were prepared for each time point of the measurement with thermal treatment (day 0, 2 ,4 ,7, 9, 11). Fresh samples were also used at each time point for comparison.*

### 2.2.3 | Proton Transfer Reaction Time-of-Flight Mass Spectrometry measurements

All measurements were performed in an automated way by using a multipurpose GC sampler (Gerstel GmbH, Mulheim am Ruhr, Germany) connected to the inlet of the PTR-ToF-MS as previously described (20). All the vials were incubated for equilibration at 50°C for 30 min before PTR-MS analysis. Each sample was measured for 60 s with an acquisition rate of one spectrum per second and a flow rate of 35 sccm. The inlet line consisted of a PEEK capillary tube (inner diameter 0.40 mm), heated at 110°C.

The measurement order was randomized and after each measurement, a waiting time of 3 minutes was set on the auto-sampler to avoid memory effects. A commercial PTR-ToF-MS 8000 instrument (Ionicon

Analytik GmbH, Innsbruck, Austria) in its standard configuration (V mode) was used for the headspace measurements. The ionization conditions in the drift tube were as follows: drift voltage=557 V, drift temperature=110°C, drift pressure=2.30 mbar affording an E/N value of 141 Townsend (1 Td=$10^{-17}$ $V^{-1}$ $cm^2$ $s^{-1}$). The mass resolution (m/Δm) was at least 3800 and data were collected for the mass range m/z 20-300.

### 2.2.4 | Data processing and peak selection

Dead time correction, internal calibration of mass spectral data and peak extraction were performed according to the procedure described by Cappellin *et al.* (21, 22). Internal calibration was performed by using mass peaks 21.0221, 29.9974 and 203.9430 corresponding respectively to protonated water, $NO^+$ and one of the fragments of 1,3-Diodobenzene, which was continuously injected as a peak reference compound into the PTR-ToF-MS drift tube through the PerMaSCal device (Ionicon, Innsbruck, Austria). Concentrations in ppbV (parts per billion by volume) were calculated according to the formula described by Lindinger *et al.* (10), using a constant reaction rate coefficient (kR=2 x $10^{-9}$ $cm^3$ $s^{-1}$) and averaging mass spectral signals over 30 spectra. This approximation introduces a systematic error for the absolute concentration of each compound that is in most cases below 30% and can be accounted for, if the actual rate coefficient is available (23). In this paper, the experimental *m/z* values are reported up to the third decimal place.

### 2.2.5 | Statistical analyses

Analysis of variance (ANOVA) with Bonferroni correction was performed for selection of mass peaks significantly higher in the AMF samples than in the blanks. $^{13}C$ isotopologues were also eliminated from the data set. On these reduced data sets, further 1-way ANOVA with Tukey's honest significant difference (HSD) and Bonferroni correction were performed to investigate packaging and thermal treatment effects. Summary tables where then built by eliminating isotopes and by selecting only mass peaks above a threshold of 0.1 ppbv. This threshold was introduced to reduce noise signals and to include just relevant VOCs into further statistical analysis. The median ± standard deviation of the five replicates for each extracted peak was then plotted for each storage point together with boxplots for each mass peak. To interpret the experiment results, principal component analysis (PCA) was conducted on the scaled and mean centred data sets. All analysis and graphs were performed with core functions of R programming language and its external packages (ChemometricsWithR, DiscriMiner, ggplot2) (24).

## 2.3 Results and discussion

### 2.3.1 | PTR-ToF-MS spectra analysis

The analysis of raw PTR-ToF-MS data of AMF samples resulted in the extraction of 396 mass peaks in the *m/z* range 21-300 as shown in Figure 2.2. After eliminating signals related to interfering ions ($NO^+$, $O_2^+$, and water clusters) at *m/z* 30, 32, 37 and 55, one-way ANOVA with Bonferroni correction (p-value < 0.01) was

performed by comparing all AMF samples with blanks. *E.g.*, the test run on AMF after 240 days of storage identified up to 183 mass peaks that were significantly higher than blanks. Further statistical analyses were performed on a reduced dataset of 129 mass peaks corresponding to peaks that were found significantly different from blanks for all storage times (120, 180, 240). The final data matrix is composed by 129 columns corresponding to the mass peaks and as rows all AMF samples (the three lots of each BIB and CT) measured in five technical replicates at the different storage points and during the different days of the thermal treatment.

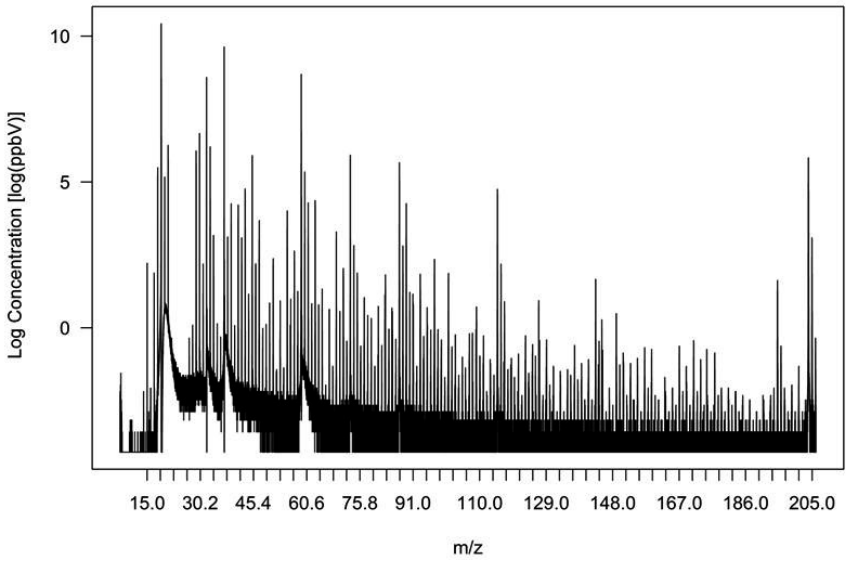



***Figure 2.2:*** *PTR-ToF-MS volatile profile in logarithmic y-scale of one of the AMF samples (321BIB) at 240 days of shelf life.*

### 2.3.2 | Effect of packaging on PTR-MS profile

PCA of the different shelf-life time points (120, 180, 240 days) of all samples at day 0 of the thermal treatment was performed as data exploration on the final matrix of mean centred and scaled data (Fig. 2.3). In most cases, BIB, CT, and FRE samples are clearly separated and a good measurement reproducibility can be observed. For example, at time 180 (Fig. 2.3B), the first principal component (PC), explaining 22.83% of the total variance, is separating BIB and CT samples while the second PC, explaining 19.21% of total variance is separating the stored samples from the fresh standards. At 120 days the standard (711FRE) is collocated near CT samples indicating a similar VOC profile.

During storage, it can be observed that while at 120 days (Fig. 2.3A) a first distinction of BIB and CT is visible, the separation appears more evident at 180 and 240 days were BIB and CT are perfectly separated. Refrigerated storage at 4°C appears to increase the differences in the volatile profile of the AMF stored in the two types of packaging. This preliminary investigation indicates that BIB packaging, thanks to its lower

$O_2$ permeability, keeps the volatile profile of the AMF more similar to the FRE samples during refrigerated storage by offering a better protection to oxidation and thus from rancidity.

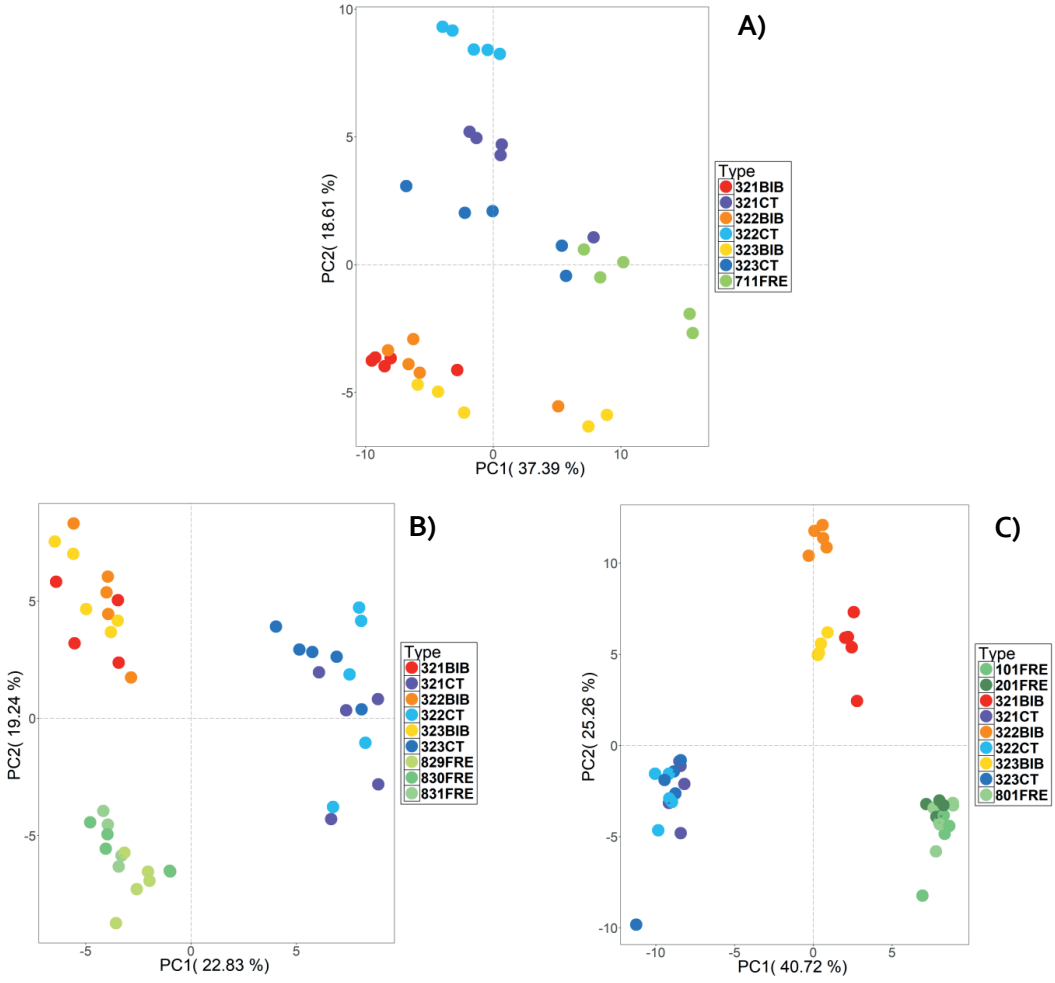



***Figure 2.3:*** *Score plots of PCA analysis for each time point at day 0 on scaled and mean centred data sets. In order from top to bottom left days 120 (Fig. 3A), 180 (Fig. 3B) and 240 (Fig. 3C).*

### 2.3.3 | Effect of storage on the PTR-MS profile

Further statistical univariate analysis was performed on the final dataset for investigating storage effects in more details. One-way ANOVA followed by Tukey HSD (with Bonferroni correction) was done on each time point (120, 180 and 240 days of refrigerated storage) to evaluate VOCs differences between the two types of packaging at day 0 of the thermal treatment. Results for the average of the three batches (321, 322, 323) for each packaging type are showed in Table 2.2 together with the tentative identification of the VOCs. All mass peaks found significantly different at least in one of the time point (p-value < .01) were

reported by starting from *m/z* = 41.038 and by taking a concentration threshold of at least 0.1 ppbV. 28 mass peaks were tentatively identified on the basis of their sum formula, fragmentation pattern and literature data (9, 12, 15, 16, 18, 24, 25).

The *post-hoc* test found significant differences between the two types of packaging at all different time points of the storage meaning that a relevant packaging effect on AMF VOCs is present. Among the 38 compounds, 25 reported higher levels in the BIB packaging than in CT for all time points. This finding may be explained by the difference in packaging: BIB does not allow exchange with the atmosphere but at the same time enhances the accumulation of volatile compounds in the AMF like secondary oxidation products generated during storage. This figure suggested that different oxygen permeability might have a direct influence on the storage and on AMF fat oxidation.

The most abundant volatiles found in the headspace of AMF were the mass peaks 73.064 and 87.080 associated respectively to 2-butanone/butanal and 2-pentanone/pentanal/2/3-methylbutanal that are key butter aroma compound (9). Other volatile compounds that were found abundant in the AMF headspace were acetaldehyde (*m/z* = 45.033), 2-propanone (*m/z* = 60.052, in this case the isotope is taken since the signal at *m/z* = 59.086 corresponding to the 2-propanone was saturated) and some unspecific fragments like the *m/z* = 43.017 that can be associated to methyl butanoate or acetic acid (12, 26).

One of the most studied volatile biomarker for dairy oxidative stress is hexanal since it is one of the major products of fat oxidation, which increases during storage. Hexanal is produced from the oxidation of n-6 polyunsaturated fatty acids, like linoleic acid (28), and is usually associated with *grassy* or metallic-like off flavour in milk and butter (29)–(31). Recently, Asaduzzaman *et al.*(2017), monitored milk oxidative stress by following hexanal evolution at 4°C by PTR-MS. The mass peaks at *m/z* 101 and *m/z* 83 corresponding to the protonated molecular ion and to the fragment obtained from the loss of a water molecule, were indicated as biomarker for hexanal determination (32). In our experiment, both these mass peaks (*m/z* = 101.096 and *m/z* = 83.085) showed a significant higher concentration in BIB samples then CT samples at all time points. At 45 days (data not shown), the AMF stored in the BIB showed already higher levels meaning that this difference was originated during the first days of the storage and is then maintained.

Beside the different oxygen permeability, it may be possible that since the BIB packaging is sealed, it allows a lower dispersion of the molecule by accumulating it into the packaging, resulting in higher levels. An increasing trend for both the hexanal mass peaks was observed during storage. These findings were in line with previous studies: Lozano *et al.* when monitoring the effect of cold storage (4°C) on sweet cream butter stored in different packaging material found a significant increase of hexanal after 6 months storage (16).

During the shelf life, almost all VOCs reported in Table 2.5.3 show increasing concentrations with a few exceptions. For both BIB and CT, the mass peak *m/z* = 49.011 ($CH_5S^+$) tentatively identified as methanethiol and *m/z* = 109.027 ($C_6H_5O_2^+$) showed decreasing concentrations during the whole storage. Methanethiol been reported to be formed from methional, which is a light-induced product of methionine (33). This

volatile sulphur compound was found both in butter samples by headspace GC (34) and in the headspace of bovine milk exposed to a fluorescent light by PTR-MS analysis (35). The latter study showed a direct dependency of methanethiol formation to light exposure. The decreasing trend observed during shelf life may be related to the compound high reactivity. In fact, it can be easily oxidized to form dimethyl disulfide and dimethyl trisulfide (36).

**Table 1.2:** Tentatively identified mass peaks in the headspace of anhydrous milk fat (AMF) at the different time points (120, 180 and 240 days) with average concentrations at the first day of the thermal treatment. Significant differences per time point are indicated with Tukey letters (one-way ANOVA).



| Measured mass (m/z) | Theoretical mass | Chemical Formula | 120 | | 180 | | 240 | | Tentative identification |
|---|---|---|---|---|---|---|---|---|---|
| | | | *BIB* | *CT* | *BIB* | *CT* | *BIB* | *CT* | |
| 41.038 | 41.0391 | C3H5+ | 15.84a | 9.99b | 16.64a | 11.81b | 53.07a | 36.74b | Alkyl fragment alcohol |
| 43.017 | 43.0184 | C2H3O+ | 23.72a | 16.11b | 37.56a | 18.77b | 111.93 | 80.57 | Acetic acid fragment |
| 45.033 | 45.0340 | C2H4OH+ | 110.31a | 45.05b | 115.57a | 41.89b | 336.72a | 122.38b | Acetaldehyde |
| 47.048 | 47.0497 | C2H7O+ | 16.43 | 22.06 | 16.11 | 11.50 | 36.86a | 19.12b | Ethanol cluster |
| 49.011 | 49.0112 | CH5S+ | 2.53 | 1.70 | 1.24 | 1.50 | 0.80a | 1.31b | Methanehtiol |
| 51.043 | 51.0435 | CH4OH*H3O+ | 3.22 | 2.20 | 1.75 | 1.89 | 11.77a | 17.35b | Methanol cluster |
| 53.001 | 53.0027 | C3OH+ | 0.16 | 0.09 | 0.22a | 0.08b | 0.70a | 0.30b | - |
| 53.038 | 53.0391 | C4H4+ | 0.73 | 0.57 | 0.75 | 0.69 | 1.64a | 1.24b | - |
| 57.069 | 57.0699 | C4H9+ | 4.06a | 2.97b | 4.07a | 2.97b | 10.73a | 7.61b | Alkyl fragment |
| 58.040 | 58.0419 | C3H5OH+ | 0.76a | 0.26b | 1.06a | 0.37b | 2.89a | 1.11b | |
| 60.052 | 60.0525 | C2[13]CH6OH+ | 42.91a | 18.53b | 61.50a | 21.14b | 190.74a | 81.20b | 2-propanone isotope |
| 63.026 | 63.0268 | C2H6SH+ | 11.61 | 13.46 | 22.69a | 15.13b | 74.62a | 61.50b | Ethanethiol / DMS |
| 67.054 | 67.0547 | C5H7+ | 0.46 | 0.33 | 0.46 | 0.35 | 1.25a | 0.97b | 2-pentanal fragment |
| 69.069 | 69.0704 | C5H8H+ | 8.26a | 3.87b | 6.94a | 3.49b | 21.71a | 11.71b | Isoprene/ 3-hexen-2-ol |
| 73.027 | 73.0284 | C3H4O2H+ | 0.01 | 0.01 | 0.22 | 0.30 | 1.19a | 1.87b | Propiolactone |
| 73.064 | 73.0653 | C4H8OH+ | 84.74 | 101.60 | 105.14 | 112.44 | 330.14a | 402.30b | 2-butanone/butanal |
| 77.058 | 77.0602 | C3H8O2H+ | 0.96a | 0.44b | 1.11a | 0.44b | 3.43a | 2.20b | Propylene glycol |
| 81.070 | 81.0699 | C6H9+ | 1.44a | 0.92b | 0.94 | 1.23 | 1.06a | 1.67b | |
| 82.067 | 82.0651 | C5H7NH+ | 0.20 | 0.21 | 0.22 | 0.29 | 0.44a | 0.76b | 1-methylpyrrole |
| 82.994 | 82.9950 | C4H3S+ | 0.60 | 0.81 | 0.91 | 1.06 | 2.52a | 3.25b | |
| 83.085 | 83.0860 | C6H11+ | 3.35a | 1.85b | 1.52 | 1.89 | 3.93 | 4.83 | Hexanal fragment |
| 84.942 | | | 0.36 | 0.55 | 0.56 | 0.69 | 1.63a | 2.12b | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 85.064 | 85.0653 | $C_5H_8OH^+$ | 0.50a | 0.32b | 0.53a | 0.36b | 1.15a | 0.87b | 2-pentenal (E)/1-penten-3-one |
| 87.041 | 87.0401 | $C_4H_6O_2H^+$ | 0.23a | 0.54b | 0.23a | 0.63b | - | 1.37 | 2,3-butanedione/γ-butyrolactone |
| 87.080 | 87.0809 | $C_5H_{10}OH^+$ | 68.20a | 19.14b | 81.48a | 21.32b | 250.23a | 84.30b | 2-pentanone/pentanal/ 2/3-methylbutanal |
| 89.060 | 89.0603 | $C_4H_8O_2H^+$ | 4.40a | 4.64a | 9.43a | 7.32a | 36.30a | 36.03a | Butanoic acid/ acetoin |
| 91.022 | 91.0217 | $C_3H_6OSH^+$ | 0.34a | 0.10b | 0.36a | 0.06b | 0.81a | 0.13b | Methylthioacetate/mercaptoacetone |
| 97.101 | 97.1012 | $C_7H_{13}$ | 2.41a | 0.86b | 2.04 | 0.90 | 6.23a | 3.00b | Heptanal fragment |
| 99.081 | 99.0810 | $C_6H_{10}OH^+$ | 0.50a | 0.31b | 0.27 | 0.30 | 0.48a | 0.62b | 2-Hexanal |
| 101.096 | 101.0966 | $C_6H_{12}OH^+$ | 1.88a | 0.73b | 1.79a | 0.72b | 4.80a | 2.37b | Hexanal |
| 103.075 | 103.0759 | $C_5H_{10}O_2H^+$ | 0.19 | 0.22 | 0.25 | 0.26 | 0.36a | 0.66b | Propanoic Acid /pentanoic acid |
| 105.071 | 105.0704 | $C_8H_8H^+$ | 0.11 | 0.14 | 0.24 | 0.24 | 0.21a | 0.36b | Styrene |
| 107.085 | 107.0497 | $C_7H_6OH^+$ | 0.28a | 0.57b | 0.45 | 0.42 | 0.42a | 1.24b | Benzaldehyde/ 1,3 dimethylbenzene |
| 109.027 | | | 0.37 | 0.35 | 0.16 | 0.18 | 0.17a | 0.26b | |
| 115.112 | 115.1117 | $C_7H_{14}OH^+$ | 28.21a | 7.93b | 22.70 | 7.84 | 67.14a | 28.65b | 2-heptanone |
| 137.134 | 137.1330 | $C_{10}H_{16}H^+$ | 0.20a | 0.39b | 0.57 | 0.75 | 0.27a | 0.90b | Mix of mononoterpenes |
| 143.145 | 143.1432 | $C_9H_{18}OH^+$ | 1.19a | 0.46b | 0.79 | 0.82 | 1.81 | 1..96 | 2-nonanone |

### 2.3.4 | Effect of thermal treatment on the PTR-MS profile: oxidation effects

For industrial production, not only the long storage at 4°C is relevant but also the one at 50°C since AMF is melted and kept at this temperature for days before the actual use of the product. For this reason the samples evaluated at 120, 180 and 240 after storage at 4°C have been evaluated also during a storage of 11 days at 50°C where oxidation effects due to heat can be observed. For further evaluation of the data, the single cold storage time points were evaluated by focusing on the effect of the storage at 50°C only. Similar trends and similar differences in concentrations were observed at all time points but in this work, as explanatory example only the data obtained at 240 days are reported. The score plots corresponding to PC1 vs PC2 for day 2, 4, 7 and 11 are shown in Figure 2.4. PCA was performed on the centred and scaled data set.

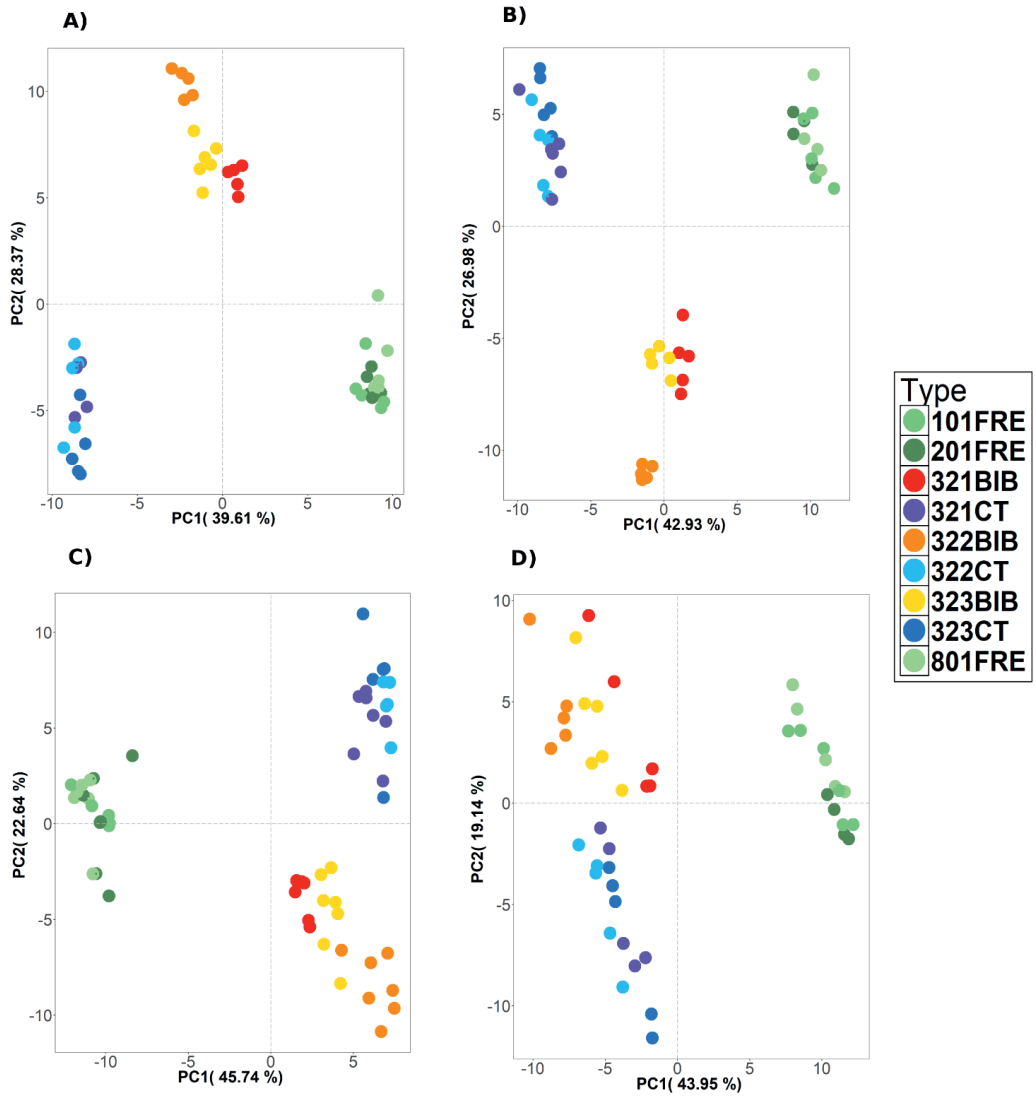



***Figure 2.4:*** *Score plots of PCA analysis for storage at 50°C at time point 240 on scaled and mean centred data sets. From top left to bottom right: day 2 (Fig. 4A), 4 (Fig. 4B), 7 (Fig. 4C) and 11 (Fig. 4D).*

In all cases the first principal component, explaining around 40% of the total variability in the data, separated the packed AMF samples from the standard ones. Especially at the beginning of the storage at 50°C, the second component, explaining around 25% of the total variance, separates the BIB from the CT samples. While the storage proceeds, the differences between the two types of packaging decreases. While at 240 days samples are still distinguishable even after 11 days of storage at 50°C, at 120 and 180 days after the first week of storage at 50°C the BIB and CT separation on the PCA score plots starts to be less clear (data not shown). This effect may be explained by the fact that during storage at 50°C AMF samples are

exposed both to light and oxygen during sample preparation and then to heating for the whole experiment. Oxygen, light and temperature are all factors that greatly affect and increase lipid oxidation processes (37). Therefore, the oxidation process in the vials may probably take over the initial VOC profile differences produced by the different packaging type. The same trend during storage at 50°C where differences between the BIB and CT samples and even the FREs are reduced, was identified when univariate analysis (1-way ANOVA, with Tukey HSD post-hoc) was performed. Results can be found in the supplementary materials (Table S2.1).

A total of 46 compounds for the BIB samples and 60 for CT ones, were found to have a significant difference in concentration at least on one of the days of the shelf life at 50°C. During the thermal treatment, for the BIB samples, a total of 25 mass peaks (54%) increased and 21 decreased (46%) their concentration while for CT samples 34 increased (57%) and 26 decreased (43%). Trends were the same in the two types of packaging for the same mass peaks. In Figure 2.5 it is possible to see some examples of these trends during storage at 50°C: four different mass peaks were selected for their importance as key butter aroma compound or in AMF quality.

Data of Figure 2.5 also indicated that at the end of the storage, the differences between the two packaging tend to decrease. This trend may be a consequence of oxidation processes induced predominantly by the exposure to heat (50°C) but also by light and air exposition during the experiment (38).

The mass peaks 93.070 (Figure 2.5a) and 89.060 (Figure 2.5c) showed a decreasing trend during the thermal treatment. The first one was tentatively identified as toluene. Toluene is an aromatic hydrocarbon highly soluble in fat. It has been previously observed by using PTR-MS in Parmigiano Cheese by Boscaini *et al* (39) and by using GC-MS by Lozano *et al* (16) in sweet cream butter when evaluating the effect of cold storage and different type of packaging on the major butter VOCs. Aromatic hydrocarbons (benzene, ethylbenzene, toluene, styrene) are considered major environmental contaminants because of the ubiquity of the sources of their emission and their harmful effects on man and animals (40). Thanks to their volatility and lipophilic nature, from the environment these compounds can penetrate food in different ways including direct absorption into lipids. Toluene and styrene can easily diffuse though a fibrous or plastic materials migrating into the food matrix and affecting food flavour (41). Standard samples at 240 days had the lowest levels of toluene probably due to the storage in glass jars while CT samples present higher concentrations than BIB samples. The same behaviour is observed for m/z = 105.072 and m/z = 107.086 corresponding to styrene and ethylbenzene respectively (data not shown) and this is true also for measurements at 120 and 180 days. Wrapping plastic around the AMF samples stored in cardboard (CT) allows a greater aromatic hydrocarbon migration in comparison to the plastic of the BIB package

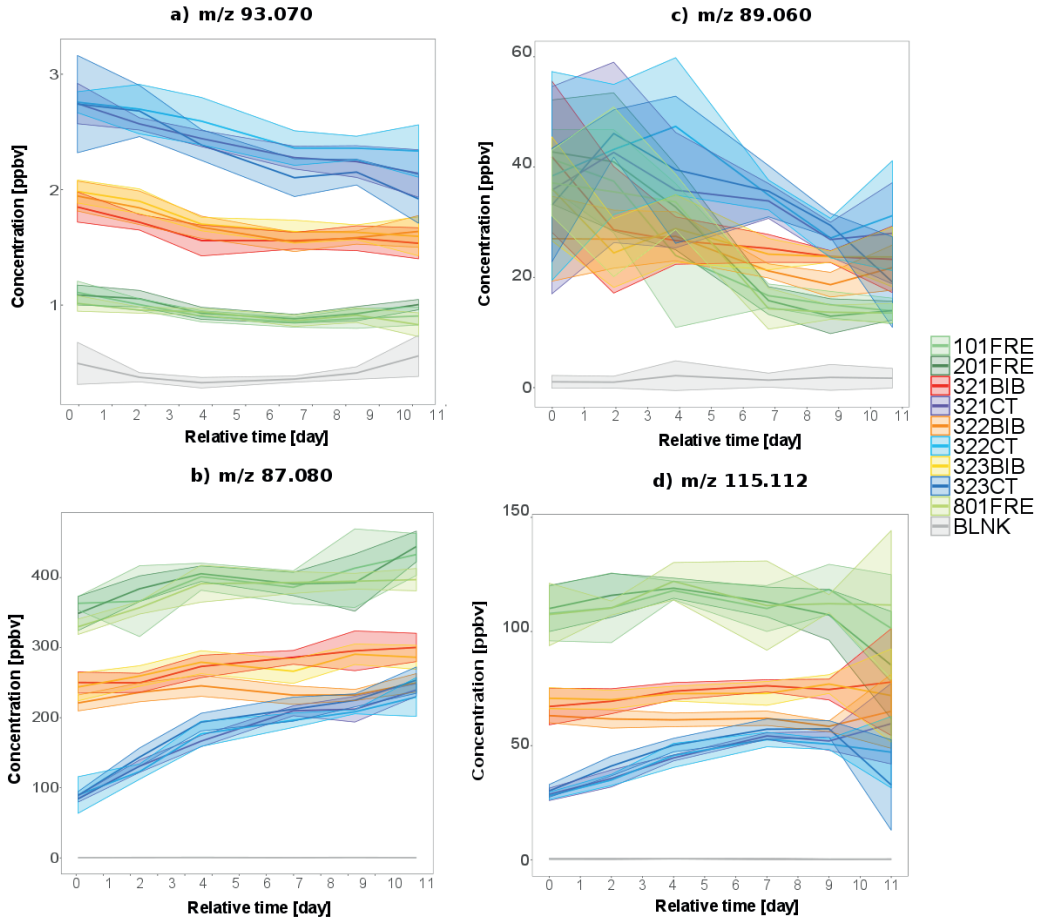



***Figure 2.5:*** *Time evolution (day 0 - day11) during storage at 50°C of the concentration of selected mass peaks: a) m/z = 93.070 ($C_7H_9^+$), T.ID. toluene, b) m/z = 89.060 ($C_4H_8O_2H^+$), T.ID. butanoic acid and/or acetoin, c) m/z = 87.080 ($C_5H_{10}OH^+$), T.ID. 2-pentanone, pentanal and/or 2/3-methylbutanal, d) m/z = 115.112 ($C_7H_{14}OH^+$), T.ID. 2-heptanone for all investigated samples at time point 240 days.*

Oxidation may explain partially the decreasing trend observed for the mass peak 89.060 corresponding to a mixture of VOCs, presumably butanoic acid and acetoin. These two compounds were both detected by GC-MS analysis in the volatile fraction of both butter (42) and butter oil (18). Butanoic acid is a short chain fatty acid that was found as one of key butter aroma compound through aroma extract dilution analysis (43) while acetoin is responsible for characteristic fatty butter flavour (44). Moreover, one of the fragments of ethyl butyrate, may also contribute to the mass peak signal (12, 26). As anticipated, the decreasing trend observed for the mass peak 89.060 may be due to oxidation accelerated by the thermal treatment: acetoin is a highly reactive compound that can undergo both oxidation to produce diacetyl (45) and esterification with acids to produce acetoin fatty acid esters (46).

The other two mass peaks presented in Figure 2.5c and 2.5d corresponding to the mass peaks *m/z* = 87.080 and *m/z* = 115.112, showing both an increasing trend in concentration during the thermal treatment. Both mass peaks showed a higher level for the samples stored in the BIB package when compared to CT. As said before, the signal of the mass peak 87.080 can be constituted by different compounds such as 2-pentanone and two different aldehydes: pentanal and 2/3-methylbutanal. All these compounds have been classified as odour compounds of butter aroma (6). Some previous studies are in line with the increasing trends detected during the thermal treatment. After 6-8 weeks of butter storage at 6°C, Mallia *et al.* (46) have found increasing levels of all these compounds through GC-MS and GC-O analysis. In a study on the effect of cold storage and packaging material on sweet cream butter (9), both 2 and 3-methylbutanal were found to increase in the first 6 months of storage at 4°C. The same was found for 2-heptanone, corresponding to the *m/z* = 115.112. This methyl ketone molecule has been identified has one of the most potent odour-active compounds in dairy (fruity, fatty flavour) (47). This compound has been identified also by GC-MS analysis by Peterson *et al*. in the headspace of heated butter (48). Moreover, the same increasing trend in concentration was observed when monitoring with PTR-MS changes induced in the headspace of bovine milk after light exposure (35). The increasing in concentration during the heat treatment may be determined by the saturated fatty acids ß-oxidation followed by decarboxylation into 2-heptanone.

## 2.4 Conclusion

In this work, PTR-ToF-MS coupled to a multipurpose autosampler was applied to verify the reliability and robustness of this rapid and non-invasive VOCs analysis as quality control of anhydrous milk fat used in food industry. AMF has been evaluated during storage at different temperatures and in different industrial packaging types. For the first time, performance of bag in-box and cardboard boxes for AMF was compared during a long-term storage (240 days) at refrigerated state (4°C) followed by 11 days storage at 50°C. Industrial sample-sets were used in this investigation. Working with these datasets had the drawback to be more challenging since both replication and standardization were more complicated. However, it had the advantage to provide a realistic representation of the industrial variability and it is a critical point to successfully apply the PTR-MS technique in the day-to-day industry applications. Statistical analysis successfully discriminated different samples, indicating the possibility to identify the effect of storage time, temperature and packaging. Moreover, the differences between fresh products and production batches were also highlighted. VOCs differences induced by different packages were also investigated and refrigerated storage increased these differences. On the contrary, differences between packaging type and production batch decreased during the storage at 50°C due to heat (photo)oxidation that in general leads to more elevated intensities of aldehydes and ketones. When performing univariate analysis on the selected mass ions it is possible to detect and follow changes in different key butter aroma compounds.

From this work the bag in box packaging was shown to have the best performance when compared to cardboard packaging: due to its lower permeability to oxygen the bag in box can offer a better protection to oxidation phenomena. Moreover, from the research it is recommended that food industries, during production should keep AMF at 50°C as short as possible due to the relevant oxidation effect induced by temperature.

The proposed methodology is a direct, rapid and reliable way for automated and standardized quality control of AMF ingredient by being able to identify the effect of all production variables: production batches, type of packaging, and storage at different temperatures and times. In general, our work demonstrates the feasibility and efficiency of rapid and non-invasive mass spectrometry analysis for quality control in agroindustry, but we envisage the possible usage of our methodology for the efficient investigation of fats oxidation. Future research in this field should be devoted to the acceptability thresholds based on sensory analysis that should be included in classification methods together with threshold values of quality routine analysis of AMF such as Rancimat, and number of peroxides.

## 2.5 References


[1] B. M. Mehta, "Ragi (Eleusine coracana L.) - a natural antioxidant for ghee (butter oil)," Int. J. Food Sci. Technol., vol. 41, pp. 86–89, 2006.

[2] D. Puravankara, V. Boghra, and R. S. Sharma, "Effect of antioxidant principles isolated from mango (Mangifera indica L) seed kernels on oxidative stability of buffalo ghee (butter-fat)," J. Sci. Food Agric., vol. 80, no. 4, pp. 522–526, 2000.

[3] R. N. Reece, "Quality Assurance Perspective of Sensory Evaluation," Food Technol., vol. 33, no. 9, p. 37, 1979.

[4] G. Bylung, Dairy processing handbook. Tetra Pak Processing Systems AB S-21 86 Lund, Sweden, 1995.

[5] C. Nerin, L. Tovar, and J. Salafranca, "Behaviour of a new antioxidant active film versus oxidizable model compounds," J. Food Eng., vol. 84, no. 2, pp. 313–320, 2008.

[6] E. Valero, M. Villamiel, B. Miralles, J. Sanz, and I. Martinez-Castro, "Changes in flavour and volatile components during storage of whole and skimmed UHT milk," Food Chem., vol. 72, no. 1, pp. 51–58, 2001.

[7] A. M. Munoz, "Sensory evaluation in quality control: an overview, new developments and future opportunities," Food Qual. Prefer., vol. 13, no. 6, pp. 329–339, 2002.

[8] F. Biasioli, C. Yeretzian, F. Gasperi, and T. D. Mark, "PTR-MS monitoring of VOCs and BVOCs in food science and technology," Trac-Trends Anal. Chem., vol. 30, no. 7, pp. 968–977, 2011.

[9] H. Maarse, C. A. Visscher, L. C. Willemsens, and M. H. Boelens, Volatile compounds in food : qualitative and quantitative data, 6th ed. Zeist: TNO-CIVO, 1989.

[10] W. Lindinger, A. Hansel, and A. Jordan, "On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) - Medical applications, food control and environmental research," Int. J. Mass Spectrom., vol. 173, no. 3, pp. 191–241, 1998.

[11] A. Jordan et al., "A high resolution and high sensitivity proton-transfer-reaction time-of-flight mass spectrometer (PTR-TOF-MS)," Int. J. Mass Spectrom., vol. 286, no. 2–3, pp. 122–128, 2009.

[12] S. M. Van Ruth et al., "Butter and butter oil classification by PTR-MS," Eur. Food Res. Technol., vol. 227, no. 1, pp. 307–317, 2008.

[13] M. Macatelli, W. Akkermans, A. Koot, M. Buchgraber, A. Paterson, and S. van Ruth, "Verification of the geographical origin of European butters using PTR-MS," J. Food Compos. Anal., vol. 22, no. 2, pp. 169–175, 2009.

[14] S. Makhoul et al., "Rapid non-invasive quality control of semi-finished products for the food industry by direct injection mass spectrometry headspace analysis: the case of milk powder, whey powder and anhydrous milk fat," J. Mass Spectrom., vol. 51, no. 9, pp. 782–791, 2016.

[15] A. J. Krause, R. E. Miracle, T. H. Sanders, L. L. Dean, and M. A. Drake, "The effect of refrigerated and frozen storage on butter flavour and texture," J. Dairy Sci., vol. 91, no. 2, pp. 455–465, 2008.

[16] P. R. Lozano, E. R. Miracle, A. J. Krause, M. Drake, and K. R. Cadwallader, "Effect of cold storage and packaging material on the major aroma components of sweet cream butter," J. Agric. Food Chem., vol. 55, no. 19, pp. 7840–7846, 2007.

[17] N. Tomlinson and B. D. Dixon, "Comparison of Wrapping Materials for Export Butter," Aust. J. Dairy Technol., vol. 32, no. 2, pp. 70–71, 1977.

[18] S. Widder, A. Sen, and W. Grosch, "Changes in the Flavour of Butter Oil during Storage - Identification of Potent Odorants," Zeitschrift Fur Leb. Und-forsch., vol. 193, no. 1, pp. 32–35, 1991.

[19] L. H. Skibsted, J. Risbo, and M. L. Andersen, Chemical deterioration and physical instability Edited by, no. 186.

[20] S. Yener et al., "PTR-ToF-MS characterisation of roasted coffees (C-arabica) from different geographic origins," J. Mass Spectrom., vol. 49, no. 9, pp. 929–935, 2014.

[21] L. Cappellin et al., "Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS," Int. J. Mass Spectrom., vol. 290, no. 1, pp. 60–63, 2010.

[22] L. Cappellin et al., "On data analysis in PTR-TOF-MS: From raw spectra to data mining," Sensors and Actuators B-Chemical, vol. 155, no. 1, pp. 183–190, 2011.

[23] L. Cappellin et al., "On Quantitative Determination of Volatile Organic Compound Concentrations Using Proton Transfer Reaction Time-of-Flight Mass Spectrometry," Environ. Sci. Technol., vol. 46, no. 4, pp. 2283–2290, 2012.

[24] R Core Team, "R Development Core Team," R: A Language and Environment for Statistical Computing, vol. 55. pp. 275–286, 2016.

[25] D. G. Peterson and G. A. Reineccius, "Characterization of the volatile compounds that constitute fresh sweet cream butter aroma," Flavour Fragr. J., vol. 18, no. 3, pp. 215–220, 2003.

[26] S. Mallia, F. Escher, and H. Schlichtherle-Cerny, "Aroma-active compounds of butter: A review," Eur. Food Res. Technol., vol. 226, no. 3, pp. 315–325, 2008.

[27] K. Buhr, S. van Ruth, and C. Delahunty, "Analysis of volatile flavour compounds by Proton Transfer Reaction-Mass Spectrometry," Int. J. Mass Spectrom., vol. 221, pp. 1–7, 2002.

[28] G. Contarini, M. Povolo, R. Leardi, and P. M. Toppino, "Influence of heat treatment on the volatile compounds of milk," J. Agric. Food Chem., vol. 45, no. 8, pp. 3171–3177, 1997.

[29] S. Panseri, S. Soncin, L. M. Chiesa, and P. A. Biondi, "A headspace solid-phase microextraction gas-chromatographic mass-spectrometric method (HS-SPME-GC/MS) to quantify hexanal in butter during storage as marker of lipid oxidation," Food Chem., vol. 127, no. 2, pp. 886–889, 2011.

[30] G. Garcia-Llatas, M. J. Lagarda, F. Romero, P. Abellan, and R. Farre, "A headspace solid-phase microextraction method of use in monitoring hexanal and pentane during storage: Application to liquid infant foods and powdered infant formulas," Food Chem., vol. 101, no. 3, pp. 1078–1086, 2007.

[31] F. Correddu, A. Nudda, M. G. Manca, G. Pulina, and T. K. Dalsgaard, "Light-Induced Lipid Oxidation in Sheep Milk: Effects of Dietary Grape Seed and Linseed, Alone or in Combination, on Milk Oxidative Stability," J. Agric. Food Chem., vol. 63, no. 15, pp. 3980–3986, 2015.

[32] M. Asaduzzaman, F. Biasioli, M. S. Cosio, and M. Schampicchio, "Hexanal as biomarker for milk oxidative stress induced by copper ions," J. Dairy Sci., vol. 100, no. 3, pp. 1650–1656, 2017.

[33] T. Wainwright, J. F. McMahon, and J. McDowell, "Formation of methional and methanethiol from methionine," J. Sci. Food Agric., vol. 23, no. 7, pp. 911–914, 1972.

[34] S. Mallia, B. Guggenbühl, S. Frapolli, B. Beisert, and D. Rauhut, "Chapter 63 – Sulfur-Containing Compounds in Butter and their Influence on Butter Aroma," in Flavour Science, 2014, pp. 337–342.

[35] J. Beauchamp, E. Zardin, P. Silcock, and P. J. Bremer, "Monitoring photooxidation-induced dynamic changes in the volatile composition of extended shelf life bovine milk by PTR-MS," J. Mass Spectrom., vol. 49, no. 9, pp. 952–958, 2014.

[36] B. C. Weimer, "Improving the flavour of cheese," Sci. Technol., p. 601, 2007.

[37] T. P. Labuza and L. R. Dugan, "Kinetics of lipid oxidation in foods," C R C Crit. Rev. Food Technol., vol. 2, no. 3, pp. 355–405, 1971.

[38] J. O. Ragnarsson and T. P. Labuza, "Accelerated shelf-life testing for oxidative rancidity in foods-A review," Food Chem., vol. 2, no. 4, pp. 291–308, 1977.

[39] E. Boscaini, S. Van Ruth, G. Biasioli, F. Gasperi, and T. D. Mark, "Gas chromatography-olfactometry (GC-O) and proton transfer reaction-mass spectrometry (PTR-MS) analysis of the flavour profile of Grana Padano, Parmigiano Reggiano, and Grana Trentino cheeses," J. Agric. Food Chem., vol. 51, no. 7, pp. 1782–1790, 2003.

[40] F. Fabietti, M. Delise, and A. P. Bocca, "Aromatic hydrocarbon residues in milk: preliminary investigation," Food Control, vol. 11, no. 4, pp. 313–317, 2000.

[41] I. Seo and H. S. Shin, "Determination of Toluene and Other Residual Solvents in Various Food Packaging Materials by Gas Chromatography/Mass Spectrometry (GC/MS)," Food Sci. Biotechnol., vol. 19, no. 6, pp. 1429–1434, 2010.

[42] M. Povolo and G. Contarini, "Comparison of solid-phase microextraction and purge-and-trap methods for the analysis of the volatile fraction of butter," J. Chromatogr. A, vol. 985, no. 1–2, pp. 117–125, 2003.

[43] P. Schieberle, K. Gassenmeier, H. Guth, A. Sen, and W. Grosch, "Character Impact Odor Compounds of Different Kinds of Butter," Food Sci. Technol. Technol., vol. 26, no. 4, pp. 347–356, 1993.

[44] Z. J. Xiao and J. R. Lu, "Generation of Acetoin and Its Derivatives in Foods," J. Agric. Food Chem., vol. 62, no. 28, pp. 6487–6497, 2014.

[45] P. L. H. McSweeney and M. J. Sousa, "Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review," Lait, vol. 80, no. 3, pp. 293–324, 2000.

[46] S. Malfia, P. Piccinali, B. Refiberger, R. Badertscher, F. Escher, and H. Schlichtherle-Cerny, "Determination of storage stability of butter enriched with unsaturated fatty acids/conjugated linoleic acids (UFA/CLA) using instrumental and sensory methods," Int. Dairy J., vol. 18, no. 10–11, pp. 983–993, 2008.

[47] L. M. L. Nollet and F. Toldrà, Handbook of Dairy Food Analysis, 1st ed. CRC Press Taylor & Francis Group, 2010.

[48] D. G. Peterson and G. A. Reineccius, "Determination of the aroma impact compounds in heated sweet cream butter," Flavour Fragr. J., vol. 18, no. 4, pp. 320–324, 2003.

## 2.6 Supplementary materials:

All peak masses found significantly different in the BIB category (p-value < 0.01 with Bonferroni correction = **0.002**) were reported by starting from m/z > 40 and by taking a concentration threshold of at least 0.5 ppbV. This time the three batches (biological replicates) were considered all together (mean) and only the BIB category is shown.

**Table S2.1:** VOCs present in the headspace of BIB anhydrous milk fat (AMF) sample during the thermal treatment at 240 days with average concentrations and standard deviation.

| MS | TREND | BIB | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 7 | 9 | 11 |
| ms41.038 | ↑ | 51.41a±2.89 | 56.11a±3.16 | 62.43b±5.33 | 63.80b±4.83 | 75.27c±6.06 | 77.17c±5.91 |
| ms42.010 | ↑ | 0.65ab±0.06 | 0.64a±0.06 | 0.69bc±0.07 | 0.70ac±0.08 | 0.73cd±0.07 | 0.78d±0.07 |
| ms42.042 | ↑ | 0.66a±0.42 | 2.55de±0.84 | 1.60bc±0.40 | 1.40b±0.59 | 3.13e±0.56 | 1.96cd±0.51 |
| ms43.018 | ↑ | 102.34a±10.62 | 106.61ab±9.78 | 120.66b±12.17 | 119.84b±13.84 | 137.20c±16.73 | 133.90c±14.14 |
| ms43.054 | ↓ | 20.36b±2.97 | 19.28b±2.08 | 20.07b±4.56 | 16.48a±1.06 | 16.09a±1.24 | 16.76a±1.45 |
| ms45.033 | ↑ | 332.72a±15.70 | 367.86ab±31.15 | 400.75b±30.45 | 405.03b±41.67 | 473.83c±48.91 | 476.58c±42.60 |
| ms47.024 | ↓ | 3.41c±0.40 | 3.20c±0.39 | 2.53b±0.18 | 2.04a±0.16 | 2.48b±0.22 | 1.99a±0.21 |
| ms49.011 | ↑ | 0.96a±0.12 | 1.52b±0.33 | 2.27c±0.31 | 2.82d±0.37 | 4.22e±0.41 | 5.24f±0.57 |
| ms51.043 | ↓ | 15.54d±3.50 | 10.34c±3.90 | 8.70bc±2.94 | 5.64ab±2.09 | 5.55ab±2.33 | 4.49a±1.81 |
| ms53.002 | ↑ | 0.72a±0.06 | 0.78ab±0.08 | 0.90c±0.10 | 0.91bc±0.11 | 1.04cd±0.14 | 1.07d±0.12 |
| ms53.039 | ↑ | 1.75a±0.14 | 1.92ab±0.13 | 2.10bc±0.07 | 2.15c±0.13 | 2.73d±0.17 | 2.74d±0.31 |
| ms55.054 | ↑ | 38.74a±2.00 | 41.20ab±2.05 | 43.91b±2.54 | 44.12b±3.34 | 50.19c±4.01 | 49.98c±6.23 |
| ms57.033 | ↑ | 2.42a±0.44 | 4.55a±0.81 | 7.81b±1.21 | 12.92c±2.17 | 18.27d±2.64 | 22.18e±2.33 |
| ms57.070 | ↓ | 9.80b±0.96 | 9.38b±0.79 | 9.09b±0.76 | 7.64a±0.75 | 7.40a±0.64 | 7.46a±0.62 |
| ms58.040 | ↑ | 2.74a±0.12 | 3.04a±0.23 | 3.61b±0.31 | 4.16c±0.39 | 4.56d±0.43 | 4.69d±0.49 |
| ms60.052 | ↑ | 181.85a±14.41 | 194.73ab±19.45 | 221.43bcd±23.92 | 217.01ac±28.19 | 242.69cd±34.22 | 240.07d±28.54 |
| ms61.028 | ↑ | 50.33ab±9.93 | 48.95a±7.04 | 54.62ac±6.35 | 55.27ac±5.35 | 59.67bc±5.44 | 59.52c±5.70 |
| ms63.027 | ↓ | 69.98c±4.68 | 65.29b±4.58 | 62.06b±4.25 | 52.84a±4.79 | 53.48a±5.52 | 50.71a±4.08 |
| ms65.022 | ↓ | 3.71c±0.17 | 3.46bc±0.34 | 3.36b±0.26 | 2.76a±0.32 | 2.96a±0.28 | 2.73a±0.26 |
| ms65.058 | ↓ | 0.86d±0.40 | 0.70cd±0.27 | 0.65bc±0.18 | 0.38ab±0.14 | 0.42ab±0.14 | 0.38a±0.13 |
| ms67.054 | ↑ | 1.25a±0.04 | 1.28a±0.07 | 1.36a±0.09 | 1.30a±0.12 | 1.53b±0.14 | 1.60c±0.11 |
| ms69.070 | ↑ | 20.64a±1.26 | 21.40ab±1.37 | 23.45bcd±1.85 | 22.72ac±2.09 | 24.44cd±2.43 | 25.67d±1.90 |
| ms71.090 | ↓ | 4.33b±0.34 | 4.13b±0.34 | 4.05b±0.27 | 3.58a±0.33 | 3.54a±0.32 | 3.53a±0.29 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ms73.027 | ↑ | 1.09a±0.22 | 2.68a±0.68 | 5.22b±0.81 | 10.10c±1.73 | 15.74d±2.31 | 18.17e±2.61 |
| ms77.058 | ↓ | 3.22c±0.82 | 2.71bc±0.56 | 2.42ac±0.51 | 1.73a±0.39 | 2.08ab±0.49 | 1.74a±0.34 |
| ms82.949 | ↓ | 2.56c±0.14 | 2.52c±0.14 | 2.41bc±0.10 | 2.23a±0.12 | 2.29ab±0.13 | 2.28ab±0.13 |
| ms83.049 | ↓ | 0.67d±0.07 | 0.52c±0.04 | 0.49ab±0.03 | 0.46a±0.03 | 0.49ac±0.04 | 0.51bc±0.06 |
| ms83.086 | ↑ | 3.82a±0.42 | 3.76a±0.30 | 3.94a±0.36 | 3.88a±0.42 | 4.17a±0.42 | 4.57b±0.45 |
| ms84.942 | ↓ | 1.65c±0.09 | 1.61c±0.08 | 1.58bc±0.10 | 1.46a±0.07 | 1.48ab±0.08 | 1.47a±0.08 |
| ms85.065 | ↑ | 1.12a±0.11 | 1.13a±0.09 | 1.21ab±0.10 | 1.37b±0.13 | 1.46c±0.12 | 1.57c±0.19 |
| ms85.100 | ↓ | 0.68c±0.05 | 0.61b±0.05 | 0.61b±0.05 | 0.53a±0.05 | 0.52a±0.04 | 0.52a±0.06 |
| ms86.071 | ↑ | 0.53a±0.05 | 0.54ab±0.04 | 0.58ac±0.05 | 0.60bc±0.06 | 0.63cd±0.08 | 0.66d±0.07 |
| ms87.041 | ↑ | 0.00a±0.02 | 0.00a±0.02 | 0.07a±0.17 | 0.14a±0.26 | 0.83b±0.29 | 1.19c±0.38 |
| ms87.080 | ↑ | 239.40a±16.46 | 248.04ab±18.26 | 268.38ac±24.64 | 262.28ac±26.50 | 278.57bc±31.95 | 280.91c±24.92 |
| ms89.0560 | ↓ | 35.36d±11.02 | 27.65cd±8.21 | 28.32bd±5.44 | 23.37abc±2.89 | 23.58ab±2.67 | 23.43a±4.80 |
| ms91.022 | ↓ | 0.80b±0.06 | 0.77b±0.07 | 0.73b±0.06 | 0.63a±0.07 | 0.63a±0.07 | 0.58a±0.06 |
| ms91.057 | ↓ | 1.15bc±0.31 | 1.25c±0.79 | 1.03ab±0.20 | 0.66a±0.12 | 0.69a±0.10 | 0.69a±0.09 |
| ms93.036 | ↓ | 4.98c±1.21 | 3.74bc±0.90 | 3.02b±0.64 | 2.03a±0.34 | 1.93a±0.34 | 1.73a±0.33 |
| ms93.069 | ↓ | 1.90b±0.12 | 1.81b±0.12 | 1.67a±0.10 | 1.56a±0.09 | 1.62a±0.09 | 1.62a±0.15 |
| ms95.085 | ↓ | 0.55d±0.05 | 0.49c±0.04 | 0.48bc±0.03 | 0.44a±0.03 | 0.48bc±0.03 | 0.45ab±0.04 |
| ms97.063 | ↑ | 0.30a±0.03 | 0.29a±0.03 | 0.32ab±0.04 | 0.41b±0.09 | 0.54c±0.13 | 0.85d±0.18 |
| ms99.081 | ↓ | 0.47b±0.05 | 0.43ab±0.03 | 0.43ab±0.04 | 0.39a±0.03 | 0.40a±0.04 | 0.41a±0.03 |
| ms101.059 | ↓ | 1.13c±0.23 | 0.76b±0.11 | 0.64ab±0.10 | 0.54a±0.09 | 0.64ab±0.08 | 0.70b±0.08 |
| ms103.075 | ↑ | 0.38a±0.06 | 0.44ab±0.03 | 0.47ab±0.04 | 0.46ab±0.05 | 0.48bc±0.06 | 0.51c±0.10 |
| ms107.085 | | 0.43b±0.07 | 0.41b±0.06 | 0.33a±0.04 | 0.40b±0.05 | 0.45b±0.06 | 0.45b±0.06 |
| ms137.134 | | 1.52c±0.40 | 1.54ac±0.29 | 1.54bc±0.25 | 1.10a±0.20 | 1.13ab±0.15 | 1.32bc±0.71 |

3

# The Good, the Bad and the Aged: predicting sensory quality of anhydrous milk fat by PTR/SRI-ToF-MS analysis and data mining

Pedrotti M., Khomenko I., Cappellin L., Fontana M., Somenzi M., Falchero L., Arveda M., Fogliano V., Biasioli F.

*International Dairy Journal 2020, 109*

*DOI:* 10.1016/j.idairyj.2020.104729

## Abstract

Due to its versatility, anhydrous milk fat (AMF) has become more popular as a food industry ingredient but its quality control remains a critical challenge. The aim of this study was to apply a direct injection mass spectrometry technique to predict sensory quality of AMF. Volatilome analysis through proton transfer reaction mass spectrometry (PTR-MS) was used to classify 39 industrial samples of AMF according to industrial sensory evaluation and to accelerated ageing. A selective reagent ionization system was used to evaluate the suitability of PTR-MS alternative ionization modes for quality control. Supervised multivariate data analysis successfully classified samples and showed that samples exposed to accelerated shelf life at 50°C presented higher intensities of most volatiles, especially for the ones derived from oxidation like aldehydes and ketones. Samples with an acceptable quality level had lower emissions of volatile compounds. PTR-MS technique is ideal to support agroindustry sensory quality programs requiring rapid on-line analytical information.

**Keywords:** anhydrous milk fat, quality control, volatilome, proton transfer reaction mass spectrometry.

## 3.1 Introduction

The global market importance of anhydrous milk fat (AMF) as a food ingredient is continuously increasing due to its high stability and longer shelf life compared with butter, mainly due to its low moisture content (0.2%). For its easy use in liquid form (above 36°C), the efficient mixing with various other dairy products and its unique sensory properties, AMF is widely used in confectionary, chocolate and ice cream manufacturing industries (1,2).

AMF may differ in quality, functionality and economic value due to i) milk quality (e.g. seasonality and origin), ii) manufacturing processes and iii) duration and conditions of storage. Different instrumental techniques have been developed to characterize physical and chemical properties of butter and AMF such as iodine value, saponification number, moisture content, cis-trans fatty acids ratio, peroxide value, anisidine value, acid value and thiobarbituric acid number (3–5). Among them, peroxide and acid values are commonly used to describe AMF quality because they provide an indication of primary oxidation and lipolysis respectively, which are the two most significant deteriorative reactions occurring during storage (3,6). These analytical methods, despite being simple and relatively inexpensive, are invasive and require potentially hazardous solvents (3). Moreover, they mainly give an indication of product oxidation and therefore about storage and manufacturing conditions. Little information is provided about secondary metabolites such as alcohols, sulphur compounds and aldehydes that could strongly affect AMF sensory characteristics and therefore consumer acceptance of the final products (7). For this reason, developments of flexible, fast, and reliable methodologies to monitor the quality focused on flavour and aroma are needed (8,9). Due to their key role in food perceived quality, volatile organic compounds (VOCs) are of primary interest in the context of monitoring and predicting product quality in relation to sensory perception (Biasioli, Yeretzian, Gasperi, & Mark, 2011).

Proton transfer reaction mass spectrometry (PTR-MS) is a direct injection mass spectrometry method that enables volatile analysis with high sensitivity and in real time (Blake, Monks, & Ellis, 2009; Lindinger, Hansel, & Jordan, 1998). This technique was applied in the past by van Ruth *et al.* (2008) for classifying butter and butter oil samples subjected to heat and off-flavour treatment. The same group also proposed the approach for discriminating the geographical origin of European butters (14). Recently, our group used a similar instrument equipped with a Time of- Flight (TOF) mass analyser (Blake, Whyte, Hughes, Ellis, & Monks, 2004; Jordan et al., 2009) to analyse the volatilome of semi-finished dairy ingredients (17) and to detect differences in VOCs release from AMF during storage in different packaging by following changes during the oxidation processes caused by storage conditions at 50°C (18). In this study, we introduce two novel factors: the sensory aspect, where VOC emissions were used to predict quality classes of AMF based on industrial sensory evaluation (quality control) and the usage of a selective reagent ionization system (SRI). The SRI system allows for the use of different precursor ions ($H_3O^+$, $NO^+$, $O_2^+$, $Kr^+$ and $Xe^+$) for ionization and

therefore extends the number of compounds that may be detected, *e.g.* to short chain alkanes, and improves the specificity, *e.g.* separating isobaric compounds as ketones and aldehydes (11,15,19). The SRI-ToF-MS system has been applied to a variety of purposes such as identification of monoterpenes (20), and identification of new drugs (21) and, in the food area, the technique was successfully applied to improve discrimination of coffee from different origins (Yener et al., 2015).

In this research, we study a fingerprinting approach based on untargeted PTR-ToF-MS volatilomics to predict different sensory classes of AMF by rapid and non-invasive headspace analysis coupled with data mining methods. Moreover, this study aims to explore if additional information provided by the SRI system can improve performances of predictive models.

## 3.2 Material and Methods

### 3.2.1 | AMF Samples

Sampling consisted of 25 AMF samples produced by five major European manufactures over two years of production (2015 and 2016). All batches were produced from pasteurized cream with 35%–40% fat content. Anhydrous milk fat was produced by applying the standard method described elsewhere (1). Samples were divided in two categories based on sensory evaluation: 16 "YES" samples that were classified as good and 9 "NO" samples that were classified as not complying (see the next section for details). Aliquots of some of the "YES" samples were subjected to storage conditions at 50°C in the dark for 5 days in a thermostatic stove to simulate aging and to create defective samples through thermal oxidation. These samples were called "AGED" (14 samples). Samples were then packed in plastic bags for shipment and transported on ice packs to the analytical laboratory at Fondazione Edmund Mach (San Michele all'Adige, Italy). Upon receipt, products were removed from shipping containers, examined for damage, and assigned to frozen storage (−20 ± 1°C) until analysis.

### 3.2.2 | Sensory analysis:

Sensory evaluation was carried out by 7 to 12 trained judges (26.8% females, age range 30-55 years old) according to the industrial partner internal evaluation protocol based on a 'difference from control' rating (23). Each AMF sample was rated by comparison with a standard. About 200 g of each sample were melted and kept at 50°C in a thermostatic stove and a spoon (around 15 g) of melted AMF was served to each participant. Panellists were instructed to evaluate the odour intensity of the samples first. Then, judges assessed flavour intensity by taking a sip of the sample. Between samples, judges rinsed their mouth with water to remove all fat residues. The difference from the standard reference sample was evaluated on a linear continuous scale from 0 to 5. For each sample average scores from the panel were obtained. Based on this average and by using a cut-off value of 1.5 established by industrial internal standards, AMF scoring

higher than 1.5 were classified as "NO" while AMF scoring lower were classified as "YES". In table 1 a list of the samples is given.

### 3.2.3 | PTR\SRI-ToF -MS analysis

According to industry practice, each sample was melted in a thermal bath (50°C) and fifteen 2.5-mL aliquots of AMF were transferred into sampling vials, which were previously conditioned for 1 day at 65°C. Vials were then closed, labelled and kept at 4°C till analysis. Empty vials were used as blanks and five replicates were measured for each reagent ion. All vials were incubated for equilibration at 50°C for at least 30 minutes before PTR-MS analysis. Each sample was measured for 60 seconds with an acquisition rate of one spectrum per second and a flow rate of 35 sccm. To avoid memory effects, measurement order was randomized and, after each measurement, a waiting time of 3 minutes was set.

All measurements were performed using a multipurpose GC sampler (Gerstel GmbH, Mulheim am Ruhr, Germany) connected to the inlet of the PTR-ToF-MS as previously described (Yener et al., 2014). The inlet line consisted of a PEEK capillary tube (inner diameter, 0.40 mm), heated at 110°C.

A PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) in its standard configuration (V mode) was used. The instrument was equipped with a switchable reagent ion system (SRI) that allowed the operation in $H_3O^+$, $NO^+$ or $O_2^+$ modes as described elsewhere (Cappellin et al., 2014; Sánchez del Pulgar et al., 2013). The current applied for the discharge in the ion source was 3.5 mA in the case of $H_3O^+$ while it was set at 5.0 mA in the case of $NO^+$ and $O_2^+$ modes. Drift conditions were the same for all three ionization conditions: drift voltage = 557 V, drift temperature = 110°C, drift pressure= 2.3 mbar (except for the $NO^+$ that was 2.8 mbar) resulting in an E/N value of 141 Td (136 Td for $NO^+$ mode). Mass resolution (m/Δm) was at least 3800, and data were collected for the mass range m/z 20 to 300.

**Table 3.1:** In the table are presented the AMF samples used in the experiment together with their sensory score, production date, supplier and classification based on the sensory test. Samples which in the sensory test scored higher than the quality control cut-off score of 1.5 were classified as "NO", while the one which scored below 1.5 as "YES". The "AGED" samples were obtained from the "YES" samples after 5 days in a thermostatic stove at 50°C to simulate aging.

| Sample number | Classification | Supplier | Production date | Sensory score |
|---|---|---|---|---|
| **12** | NO | A | 20-03-15 | 3 |
| **18** | NO | A | 26-01-15 | 1.7 |
| **20** | NO | B | 03-06-15 | 3.3 |
| **22** | NO | C | 03-06-15 | 1.5 |
| **A** | NO | ND | ND | 1.6 |
| **B** | NO | ND | ND | 1.6 |
| **C** | NO | ND | ND | 1.7 |
| **D** | NO | ND | ND | 1.6 |
| **1** | NO | ND | ND | 1.0 |
| **2** | YES | ND | ND | 1.5 |
| **3** | YES | ND | ND | 1.1 |
| **46** | YES | A | 31-08-16 | 0.9 |
| **47** | YES | C | 30-10-16 | 0.6 |
| **48** | YES | D | 07-11-16 | 0.9 |
| **49** | YES | A | 07-11-16 | 0.8 |
| **50** | YES | C | 20-12-16 | 0.6 |
| **51** | YES | A | 09-01-17 | 0.9 |
| **45** | YES | A | 14-11-16 | 1.0 |
| **38** | YES | C | 01-08-16 | 0.7 |
| **39** | YES | A | 30-08-16 | 1.1 |
| **40** | YES | A | 06-07-16 | 0.9 |
| **41** | YES | C | 11-07-16 | 0.7 |
| **43** | YES | E | 27-07-16 | 1.0 |
| **52** | YES | D | 30-07-16 | 1.3 |
| **53** | YES | A | 27-02-17 | 0.8 |
| **46-i** | AGED | A | 31-08-16 | 1.5 |
| **47-i** | AGED | C | 30-10-16 | 1.5 |
| **48-i** | AGED | D | 07-11-16 | 1.5 |
| **49-i** | AGED | A | 07-11-16 | 1.6 |
| **50-i** | AGED | C | 20-12-16 | 1.3 |
| **51-i** | AGED | A | 09-01-17 | 1.7 |
| **45-i** | AGED | A | 14-11-16 | 2.2 |
| **38-i** | AGED | C | 01-08-16 | 1.5 |
| **39-i** | AGED | A | 30-08-16 | 1.7 |
| **40-i** | AGED | A | 06-07-16 | 2.5 |
| **41-i** | AGED | C | 11-07-16 | 1.3 |
| **43-i** | AGED | E | 27-07-16 | 1.4 |
| **52-i** | AGED | D | 30-07-16 | 1.5 |
| **53-i** | AGED | A | 27-02-17 | 1.6 |

### 3.2.4 | Data processing

Data processing of ToF spectra included dead time correction, internal calibration of mass spectral data and peak extraction (Cappellin et al., 2010, 2011) to reach a mass accuracy sufficient for determining sum formula of volatile compounds. In this paper, the experimental *m/z* values are reported up to the third decimal place. The m/z axis internal calibration was performed by using *m/z* 21.0221, 29.9974, and 203.9430 corresponding to protonated water, $NO^+$, and one of the fragments of 1,3-Diodobenzene, which was continuously injected as a reference compound through the PerMaSCal device (Ionicon, Innsbruck, Austria). Peak intensities from the absolute mass spectra were converted to concentrations in ppbV (parts per billion by volume) according to the procedure reported in Lindinger *et al*. (1998), assuming a constant reaction rate coefficient ($kR = 2 \times 10^{-9}$ $cm^3$ $s^{-1}$) and by averaging mass spectral signals over 40 spectra. This approximation introduces a systematic error for the absolute concentration of each compound that is in most cases below 30% (Biasioli et al., 2006; Cappellin et al., 2012).

The data extraction of PTR\SRI-ToF-MS spectra provided 519 mass peaks for $H_3O^+$, 445 for $O_2^+$ and 461 for $NO^+$. The matrix consisting of concentration estimation for each sample and each peak ("raw data"), was further processed to extract relevant information and reduce the noise signals associated with PTR-MS measurements. A preliminary reduction of the data set was obtained by excluding i) all peaks with a concentration significantly lower than blanks (t.test with $p < .01$ after Bonferroni correction for multiple tests), ii) $^{13}C$ isotopologues. and iii) signals related to interfering ions at m/z 29.997 ($NO^+$), 32.998 ($O_2^+$ isotope), 37.032, 55.039, 73.050 (all water clusters) and 51.043 (methanol cluster). This procedure allowed to select 120 mass peaks for $H_3O^+$, 73 for $O_2^+$ and 84 for $NO^+$. If not stated differently, statistical analyses were performed on these "reduced data" sets.

### 3.2.5 | Data analysis

Preliminary principal components analysis (PCA) was performed after logarithmic transformation and scaling (mean centring and unit variance). After checking assumptions, one-way ANOVA with Tukey honest significant difference (HSD) and Bonferroni corrections were performed to find the mass peaks that were significantly different among the AMF sensory classes. Of these, the ones with *m/z*>33 and with a concentration threshold equal or above to 0.5 ppbV in at least two classes, were selected to build summary tables. Tentative peak identification was performed using an in-house library developed by the authors and available literature (13,17,18,31–34).

A spider plot for the log transformed data obtained with $H_3O^+$ as reagent ion was built based on the mass peaks selected by univariate data analysis and with a concentration higher than 1 ppbV. Total Ion Count (TIC) obtained by summing the selected mass peaks for each sample was included the in the graph as well.

Finally, a supervised classification method - namely partial least square regression-discriminant analysis (PLS-DA) - was carried out for samples classification. The analysis was applied to the datasets obtained with

the three different reagent ions on both "raw data" and "reduced data" resulting from the data elaboration process. Each dataset was firstly divided into training and test sets with a proportion of 80% and 20% of the samples, respectively. Then, by using the training set, the model was trained via applying a three-fold cross-validation procedure iterated 100 times to extract classification error rates to adjust the discrimination method and chose the models' number of components. The trained models were then used to predict the samples sensory classes of the corresponding test sets. The whole procedure was iterated 1000 times for all datasets. Results were evaluated using mean classification errors, mean balanced error rates (BER) and confusion matrices of each model. The BER is used when the samples classes are unbalanced since it calculates the average proportion of wrongly classified samples in each class, weighted by the number of samples in each class (35). In addition to the individual analysis of the three precursor ions, the same data was merged into a multi-precursor dataset, following a data fusion strategy (36). Particularly, a "low-level" fusion was applied: data from the 3 different ionization modes were concatenated sample-wise into a single matrix. This was done separately for data sets of "raw data" and for "reduced data". As for the PLS-DA performed previously, the matrixes were pre-processed through auto-scaling. The prediction models were run on all the three classes and as well by only considering the "YES *vs* NO" case.

All analyses and graphs were performed with core functions of R programming language and its external packages (37) (ChemometricsWithR, mixOmics, multcomp, vegan, matrixStats, ggplot2) and Excel (version 14.0.7224.5000).

## 3.3 Results and Discussion

### 3.3.1 | Samples classification: sensory and aging effect

The mass peaks in the "reduced data" sets were chosen as variables, and two principal components (PC) were produced. The score plots obtained for PC1 and PC2 corresponding to each ionization mode ($H_3O^+$, $NO^+$ and $O_2^+$) are shown in Figure 3.1.

As presented in Figure 3.1, for all PCAs the first two PCs explain more than 50% of the total variability and the three different sample classes (YES, NO and AGED) are represented with different colours. In the case of $H_3O^+$, the PCA indicates the presences of some clusters: the PC1 separates the AGED samples from the original ones (especially when looking at Figure 3.1A) while the PC2 separates the "YES" class from the "NO" class. This latter distinction is even better when the $NO^+$ mode was used (Figure 3.1B). It is also possible to notice some internal structure in the "YES" class for the $H_3O^+$ mode that is associated to the different suppliers but it is not considered here.

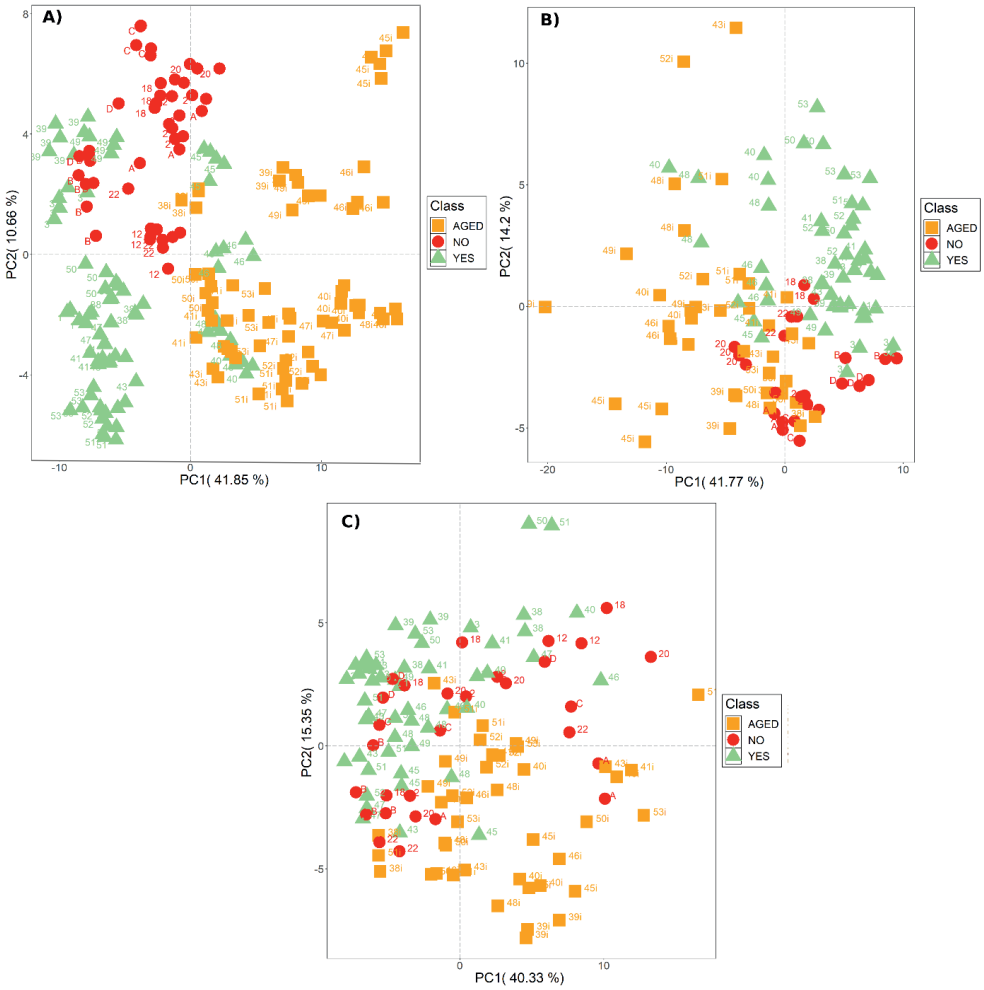



***Figure 3.1:*** *Score plots of principal component analysis (PCA) for the 3 ionization modes. Panel A: $H_3O^+$ mode. Panel B: $NO^+$ mode. Panel C: $O_2^+$ mode. The first two principal components are shown. The different colours and the different shapes indicate the different AMF classes. For each sample the five replicates are shown.*

One-way ANOVA followed by Tukey HSD (with Bonferroni correction) was conducted to further evaluate differences in VOCs among the three sample classes for all the reagent ions. Mass peaks that were significantly different for at least one class ($p < .01$) were reported in Table 3.2. Of the 49 mass peaks listed, 36 were tentatively identified based on their sum formula, isotopic and fragmentation pattern, our internal database and literature data. Results for $O_2^+$ and $NO^+$ are reported in supplementary materials (see table S1 and S2).

**Table 3.2:** Tentatively identified mass peaks from the headspace analysis of anhydrous milk fat (AMF). The mass peaks that were found significantly different among the classes ($p < .01$) and having a concentration above 0.5 ppbV in at least two classes were selected.

| Mass | Theoretical Mass | Chemical Formula | AGED | NO | YES | Tentative Identification |
|---|---|---|---|---|---|---|
| ms33.033 | 33.0339 | $CH_4OH^+$ | 33.5±26.9a | 217±261.3c | 71±153.9b | Methanol |
| ms41.038 | 41.0391 | $C_3H_5^+$ | 29.8±25.9ab | 23.7±119.1b | 13.5±12.5a | Alkyl fragment alcohol |
| ms42.034 | 42.0344 | $C_2H_3NH^+$ | 1.2±1a | 5.8±14b | 2.1±1.9a | Acetonitrile |
| ms43.018 | 43.0184 | $C_2H_3O^+$ | 61±27.8b | 32.7±9.6a | 24.1±25.3a | Acetic acid fragment |
| ms43.054 | 43.0548 | $C_3H_7^+$ | 8.6±2.9a | 13.4±128.4b | 7±4.4a | Alkyl fragment |
| ms45.033 | 45.0340 | $C_2H_4OH^+$ | 166.6±74.1b | 71.9±17.9a | 39.6±37.9a | Acetaldehyde |
| ms45.992 | 45.9871 | $CHSH^+$ | 1.22±0.06b | 1.17±0.04a | 1.19±0.05a | - |
| ms46.029 | 46.0287 | $CH_3NOH^+$ | 1.3±0.5b | 1.5±0.3b | 1.3±0.2a | - |
| ms47.013 | 47.0128 | $CH_2O_2H^+$ | 9.5±5.4b | 9.4±2.5ab | 8.8±1.7a | Formaldehyde |
| ms47.025 | 47.0245 | $H_2N_2OH^+$ | 3±0.3b | 2.8±0.2a | 3.1±0.3b | - |
| Ms47.049 | 47.0497 | $C_2H_6OH^+$ | 24.78±41.05ab | 57.34±64.42a | 26.63±74.45b | Ethanol |
| ms49.011 | 49.0112 | $CH_5S^+$ | 2.4±1.5c | 0.7±0.5a | 1.4±0.8b | Methanehtiol |
| ms53.039 | 53.0391 | $C_4H_4^+$ | 1.1±0.4b | 0.6±0.2a | 0.5±0.2a | - |
| ms55.055 | 55.0548 | $C_4H_6H^+$ | 25.2±8.4b | 13.7±4.2a | 10.1±4.8a | Butanal fragment |
| ms57.034 | 57.0340 | $C_3H_4OH^+$ | 5.6±8.5b | 1.4±1.5a | 1.1±0.8a | 2-propenal/ acetyl fragment |
| ms57.07 | 57.0699 | $C_4H_9^+$ | 3.7±1.4b | 3.4±1.5ab | 3.1±0.9a | Common fragment (alcohol, ester) |
| ms58.041 | 58.0419 | $C_3H_5OH^+$ | 2.1±0.9b | 0.7±0.3a | 0.3±0.4a | - |
| ms60.053 | 60.0525 | $C_2[13]CH_6OH^+$ | 87.7±29.7b | 15.7±12.5a | 21.5±21.3a | 2-propanone isotope |
| ms61.028 | 61.0290 | $C_2H_4O_2H^+$ | 37.5±38.2b | 29.7±14.7ab | 20.3±35.1a | Acetic acid/acetate |
| ms63.027 | 63.0268 | $C_2H_6SH^+$ | 9.1±3.5b | 2.4±0.8a | 9.8±5.7b | Ethanethiol |
| ms67.055 | 67.0547 | $C_5H_7^+$ | 0.9±1b | 0.6±0.3a | 0.5±0.5a | 2-pentanal fragment |
| ms69.07 | 69.0704 | $C_5H_8H^+$ | 14.8±27.1b | 7.9±8.9a | 4.8±15.5a | Isoprene/ 3-hexen-2-ol |
| ms71.049 | 71.0491 | $C_4H_6OH^+$ | 3.2±1.5b | 1.7±0.6a | 1.4±0.5a | 2-butenal/2,3-butadien-1-ol/2-butenal |
| ms71.086 | 71.0855 | $C_5H_{10}H^+$ | 1.3±0.2b | 0.8±0.3a | 0.9±0.3a | 1(2)-pentene |
| ms73.028 | 73.0284 | $C_3H_4O_2H^+$ | 1.1±0.4b | 0.7±0.2a | 0.7±0.1a | Propiolactone |
| ms73.065 | 73.0653 | $C_4H_8OH^+$ | 147.7±26.8c | 66.9±17.5a | 97.2±30.3b | 2-butanone/butanal |
| ms75.029 | 75.0263 | $C_3H_6SH^+$ | 1.9±0.3a | 2±0.2b | 1.8±0.3a | - |
| ms75.044 | 75.0441 | $C_3H_6O_2H^+$ | 3.1±3.1b | 3.6±3.2b | 1.7±1a | Propanoic acid |
| ms79.054 | 79.0542 | $C_6H_6H^+$ | 0.8±0.2b | 0.9±0.2c | 0.7±0.1a | Benzene |

| | | | | | | |
|---|---|---|---|---|---|---|
| ms82.945 | 82.9950 | $C_4H_3S^+$ | 0.6±0.2b | 0.5±0.1a | 0.6±0.2b | - |
| ms83.087 | 83.0860 | $C_6H_{11}^+$ | 3.4±2.8b | 2.1±1.6a | 1.9±1.4a | Hexanal fragment |
| ms85.066 | 85.0653 | $C_5H_8OH^+$ | 1.1±0.8b | 0.6±0.2a | 0.4±0.2a | 2-pentenal (E)/1-penten-3-one |
| ms87.044 | 87.0401 | $C_4H_6O_2H^+$ | 4.2±1.9b | 1.1±0.8a | 1.2±0.8a | 2,3-butanedione/γ-butyrolactone |
| ms87.081 | 87.0809 | $C_5H_{10}OH^+$ | 143.3±46.9b | 51.3±27.7a | 23.7±33.6a | 2-pentanone/2/3-methylbutanone |
| ms89.061 | 89.0603 | $C_4H_8O_2H^+$ | 9.7±5.1c | 7.5±2.7b | 5±2.9a | Butanoic acid/ acetoin |
| ms90.95 | | | 1.7±0.1b | 1.8±0.1b | 1.7±0.1a | - |
| ms93.071 | 93.0704 | $C_7H_8H^+$ | 1.3±0.9a | 2.1±2.8b | 1.2±3.6ab | Toluene (=Methyl Benzene) |
| ms96.961 | 96.9612 | $C_2H_2Cl_2H^+$ | 0.1±0.6a | 1.1±0.7b | 0.1±0.3a | Dichloroethylene |
| ms97.103 | 97.1012 | $C_7H_{13}^+$ | 5.8±2b | 2.9±1.2a | 1.5±1.3a | Heptanal fragment |
| ms101.061 | 101.0597 | $C_5H_9O_2H^+$ | 1±0.4b | 0.8±0.1a | 0.7±0.1a | 2,3-pentanedione |
| ms101.097 | 101.0966 | $C_6H_{12}OH^+$ | 3.3±1.3b | 1.6±0.6a | 0.7±0.9a | Hexanal |
| ms107.087 | 107.0497 | $C_7H_6OH^+$ | 0.4±0.3a | 0.8±1.2b | 0.5±0.3a | Benzaldehyde/ 1,3 dimethylbenzene |
| ms108.957 | | | 0.89±0.05b | 0.86±0.04a | 0.91±0.04b | - |
| ms109.103 | 109.1012 | $C_8H_{13}^+$ | 0.49±0.11ab | 0.5±0.22b | 0.47±0.12a | - |
| ms115.085 | 115.0867 | $C_5H_{10}N_2OH^+$ | 0.6±0.2b | 0.4±0.1a | 0.4±0.1a | - |
| ms115.114 | 115.1117 | $C_7H_{14}OH^+$ | 63.4±22.5b | 27.9±14.9a | 12.4±16.2a | 2-heptanone |
| ms143.147 | 143.1432 | $C_9H_{18}OH^+$ | 1.5±0.5b | 0.9±0.3a | 0.8±0.3a | 2-nonanone |

From the table it is possible to notice that the intensity of most peaks is higher for AGED samples compared to the other classes (59%) (Table 3.2). The same results were found for the measurements using $O_2^+$ and $NO^+$ as precursor ions, indicating the importance of oxidation phenomena during heat treatment in increasing the concentration of different VOCs (Table S1 and S2 in supplementary materials).

The most abundant mass peaks found in the headspace of the "AGED" AMFs, measured with $H_3O^+$ ion, were *m/z* 45.033, 73.065 and 87.081 tentatively identified as acetaldehyde, 2-butanone/butanal and 2-pentanone/diacetyl/2-/3-methylbutanal, respectively. Such compounds were all described as key butter aroma compounds (38–40) and were previously found in a similar experiment (18). In particular, 2-butanone and acetone in milk have been reported to originate from cows' feed (41) while acetaldehyde, diacetyl, 2- and 3-methylbutanal were found as important contributors of the aroma in sweet cream butter (42) and some of them were reported to increase during storage at 4°C (43). The mass peak *m/z* 73.065, was the most concentrated compound as well in the "YES" class. This class had a significantly lower concentration of *m/z* 73.065 than the "AGED" class but was significantly higher than the "NO" class. In this case, the SRI analysis can add relevant information where, by using $NO^+$ as reagent ion, is possible to achieve a separation of

aldehydes and ketones since different reactions occurs (aldehydes undergo mostly a charge-transfer reaction with $NO^+$ while ketones are subjected to ion-molecule association reactions).

This separation is shown in the boxplots in Figure 3.2 for mass peak *m/z* 73.065: the aldehyde proportion (corresponding to butanal) at *m/z* 71.049 and the ketone (2-butanone) at *m/z* 102.054. A different trend is observed for the two compounds: the ketone in the "YES" class has higher concentrations ($p < .01$) than the "NO" class, while for the aldehyde the "YES" class has lower levels although only marginally significant ($p < .03$). Different levels of 2-butanone may be due to the different type of feeding that was provided to the cows (41,44). This data indicates that the 2-butanone may be recognized by the panellists as a marker of freshness since it has been associated with *buttery* odour (45). The difference in concentration for the different classes, makes the ketone a potential quality marker; the compound needs to be over a certain concentration to meet industrial quality criteria but may be critical when samples reach higher concentrations possibly induced by thermo-oxidation processes. $NO^+$ ionization mode also led to a separation of the mass peak *m/z* 115.113 tentatively identified as a mixture of heptanal and 2-heptanone, a potent odour-active compounds in dairy products (18). In this case the aldehyde and the ketone did not have different trends in the AMF classes but 2-heptanone had higher concentrations than the aldehyde. 2-heptanone, has been reported in many different studies on both butter and butter oil aroma (14,42,45) and has been described as having a *dairy-like* odour (40).

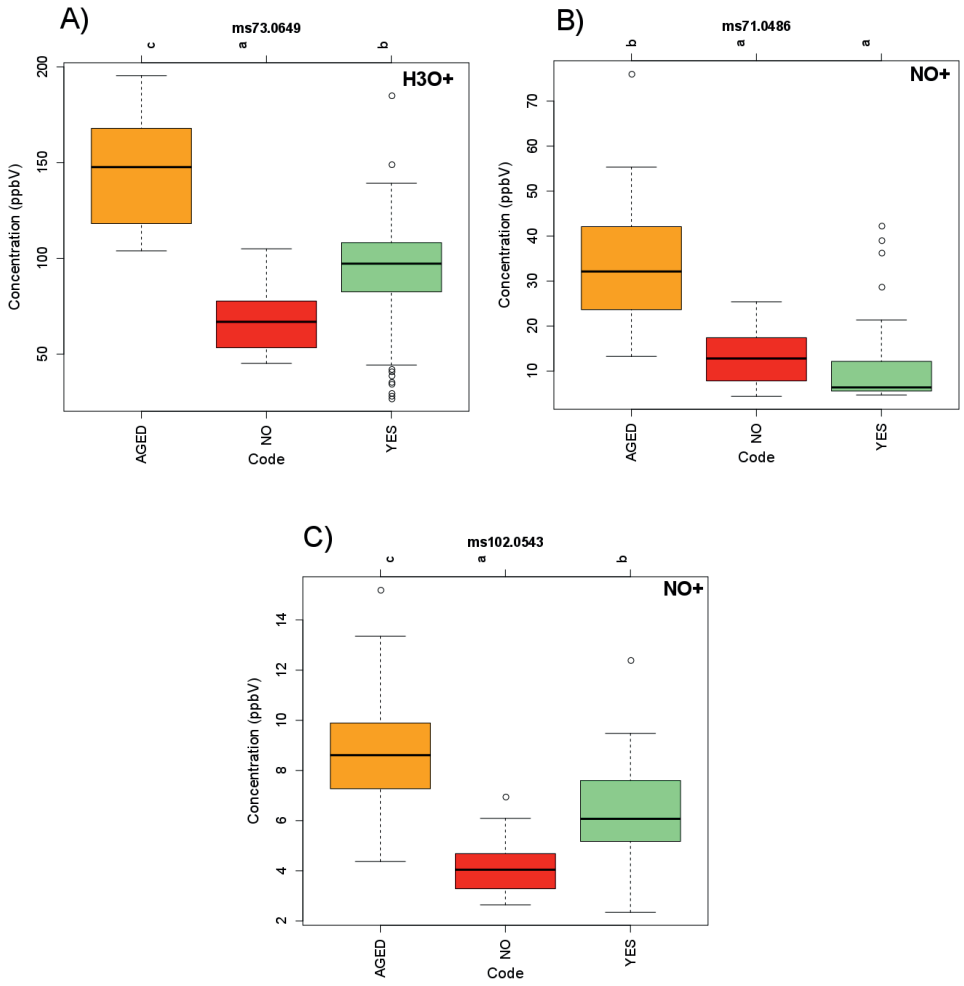



***Figure 3.2**: Boxplots of relevant AMF mass peaks. A) m/z = 73.065 - $C_4H_8OH^+$ obtained with $H_3O^+$ as reagent ion, tentatively identified as a mixture of 2-butanone and butanal. B) m/z = 71.049 – $C_4H_7O^+$ tentatively identified as butanal resulting from the hydride ion transfer reaction obtained with NO+ as reagent ion. C) m/z = 102.054 – $C_4H_8ONO^+$ tentatively identified as 2-butanaone resulting from the ion-molecule association obtained with NO+ as reagent ion.*

The spider plot in Figure 3.3 summarizes the profile of significant ($p < .05$) VOCs with concentration greater than 1.0 ppbV for the three different classes of AMF samples for the $H_3O^+$ mode. As already seen in the table, the "AGED" class presented more compounds with higher concentrations due to oxidation induced by the thermal treatment. This is summarized also by the TIC in the spider plot obtained by summing the signal of all these mass peaks: the "AGED" class has the highest levels, followed by the "NO" samples, which also have some mass peaks with the highest levels.

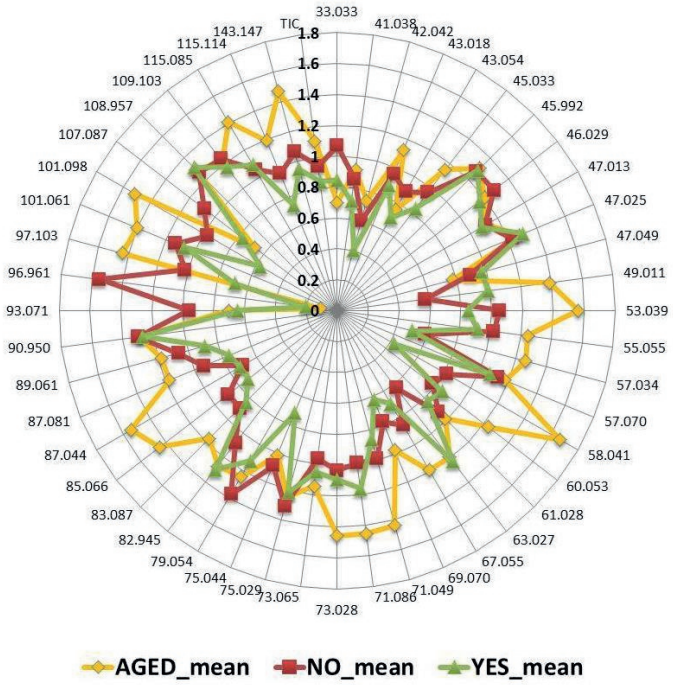



***Figure 3.3:*** *$H_3O^+$ mode. Spider plot of mass peaks selected from 1-way ANOVA and using a threshold value of 0.5 ppbV. Values were centred and log scaled before being plotted.*

An illustrative example of this trend is *m/z* 101.098 tentatively identified as hexanal, one of the most studied volatile biomarkers for dairy product oxidation. Several studies confirmed that this compound – that has been associated with *grassy/metallic-like* off-flavour in butter and milk - increases during storage (46–49). Hexanal is produced from the auto-oxidation of n-6 polyunsaturated fatty acids (50) and therefore it is not surprising that storage at 50°C boosted its formation.

In general, the "AGED" class had the highest concentration for most of the mass peaks, followed by the "NO" class which had higher levels than the "YES" class. This is in agreement with sensory analysis results of the industrial partner where the "gold" standard for a raw material is to have a neutral flavour as much as possible, without any off-notes. Therefore, samples are penalized when they present strong flavours.

The "NO" class presented higher levels for *m/z* 33.033 that was tentatively associated with methanol. To the authors knowledge no previous GC-MS studies on milk fat or butter reported the presence of this compound in the food matrixes. This may be due to a different number of analytical factors related to the GC-MS methods. For example, some SPME fibres do not have a high affinity for this compound, methanol can be used as a solvent for extractions of VOCs or, due to its psychochemical characteristics (i.e. low molecular weight, high volatility), the compound may elute under the air peak or at the beginning of the chromatogram and then is not been considered in the analysis. On the other hand, PTR-MS has found

methanol in both AMF and milk powders (Liu, Koot, Hettinga, de Jong, & van Ruth, 2018; Makhoul et al., 2016; Pedrotti et al., 2018). Methanol formation from pectin has been reported in cow's rumen fermentation from the hydrolysis of methyl esters by rumen microorganisms (52). The methanol may then be transferred to the milk and then to the AMF although at very low concentration. In the same study, it was also suggested that diets with different pectin contents may lead to differences in methanol formation. In our case the "NO" samples may then come from cows which were fed diets containing higher pectin contents. Other mass peaks that have a higher concentration (but still < 5.0 ppbV) in the headspace of the "NO" class are those associated to *m/z* 79.054 and 93.071, tentatively associated to aromatic hydrocarbons like benzene and toluene and possibly fragments of higher aromatic compounds or of other hydrocarbons (i.e. ethylbenzene, n-ethyltoluene, propylbenenze) (53). Aromatic hydrocarbons are known environmental contaminants in food due to their high volatility and attraction to fats. Benzene and toluene have been reported in different food volatile mixtures (54) and have been found in traces in butter, heated butter and milk in previous investigations with different analytical techniques (45,55–58). As well, Villenueve *et al.* (2013) found that pasture-fed cows produced milk with a higher level of toluene then cows fed hay and silage. Toluene could be a product of β-carotene degradation (59). The processing of hay and silage forages are known to degrade carotenoids and so the diet type may lead to differences in milk concentrations of carotenoid, leading to differences in toluene levels in the milk (60). In our case, these differences in toluene concentrations have been transferred to AMF and stress again the preeminent role of cow diet on the quality of the processed product. Finally, another mass peak that had higher levels in the "NO" class and that can be used as another quality marker in the model definition is the *m/z* 96.961. This mass peak has been associated with a chloride compound ($C_2H_2Cl_2H^+$) based on *m/z* value and its isotopic ratios. The compound may be a fragment of one or more organochlorine pesticides residues like chlorinated derivatives of diphenyl ethane, the groups of hexachlorobenzene, hexachlorocyclohexanes and cyclodienes (61). Despite these pesticides has been banished for usage in agriculture in most countries worldwide, occurrence in different milk samples have been registered due to water contamination, use of pesticides in the control of ectoparasites directly in the animal and consumption of contaminated pastures and/or rations since the rates of reduction in organochlorine levels in the terrestrial environment are slow (61,62).

The mentioned mass peaks may then be of primary importance for industrial quality control: they can help in discriminating samples of lower sensory quality although the link with sensory attribute cannot be direct. In fact, these defects may originate during the different steps of the supply chain or they may be the result of different types of the cow's feeds (58). Also, they may come from latter stages during the raw material processes at the manufacturer level. Finally, non-optimal storage conditions and different packaging materials (18) may also led to increasing concentrations of these VOCs. Some of the highlighted markers may be directly correlated to the sensory perception and evaluation of the samples (*e.g.* hexanal, 2/3-methyl butanone) while some others may not be perceivable to the panel.

### 3.3.2 | PLS-DA classification

#### 3.3.2.1 YES vs NO vs AGED:

Cross-validation of the models resulted in classification performances between 9 and 22% with the lowest error for the $NO^+$ mode (see table S3 in the supplementary materials). Data obtained with $H_3O^+$ and $O_2^+$ ionization modes presented a BER like the aggregate data ("reduced data") matrix. Using the "reduced data" provided better classification results for the $NO^+$ and the $O_2^+$ ionization mode while it was the opposite for the $H_3O^+$ mode. In Table 3.3 are presented the confusion matrixes for the "YES" vs "NO" comparison for the 3 ionization modes and the aggregate data matrix. The table contains the classification rates averages for each classification model. In table S3.4 (supplementary materials) can be found the same data for the comparison also with the "AGED" samples.

#### 3.3.2.2 YES vs NO:

While the "AGED" class is relevant for observing how thermo-oxidation phenomena can affect the aroma profile, for industrial quality control purposes the "YES" *vs* "NO" classes comparison is more relevant. For this reason, all statistical analyses have been repeated by considering just this case.

PLS-DA with two classes "YES" and "NO" has a considerably higher classification rate reaching a minimum average BER of 4% for $NO^+$ mode when all mass peaks were considered (Table S3.3). Confusion matrixes in Table 3.3 indicate that for this ionization mode, when "raw data" were included in the model, just 3% of the samples were misclassified on average. The $NO^+$ ionization mode has the best performance also when using "reduced data set". The reason for a better performance of the $NO^+$ mode may lay in the fact that this ionization mode provides an enhanced separation of aldehydes and ketones that are known to be critical in determining perceived flavour of food (63). It may then be interesting from an industrial point of view to have a closer look at the SRI system for quality control purposes. For the other ionization modes and the aggregated data matrix, the "NO" class, the most critical class for a quality control program, had a slighter higher chance to be misclassified then the "YES" class. When looking more in details at the misclassified samples, the sample "B" (sensory score 1.6), followed by sample "3", "49" and "18" (sensory score: 1.1, 0.8, 1.7) were the ones that, across all models, had the highest rate of misclassification. Most of these samples have scores near the acceptability threshold of 1.5 suggesting a good reliability of the predictive models built from PTR/SRI-TOF-MS data.

**Table 3.3:** Confusion matrixes of the different test sets. Average values were obtained by a 1000-time prediction. Classification rates are reported in %. In the case of the aggregate matrix, the all masses dataframe was obtained by selecting all mass peaks that had a concentration above 1 ppbV.

| Predicted vs original | Reduced data (selected masses) | | Raw data (all masses) | |
|---|---|---|---|---|
| **$H_3O^+$** | NO | YES | NO | YES |
| NO | 82 | 18 | 84 | 16 |
| YES | 11 | 89 | 8 | 92 |
| **$NO^+$** | NO | YES | NO | YES |
| NO | **97** | **3** | **97** | **3** |
| YES | **6** | **94** | **3** | **97** |
| **$O_2^+$** | NO | YES | NO | YES |
| NO | 89 | 11 | 79 | 21 |
| YES | 12 | 88 | 16 | 84 |
| **Aggregate matrix** | NO | YES | NO | YES |
| NO | 88 | 12 | 81 | 19 |
| YES | 11 | 89 | 7 | 93 |

Univariate data analysis confirms that for $H_3O^+$ mode "NO" samples presented a higher portion of mass peaks with higher concentrations than "YES" samples (75%, see Table 3.2). On the other hand, some markers with a significantly higher concentration in the "YES" class were identified ($p > .001$). When looking at compounds with relevant concentrations (> 1 ppbV) two mass peaks were identified: 63.027 and 73.065 tentatively identified as ethanethiol/dimethyl sulphide and 2-butanone/butanal. As previously highlighted by the $NO^+$ data, the "YES" category has a higher concentration of 2-butanone and it is probably recognized by panellists as a marker for good quality of the product. For ethanethiol/ dimethyl sulphide the finding is counterintuitive. Ethanethiol, is well known for its sulphurous vegetable-like aroma (64). Nevertheless it has been shown to have a positive impact on both beer and wine flavour (Liu, 2015), its higher concentration in the "YES" class was unexpected. It may be possible that this marker at low concentrations [< 10 ppbV] has a positive contribution to the quality perceived by the panel. However, more investigation is needed in this direction to clarify the contribution of this sulphur volatile compounds to the whole AMF aroma.

## 3.4 Conclusions

This research implemented a rapid non-invasive mass spectrometry method to classify industrial anhydrous milk fat (AMF) samples according to the indications of an industrial sensory quality assessment. The 39 AMF samples from different suppliers were classified based on sensory analysis conducted according to the industrial partner internal protocol and according to thermal treatment. PTR/SRI-ToF-MS method was applied to screen VOC emissions of these samples and both unsupervised and supervised multivariate

data analysis showed promising results in discriminating the three different classes ("YES", "NO" and "AGED").

Univariate data analysis revealed some quality markers for each class giving indications about possible key compounds that should be monitored carefully during VOC headspace analysis. The implementation of other reagent ions added information for identification of these markers and provides better classification models. Particularly, samples that underwent thermal treatment ("AGED") had the highest concentrations of VOCs, due to thermal oxidation. The "NO" class, which contained samples with sensory defects accumulated during the different steps of the food supply chain, presented few mass peaks at significantly higher levels than the other classes which may be used as quality markers. On the other hand, "YES" samples are, in general, characterized by lower VOC release indicating that a higher concentration of VOCs penalized the samples sensory evaluation.

The more relevant comparison for industrial purposes, namely the one between "YES" and "NO" samples, PLS-DA showed a correct classification rate

between 80-97%. It is reasonable to speculate that with more representative sampling and with a more accurate definition of sensory data the volatilome analysis with the described method can be implemented profitably in industrial quality control programs.

Future studies in this direction with larger sampling, should test the reliability of our model on a larger dataset to increase the classification rate by also selecting a pool of critical mass peaks and by further investigating the origin of the mentioned quality markers. These markers may be related to one or more stages in the food supply chain such as for example dairy cow feed, storage of milk samples and the manufacturing process. As well, more effort should be dedicated towards improving uncertainties linked to the industrial sensory evaluation, where an arbitrary score threshold was assigned to classify samples quality and an accurate definition of the evaluation error is missing.

## 3.5 Acknowledgments and declaration of interest

The authors thank Dr. Kasper Hettinga for the useful discussions about the possible origin of different quality markers in milk and anhydrous milk fat. LF, MA, MF and MS are employees of Soremartec S.r.l. which provided the samples. Support of Autonomous Province of Trento is acknowledged (AdP 2020).

## 3.6 References


1. Bylung G. Dairy processing handbook. AB T, editor. Tetra Pak Processing Systems AB S-21 86 Lund, Sweden; 1995.
2. Wang Y, Yuan D, Li Y, Li M, Wang Y, Li Y, et al. Thermodynamic and whipping properties of milk fat in whipped cream: A study based on DSC and TD-NMR. Int Dairy J. 2019;97:149–57.
3. Koczoñ P, Gruczyñska E, Kowalski B. Changes in the acid value of butter during storage at different temperatures as assessed by standard methods or by FT-IR spectroscopy. Am J Food Technol. 2008;3(3):154–63.
4. Ismail AA, van de Voort FR, Emo G, Sedman J. Rapid quantitative determination of free fatty acids in fats and oils by fourier transform infrared spectroscopy. J Am Oil Chem Soc. 1993;70(4):335–41.
5. Abrahamsen RK. Butter and Related Products – Product Characteristics, Production Technology and Quality Aspects. Int Dairy J. 2013;31(2):92.
6. FAO. INFLUENCE OF QUALITY OF RAW MATERIALS ON TASTE OF MILK INTENDED FOR CONSUMPTION [Internet]. 1984 [cited 2018 Dec 10]. Available from: http://www.fao.org/docrep/003/x6537e/X6537E03.htm
7. Murray JM, Baxter IA. SENSORY EVALUATION | Food Acceptability and Sensory Evaluation. Encycl Food Sci Nutr [Internet]. 2003 Jan 1 [cited 2018 Oct 25];5130–6. Available from: https://www.sciencedirect.com/science/article/pii/B012227055X013729
8. Munoz AM. Sensory evaluation in quality control: an overview, new developments and future opportunities. Food Qual Prefer. 2002;13(6):329–39.
9. Reece RN. Quality Assurance Perspective of Sensory Evaluation. Food Technol. 1979;33(9):37.
10. Biasioli F, Yeretzian C, Gasperi F, Mark TD. PTR-MS monitoring of VOCs and BVOCs in food science and technology. Trac-Trends Anal Chem. 2011;30(7):968–77.
11. Lindinger W, Hansel A, Jordan A. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) - Medical applications, food control and environmental research. Int J Mass Spectrom. 1998;173(3):191–241.
12. Blake RS, Monks PS, Ellis AM. Proton-Transfer Reaction Mass Spectrometry. Chem Rev. 2009;109(3):861–96.
13. Van Ruth SM, Koot A, Akkermans W, Araghipour N, Rozijn M, Baltussen M, et al. Butter and butter oil classification by PTR-MS. Eur Food Res Technol [Internet]. 2008;227(1):307–17. Available from: http://dx.doi.org/10.1007/s00217-007-0724-7
14. Macatelli M, Akkermans W, Koot A, Buchgraber M, Paterson A, van Ruth S. Verification of the geographical origin of European butters using PTR-MS. J Food Compos Anal [Internet]. 2009;22(2):169–75. Available from: http://ac.els-cdn.com/S0889157508001890/1-s2.0-S0889157508001890-main.pdf?_tid=e55ea302-f85d-11e6-a378-00000aab0f6c&acdnat=1487699464_a366a2c5ca158628137c9ed5519eb7a6
15. Jordan A, Haidacher S, Hanel G, Hartungen E, Mark L, Seehauser H, et al. A high resolution and high sensitivity proton-transfer-reaction time-of-flight mass spectrometer (PTR-TOF-MS). Int J Mass Spectrom. 2009;286(2–3):122–8.
16. Blake RS, Whyte C, Hughes CO, Ellis AM, Monks PS. Demonstration of proton-transfer reaction time-of-flight mass spectrometry for real-time analysis of trace volatile organic compounds. Anal Chem. 2004;76(13):3841–5.
17. Makhoul S, Yener S, Khomenko I, Capozzi V, Cappellin L, Aprea E, et al. Rapid non-invasive quality control of semi-finished products for the food industry by direct injection mass spectrometry headspace analysis: the case of milk powder, whey powder and anhydrous milk fat. J Mass Spectrom [Internet]. 2016;51(9):782–91. Available from: http://onlinelibrary.wiley.com/store/10.1002/jms.3801/asset/jms3801.pdf?v=1&t=j5v4huhd&s=220736ofa980d515ec2f3cbacb19076da772621e
18. Pedrotti M, Khomenko I, Cappellin L, Fontana M, Somenzi M, Falchero L, et al. Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. J Mass Spectrom.

2018;53(9):753–62.

19. Karl T, Hansel A, Cappellin L, Kaser L, Herdlinger-Blatt I, Jud W. Selective measurements of isoprene and 2-methyl-3-buten-2-ol based on NO+ ionization mass spectrometry. Atmos Chem Phys. 2012;12(24):11877–84.
20. Materić D, Lanza M, Sulzer P, Herbig J, Bruhn D, Gauci V, et al. Selective reagent ion-time of flight-mass spectrometry study of six common monoterpenes. Int J Mass Spectrom. 2017;421:40–50.
21. Lanza M, Acton WJ, Sulzer P, Breiev K, Jürschik S, Jordan A, et al. Selective reagent ionisation-time of flight-mass spectrometry: A rapid technology for the novel analysis of blends of new psychoactive substances. J Mass Spectrom. 2015;50(2):427–31.
22. Yener S, Romano A, Cappellin L, Granitto PM, Aprea E, Navarini L, et al. Tracing coffee origin by direct injection headspace analysis with PTR/SRI-MS. Food Res Int. 2015;69:235–43.
23. Lawless HT, Heymann H. Sensory Evaluation of Food. Elementary Food Science. 2010. 600 p.
24. Yener S, Romano A, Cappellin L, Mark TD, del Pulgar JS, Gasperi F, et al. PTR-ToF-MS characterisation of roasted coffees (C-arabica) from different geographic origins. J Mass Spectrom [Internet]. 2014;49(9):929–35. Available from: http://onlinelibrary.wiley.com/store/10.1002/jms.3455/asset/jms3455.pdf?v=1&t=j488vtqk&s=5e5f068e2b46bbd362bfc98556585ea01039f6a8
25. Cappellin L, Makhoul S, Schuhfried E, Romano A, Sanchez Del Pulgar J, Aprea E, et al. Ethylene: Absolute real-time high-sensitivity detection with PTR/SRI-MS. the example of fruits, leaves and bacteria. Int J Mass Spectrom. 2014;365–366:33–41.
26. Sánchez del Pulgar J, Soukoulis C, Carrapiso AI, Cappellin L, Granitto P, Aprea E, et al. Effect of the pig rearing system on the final volatile profile of Iberian dry-cured ham as detected by PTR-ToF-MS. Meat Sci. 2013;93(3):420–8.
27. Cappellin L, Biasioli F, Granitto PM, Schuhfried E, Soukoulis C, Costa F, et al. On data analysis in PTR-TOF-MS: From raw spectra to data mining. Sensors and Actuators B-Chemical. 2011;155(1):183–90.
28. Cappellin L, Biasioli F, Fabris A, Schuhfried E, Soukoulis C, Mark TD, et al. Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS. Int J Mass Spectrom. 2010;290(1):60–3.
29. Cappellin L, Karl T, Probst M, Ismailova O, Winkler PM, Soukoulis C, et al. On Quantitative Determination of Volatile Organic Compound Concentrations Using Proton Transfer Reaction Time-of-Flight Mass Spectrometry. Environ Sci Technol. 2012;46(4):2283–90.
30. Biasioli F, Gasperi F, Aprea E, Endrizzi I, Framondino V, Marini F, et al. Correlation of PTR-MS spectral fingerprints with sensory characterisation of flavour and odour profile of "Trentingrana" cheese. Food Qual Prefer. 2006;17(1–2):63–75.
31. Widder S, Sen A, Grosch W. Changes in the Flavour of Butter Oil during Storage - Identification of Potent Odorants. Zeitschrift Fur Leb Und-forsch [Internet]. 1991;193(1):32–5. Available from: http://download.springer.com/static/pdf/581/art%253A10.1007%252FBF01192013.pdf?originUrl=http%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252FBF01192013&token2=exp=1475668381~acl=%252Fstatic%252Fpdf%252F581%252Fart%2525253A10.1007%2525252FBF01192013.pdf%253ForiginUrl%253
32. Krause AJ, Miracle RE, Sanders TH, Dean LL, Drake MA. The effect of refrigerated and frozen storage on butter flavour and texture. J Dairy Sci [Internet]. 2008;91(2):455–65. Available from: http://www.sciencedirect.com/science/article/pii/S0022030208713870
33. Peterson DG, Reineccius GA. Characterization of the volatile compounds that constitute fresh sweet cream butter aroma. Flavour Fragr J [Internet]. 2003;18(3):215–20. Available from: http://onlinelibrary.wiley.com/store/10.1002/ffj.1192/asset/1192_ftp.pdf?v=1&t=j5b155zh&s=a31d68752f0b7d5c2c05d77963e42bbfa1450b4c
34. Beauchamp J, Zardin E, Silcock P, Bremer PJ. Monitoring photooxidation-induced dynamic changes in the volatile composition of extended shelf life bovine milk by PTR-MS. J Mass Spectrom [Internet]. 2014;49(9):952–8. Available from:

http://onlinelibrary.wiley.com/store/10.1002/jms.3430/asset/jms3430.pdf?v=1&t=j5b2bzy9&s=d893aef031c4b635db87264be6644d5634fd39b2

35. Rohart F, Gautier B, Singh A, Lê Cao KA. mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS Comput Biol. 2017;13(11).
36. Borràs E, Ferré J, Boqué R, Mestres M, Aceña L, Busto O. Data fusion methodologies for food and beverage authentication and quality assessment - A review. Analytica Chimica Acta. 2015.
37. R Core Team. R Development Core Team [Internet]. Vol. 55, R: A Language and Environment for Statistical Computing. 2016. p. 275–86. Available from: https://www.r-project.org/
38. Maarse H, Visscher CA, Willemsens LC, Boelens MH. Volatile compounds in food : qualitative and quantitative data. 6th ed. Zeist: TNO-CIVO; 1989. 3 vol. (1377 p.).
39. Schieberle P, Gassenmeier K, Guth H, Sen A, Grosch W. Character Impact Odor Compounds of Different Kinds of Butter. Food Sci Technol Technol. 1993;26(4):347–56.
40. Mallia S, Escher F, Schlichtherle-Cerny H. Aroma-active compounds of butter: A review. Eur Food Res Technol. 2008;226(3):315–25.
41. Marsili R. Flavours and off-flavours in dairy foods. Encycl Dairy Sci Second Ed. 2011;(July 2015):533–51.
42. Peterson DG, Reineccius GA. Determination of the aroma impact compounds in heated sweet cream butter. Flavour Fragr J. 2003;18(4):320–4.
43. Lozano PR, Miracle ER, Krause AJ, Drake M, Cadwallader KR. Effect of cold storage and packaging material on the major aroma components of sweet cream butter. J Agric Food Chem [Internet]. 2007;55(19):7840–6. Available from: http://pubs.acs.org/doi/pdfplus/10.1021/jf071075q
44. Villeneuve M-P, Lebeuf Y, Gervais R, Tremblay GF, Vuillemard JC, Fortin J, et al. Milk volatile organic compounds and fatty acid profile in cows fed timothy as hay, pasture, or silage. J Dairy Sci [Internet]. 2013;96(11):7181–94. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030213006206
45. O'Callaghan TF, Faulkner H, McAuliffe S, O'Sullivan MG, Hennessy D, Dillon P, et al. Quality characteristics, chemical composition, and sensory properties of butter from cows on pasture versus indoor feeding systems. J Dairy Sci [Internet]. 2016;99(12):9441–60. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030216307135
46. Panseri S, Soncin S, Chiesa LM, Biondi PA. A headspace solid-phase microextraction gas-chromatographic mass-spectrometric method (HS-SPME-GC/MS) to quantify hexanal in butter during storage as marker of lipid oxidation. Food Chem [Internet]. 2011;127(2):886–9. Available from: http://ac.els-cdn.com/S030881461100063X/1-s2.0-S030881461100063X-main.pdf?_tid=5e613244-6bd9-11e7-b51d-00000aab0f27&acdnat=1500396879_b3b78b5cc37e45358dca15e893c16877
47. Garcia-Llatas G, Lagarda MJ, Romero F, Abellan P, Farre R. A headspace solid-phase microextraction method of use in monitoring hexanal and pentane during storage: Application to liquid infant foods and powdered infant formulas. Food Chem. 2007;101(3):1078–86.
48. Correddu F, Nudda A, Manca MG, Pulina G, Dalsgaard TK. Light-Induced Lipid Oxidation in Sheep Milk: Effects of Dietary Grape Seed and Linseed, Alone or in Combination, on Milk Oxidative Stability. J Agric Food Chem. 2015;63(15):3980–6.
49. Asaduzzaman M, Biasioli F, Cosio MS, Schampicchio M. Hexanal as biomarker for milk oxidative stress induced by copper ions. J Dairy Sci [Internet]. 2017;100(3):1650–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28088419
50. Contarini G, Povolo M, Leardi R, Toppino PM. Influence of heat treatment on the volatile compounds of milk. J Agric Food Chem. 1997;45(8):3171–7.
51. Liu N, Koot A, Hettinga K, de Jong J, van Ruth SM. Portraying and tracing the impact of different production systems on the volatile organic compound composition of milk by PTR-(Quad)MS and PTR-(ToF)MS. Food Chem. 2018;239:201–7.
52. Vantcheva ZM, Pradhan K, Hemken RW. Rumen Methanol in Vivo and in Vitro. J Dairy Sci. 2010;53(10):1511–4.
53. Gueneron M, Erickson MH, Vanderschelden GS, Jobson BT. International Journal of Mass Spectrometry PTR-

MS fragmentation patterns of gasoline hydrocarbons. Int J Mass Spectrom [Internet]. 2015;379:97–109. Available from: http://dx.doi.org/10.1016/j.ijms.2015.01.001

54. McNeal TP, Nyman PJ, Diachenko GW, Hollifield HC. Survey of benzene in foods by using headspace concentration techniques and capillary gas chromatography. J AOAC Int. 1993;76(6):1213–9.
55. Forss DA. Mechanisms of formation of aroma compounds in milk and milk products. J Dairy Res [Internet]. 06/01. 2009;46(4):691–706. Available from: https://www.cambridge.org/core/article/mechanisms-of-formation-of-aroma-compounds-in-milk-and-milk-products/5864321C2E5D0E7A05DE046E3D117B84
56. Fabietti F, Delise M, Bocca AP. Aromatic hydrocarbon residues in milk: preliminary investigation. Food Control. 2000;11(4):313–7.
57. Lee SR, Macku C, Shibamoto T. Isolation and Identification of Headspace Volatiles Formed in Heated Butter. J Agric Food Chem. 1991;39(11):1972–5.
58. Faulkner H, Callaghan TFO, Mcauliffe S, Hennessy D, Stanton C, Sullivan MGO, et al. Effect of different forage types on the volatile and sensory properties of bovine milk. J Dairy Sci [Internet]. 2018;101(2):1034–47. Available from: http://dx.doi.org/10.3168/jds.2017-13141
59. Daun H. Produce color and appearance. In: Produce Degradation: Pathways and Prevention. 2005.
60. Nozière P, Graulet B, Lucas A, Martin B, Grolier P, Doreau M. Carotenoids for ruminants: From forages to dairy products. Animal Feed Science and Technology. 2006.
61. Rêgo ICV, Dos Santos GNV, Dos Santos GNV, Ribeiro JS, Lopes RB, Dos Santos SB, et al. Organochlorine pesticides residues in commercial milk: a systematic review. Acta Agronómica. 2019;
62. Bajwa U, Sandhu KS. Effect of handling and processing on pesticide residues in food- A review. Journal of Food Science and Technology. 2014.
63. Diez-Simon C, Mumm R, Hall RD. Mass spectrometry-based metabolomics of volatiles as a new tool for understanding aroma and flavour chemistry in processed food products. Vol. 15, Metabolomics. 2019.
64. Parker JK. Introduction to aroma compounds in foods. In: Flavour Development, Analysis and Perception in Food and Beverages. 2014. p. 3–30.
65. Liu SQ. Impact of yeast and bacteria on beer appearance and flavour. In: Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste. 2015. p. 357–74.

## 3.7 Supplementary materials

**Table S3.1**: Mass peaks in the headspace of anhydrous milk fat (AMF) analysed with $O_2^+$ as reagent ion. The mass peaks that were found significantly different among the classes ($p < .01$) and having a concentration above 0.5 ppbV in at least two classes were reported.

| Mass | AGED | NO | YES |
|---|---|---|---|
| ms29.042 | 1.24±0.51 | 0.73±0.35 | 0.67±0.32 |
| ms33.036 | 4.21±2.14 | 3.33±2.44 | 2.19±1.04 |
| ms36.019 | 11.55±0.24 | 11.44±0.24 | 11.67±0.22 |
| ms37.028 | 393.87±73.46 | 376.35±42.88 | 420.76±66.3 |
| ms38.033 | 0.58±0.11 | 0.55±0.06 | 0.62±0.1 |
| ms39.024 | 7.32±1.99 | 6.85±2.44 | 5.47±1.7 |
| ms41.039 | 12.73±4.41 | 11.48±4.89 | 8.76±3.44 |
| ms42.01 | 4.31±1.61 | 2.57±0.94 | 2.25±0.94 |
| ms42.045 | 2.03±0.61 | 2.65±1.48 | 1.93±0.81 |
| ms44.021 | 25.84±12.14 | 9.5±5.45 | 8.65±5.03 |
| ms55.017 | 1.42±0.82 | 0.94±0.26 | 0.7±0.15 |
| ms57.031 | 10.38±5.11 | 4.55±2.51 | 4.74±2.27 |
| ms58.039 | 45.97±26.85 | 17.49±14.05 | 12.4±9.45 |
| ms59.046 | 16.48±12.72 | 11.01±16.21 | 5.18±3.65 |
| ms61.026 | 0.7±0.58 | 1.57±1.19 | 0.49±0.32 |
| ms67.052 | 1.18±0.45 | 1±0.51 | 0.88±0.31 |
| ms71.047 | 14.73±9.16 | 6.52±5.64 | 4.61±3.64 |
| ms72.053 | 3.07±1.46 | 1.62±0.87 | 1.56±0.74 |
| ms73.06 | 0.94±0.54 | 0.54±0.26 | 0.6±0.33 |
| ms75.944 | 1.32±0.13 | 1.21±0.13 | 1.31±0.13 |
| ms80.987 | 0.24±0.44 | 1.37±0.94 | 0.22±0.28 |
| ms81.067 | 0.61±0.27 | 0.48±0.19 | 0.4±0.13 |
| ms85.062 | 1.51±1.05 | 0.84±0.72 | 0.52±0.41 |
| ms86.069 | 6.39±3.75 | 2.52±2.18 | 1.82±1.48 |
| ms87.075 | 1.12±0.95 | 0.45±0.41 | 0.31±0.28 |
| ms88.035 | 0.95±0.65 | 0.25±0.21 | 0.26±0.18 |
| ms89.945 | 0.55±0.04 | 0.57±0.04 | 0.53±0.04 |
| ms90.945 | 1.03±0.06 | 1±0.05 | 0.98±0.05 |
| ms125.954 | 1.37±0.09 | 1.35±0.08 | 1.41±0.08 |

**Table S3.2:** Mass peaks in the headspace of anhydrous milk fat (AMF) analysed with $NO^+$ as reagent ion. The mass peaks that were found significantly different among the classes ($p < .01$) and having a concentration above 0.5 ppbV in at least two classes were selected.

| Mass | AGED | NO | YES |
|---|---|---|---|
| ms33.033 | 9.26±3.37 | 12.34±5.57 | 9.79±2.99 |
| ms43.017 | 108.57±39.68 | 44.54±13.27 | 40±22.06 |
| ms43.054 | 16.17±6.78 | 12.28±5.19 | 9.32±3.53 |
| ms44.022 | 2.8±1.03 | 1.33±0.38 | 1.1±0.59 |
| ms44.056 | 0.77±0.31 | 0.55±0.2 | 0.43±0.17 |
| ms46.991 | 13.57±1.01 | 12.14±0.37 | 13.98±1.44 |
| ms48.007 | 252.47±120.28 | 190.77±28.04 | 279.15±75.66 |
| ms49.006 | 1.29±0.55 | 0.96±0.14 | 1.41±0.37 |
| ms50.006 | 1.71±0.42 | 1.46±0.12 | 1.84±0.31 |
| ms53.038 | 0.73±0.17 | 0.52±0.13 | 0.46±0.09 |
| ms61.028 | 1.29±0.88 | 0.89±0.37 | 0.67±0.52 |
| ms62.018 | 4.15±2.28 | 1.1±0.34 | 4.81±3.14 |
| ms64.002 | 3.86±2.43 | 2.57±0.49 | 4.44±1.51 |
| ms68.058 | 2.99±0.83 | 2.12±0.63 | 2±0.57 |
| ms69.069 | 3.04±1.04 | 2.59±1.04 | 2.38±0.71 |
| ms71.085 | 3.01±1.2 | 2.15±0.79 | 1.95±0.71 |
| ms78.047 | 0.76±0.21 | 0.67±0.14 | 0.5±0.16 |
| ms81.07 | 3.36±1.04 | 2.19±0.54 | 1.94±0.59 |
| ms82.044 | 0.64±0.21 | 0.57±0.1 | 0.4±0.09 |
| ms82.075 | 1.11±0.31 | 0.94±0.27 | 0.83±0.28 |
| ms83.049 | 2.38±1.74 | 1.15±0.5 | 0.82±0.34 |
| ms84.081 | 4.74±2.02 | 2.31±1.11 | 1.87±1.3 |
| ms85.064 | 16.79±11.67 | 11.76±10.87 | 7.76±9.81 |
| ms85.102 | 1.31±0.95 | 0.84±0.46 | 0.76±0.56 |
| ms86.071 | 9.81±3.92 | 3.98±1.82 | 3.48±2.82 |
| ms88.039 | 52.37±25.41 | 10.74±7.49 | 17.58±15.57 |
| ms90.021 | 1.15±0.83 | 0.59±0.3 | 0.58±0.63 |
| ms91.055 | 1.02±0.37 | 1.83±1.02 | 1.16±0.63 |
| ms95.085 | 1.17±0.33 | 0.78±0.16 | 0.77±0.19 |
| ms98.061 | 4.86±2.04 | 2.4±1.14 | 1.88±1.36 |
| ms99.054 | 0.92±0.29 | 0.55±0.14 | 0.54±0.19 |
| ms99.08 | 3.79±1.86 | 2.08±0.85 | 1.7±1.06 |
| ms100.079 | 0.79±0.24 | 0.57±0.13 | 0.56±0.19 |
| ms102.054 | 8.8±2.08 | 4.2±1.09 | 6.06±2.02 |
| ms106.078 | 1.34±0.82 | 3.52±2.87 | 1.35±0.65 |
| ms112.079 | 0.53±0.22 | 0.36±0.24 | 0.26±0.13 |
| ms113.096 | 4.75±2.23 | 2.17±1.02 | 1.8±1.43 |
| ms114.102 | 1.96±0.95 | 0.97±0.48 | 0.74±0.61 |
| ms115.114 | 1.13±0.8 | 0.33±0.22 | 0.38±0.47 |
| ms116.07 | 24.8±10.4 | 8.7±4.84 | 7.85±7.23 |
| ms118.052 | 0.93±0.4 | 1.1±0.34 | 0.69±0.19 |
| ms126.092 | 2.3±1.08 | 1.09±0.57 | 0.82±0.69 |
| ms127.101 | 1.3±0.56 | 0.65±0.28 | 0.55±0.37 |
| ms144.101 | 13.22±6.81 | 5.54±3.18 | 4.43±4.34 |

**Table S3.3:** Balanced Error Rate (%) obtained by Partial Least Square Discriminant Analysis (PLS-DA) for the 3 ionization modes and the aggregate matrix resulting from the data fusion. In the brackets the number of components selected for each classification model is indicated.

| Data | YES vs NO vs AGED BER (n. components) | YES vs NO BER (n. components) |
|---|---|---|
| *$H_3O^+$ selected* | 22% (6) | 14% (4) |
| *$H_3O^+$ all masses* | 15% (7) | 11% (5) |
| *$NO^+$ selected* | **9%** (3) | 7% (3) |
| *$NO^+$ all masses* | 11% (6) | **4%** (6) |
| *$O_2^+$ selected* | 17% (5) | 14% (4) |
| *$O_2^+$ all masses* | 20% (5) | 20% (3) |
| *All df selected masses* | 20% (3) | 13% (2) |
| *All df all masses > 1 ppbV* | 16% (3) | 16% (2) |

**Table S3.4:** Confusion matrixes of the different test sets. Average values were obtained by a 1000-time prediction. Classification rates are reported in %. In the case of the aggregate matrix, the all masses dataframe was obtained by selecting all the mass peaks that had a threshold above of 1 ppbV.

| **Predicted vs original** | **Selected masses** | | | **All masses** | | |
|---|---|---|---|---|---|---|
| **H3O+** | AGED | NO | YES | AGED | NO | YES |
| AGED | 94 | 0 | 6 | 93 | 1 | 7 |
| NO | 13 | 59 | 28 | 7 | 69 | 24 |
| YES | 4 | 30 | 66 | 14 | 17 | 69 |
| **NO+** | AGED | NO | YES | AGED | NO | YES |
| AGED | 90 | 3 | 7 | 80 | 9 | 11 |
| NO | 3 | 71 | 26 | 7 | 81 | 12 |
| YES | 11 | 20 | 69 | 15 | 18 | 68 |
| **O2+** | AGED | NO | YES | AGED | NO | YES |
| AGED | 91 | 9 | 0 | 87 | 10 | 3 |
| NO | 6 | 88 | 6 | 1 | 86 | 13 |
| YES | 3 | 11 | 87 | 13 | 20 | 67 |
| **Aggregate matrix** | AGED | NO | YES | AGED | NO | YES |
| AGED | 97 | 3 | 1 | 88 | 0 | 12 |
| NO | 2 | 79 | 19 | 1 | 81 | 18 |
| YES | 0 | 21 | 79 | 10 | 16 | 74 |

For $H_3O^+$ and $NO^+$ both the NO and the AGED class were mostly confused with the YES class while the YES class was mostly confused with the NO class. The same is true for the aggregate matrix. For $O_2^+$ the AGED class was mostly confused with the NO class while the YES class was mostly confused with the NO class both when using all the features and the selected ones.

4

# Application of PTR-ToF-MS for the quality assessment of lactose-free milk: effect of storage time and employment of different lactase preparations.

Bottiroli R., Pedrotti M., Aprea E., Biasioli B., Fogliano V., Gasperi F.

*Journal of Mass Spectrometry*, 2020 e4505

DOI: 10.1002/jms.4505

## Abstract

Lactose-free dairy products undergo several chemical modifications during shelf life due to the reactivity of glucose and galactose produced by the lactose enzymatic hydrolysis. In this study, proton transfer reaction-mass spectrometry (PTR-MS), coupled with a time-of-flight (ToF) mass analyser, was applied to get an insight on the phenomena occurring during the shelf life of ultra-high-temperature (UHT) lactose-free milk (LFM). UHT LFMs produced by three different commercial lactase preparations were evaluated during storage at 20°C over a 150 days period, sampling the milk every 30 days. Production was repeated three times, on three consecutive weeks, to take milk variability into consideration. Principal component analysis applied to the whole "volatilome" data demonstrated the capability of PTR-ToF-MS in detecting the milk batch-to-batch variability: freshly produced milk samples were distinguished based on the week of production at the beginning of shelf life. Additionally, a clear evolution of the volatiles organic compounds (VOCs) profiling during storage was highlighted. Further statistical analysis confirmed VOCs temporal evolution, mostly due to changes in methyl ketones concentration. Differences caused by the commercial lactases did not emerged, except for benzaldehyde. All together data demonstrated PTR-ToF-MS analysis as a valuable and rapid method for the detection of changes in the VOCs profiling of ultra-high-temperature lactose-free milk.

**Keywords:** lactose-free milk, lactose hydrolysis, PTR-MS, shelf life, VOCs.

## 4.1. Introduction

Complications related to lactose malabsorption have gathered the attention of both scientists and consumers in the recent past(1). The increasing demand of healthier foods may push the preference for lactose-free milk (LFM) over conventional milk even among consumers which are not lactose-intolerant (2). In this scenario, LFM have experienced a valuable increase in market shares (3) and today represents a staple product in the diet of many consumers.

Milk sensory quality is driven by absence of odour and aftertaste, yet rejection might arise due unpleasant reaction occurring during shelf life(4). Focusing on LFM, manufacturing is not trivial and it poses additional challenges. For example, even though heating the milk at ultra-high-temperature (UHT) guarantees a prolonged shelf life (5), the presence of free glucose and galactose produced by lactose hydrolysis renders the product susceptible to Maillard reaction (6). Moreover, most UHT LFMs are produced by adding free soluble β-1,4-galactosidase (lactase) to milk (3). The commercially available options were found to contain arylsulfatase and proteolytic side activities which can potentially modify the sensory quality of milk during shelf life(2,7). In a study conducted by Stressler and co-workers (2016) for example, arylsulfatases from the β-1,4-galactosidase altered the odour of milk imparting a defect recognized as "cowshed-like"(8). Differently, proteolysis leads to the release of peptides and free amino acids in milk. The phenomena may confer bitterness and astringency to the product (9), as well as provide further substrate for formation of volatiles and non-volatile compounds by Maillard reaction (10).

Therefore, the importance of volatiles organic compounds (VOCs) in defining the quality of UHT LFM during shelf life is unquestionable. VOCs are responsible for the complexity of food flavour and aroma, as their release occur at each step of production, storage and consumption (11). In this frame, it appears relevant to develop methodologies that allow a fast and reliable analysis of VOCs to monitor the quality of food products (11). Proton transfer reaction-mass spectrometry (PTR-MS) was reported as a successful methodology for rapid, non-invasive, sensitive assessment of VOCs in food science (11,12). The instrument exploits the ionization of VOCs by proton transfer from $H_3O^+$ and registers a mass spectrum in which the measured signal and the mass/charge ratio (*m/z*) of the ions are plotted together (13). In the dairy sector, applicability of PTR-MS has been previously verified on various products such as grana-type cheeses (14,15), butter (16) and yoghurt (17). When PTR is coupled with a quadrupole detector, distinction of molecules by PTR-MS is limited (18). Better resolution and detection were achieved when the instrument was coupled with a time-of-flight (ToF) mass spectrometer (17).

Different approaches have been previously applied to evaluate the VOCs profiling of UHT LFM during shelf life. For example, dynamic headspace (DHS) GC-MS was applied by Jansson and co-workers (2014) to investigate differences in VOCs evolution between UHT LFM and conventional UHT milk over a 9-month storage (19). Fifteen VOCs were quantified using external standards and a sharper increase in concentration

was registered by the lactose-free. Together with other analysis, the study demonstrated stronger occurrence of Maillard reaction in LFM. As a solution, the same research group published an article proposing the addition of green tea extract (GTE) to UHT LFM to inhibit the proceeding of the reaction (6). They studied the volatiles profile of UHT LFM added with GTE again by DHS GC-MS and a significant decrease of specific VOCs was demonstrated. Troise and co-workers (2016) applied solid-phase micro extraction (SPME) GC-MS for the evaluation of VOCs in commercial UHT LFM samples (7). In that case, external standards were injected and the analysis was limited to specific VOCs. The results shown different temporal trend in the evolution of the compounds depending on the commercial samples. Different degrees of proteolytic activity in the lactases used by producers was suggested as main responsible for such variations.

From the studies mentioned above it was clear that UHT LFMs is highly sensitive to changes in the "volatilome" during shelf life. The production process, the lactase preparations and storage conditions play a crucial role in the definition of the final product quality. In this frame, the present study aims to investigate the application of PTR-ToF-MS to characterize how the VOCs profile of UHT LFM evolves during shelf life at 20°C. By pairing PTR-ToF-MS with an auto-sampling system, we attempt a rapid characterization of the milk thanks to the high speed of the analysis, which allowed us to measure one sample per minute. To our knowledge, despite relevant published literature on the VOCs formation in UHT LFM during shelf life, this is the first time in which the task is performed by the rapid PTR-MS technique.

## 4.2. Materials and Methods

### 4.2.1 | Chemicals

4-methyl-2-pentanone (purity ≥ 99%) used as internal standard for gas chromatography mass spectrometry (GC-MS) analysis was purchased from Sigma-Aldrich (Steinheim, Germany).

### 4.2.2 | Preparation of the lactose-free milk samples

Manufacturing of UHT LFM was carried out industrially on three consecutive weeks to include the milk batch-to-batch variability in the experimental design. A schematic drawing of each week of production is illustrate in Figure 4.1. Three different commercial lactase preparations were purchased and employed for the experiment (Lac1, Lac2 and Lac3). Semi-skimmed milk was firstly pasteurized and employed for the production of the samples. The commercial lactase was added to the milk and lactose conversion lasted for about 25-35 hours at refrigerated conditions. UHT treatment was then applied and the milk was packed aseptically. A 150-days shelf life simulation was performed, storing the samples in a climate chamber at 20°C under controlled conditions. Samples were collected at time 0 and every 30 days and stored at -80°C until the analysis was performed. A 5 mL of each UHT LFM was placed into 20 mL glass vials (Supelco, Bellefonte, PA, USA). Prior to analysis, samples were thawed at room temperature until thoroughly

defrosted. Empty vials were used as blanks while the repeated measurement of a reference milk was used as quality control.

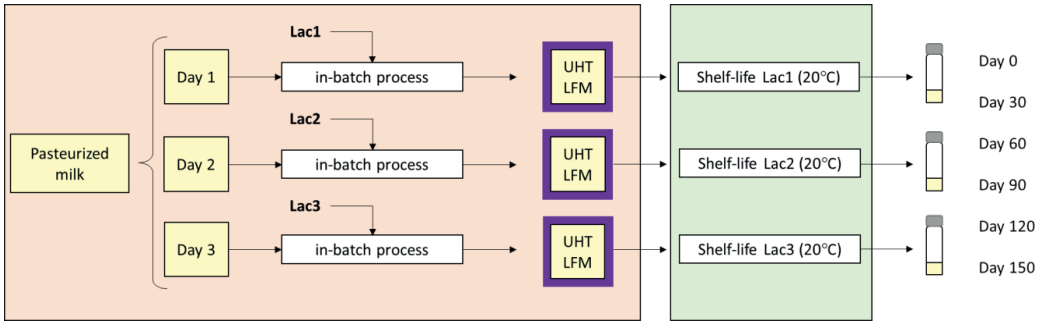



***Figure 4.1:*** *Simplified representation of the experimental design. UHT LFM was produced adding the three different commercial lactases (Lac1, Lac2, Lac3) prior to UHT treatment. Manufacturing of the three LFM occurred on three consecutive days within the same week of production. The experiment was replicated three times (namely on three different weeks of production) in the same manner to have three production replicates for each UHT LFM and take the milk batch-to-batch variability into consideration. All the LFM samples were stored at 20°C under controlled conditions for the shelf life study. As soon as after production (0 days of storage), sampling was then performed every 30 days until 150 days of storage.*



### 2.3 | GC-MS measurements

In concomitance with the PTR-ToF-MS measurements, to identify the compounds responsible for the mass peaks observed, headspace solid phase microextraction (HS-SPME) GC-MS analysis has been carried out. The analysis was carried out both at the beginning and at the end of the shelf life study (namely at 0 and 150 days of storage) on the UHT LFMs from the first week production. Measurements were based on the methodology already applied by our research group in previous studies (20). VOCs extraction was performed at 40°C for 60 min with a 2 cm DVB-Carboxen–PDMS SPME fiber. Volatiles compounds were desorbed at 250°C in the injector port of a GC interfaced with a mass detector operating in an electron ionization mode (70 eV). Mass scan ranged from *m/z* 33 to 300 (GC Clarus 500, PerkinElmer, Norwalk, CT). An auto-sampling system (CTC combiPAL, CTC Analysis AG, Zwingen, Switzerland) was used to manage the measurement in an automatic manner. A HP-Innowax fused-silica capillary column (30 m, 0.32 mm inner diameter, 0.5 µm film thickness; Agilent Technologies, Palo Alto, CA) was used for the separation of the compounds. Linear retention indices (RIs) were calculated and, using the NIST-2014/Wiley 7.0 libraries, an identification of VOCs was provided.

### 4.2.4 | PTR-TOF-MS measurements

The set-up of the measuring conditions was based on PTR-MS procedures described elsewhere(21). Briefly, a PTR-TOF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) operating in V mode (standard

configuration of the instrument) was used for measuring the samples headspace. The following ionization conditions were set in the drift tube: extraction voltage of 24.3 V, drift voltage of 628 V, drift temperature of 110°C, and drift pressure 2.78 mbar corresponding to an E/ N value of 128 Townsend (1 Td = $10^{-21}$ V·m²). The mass resolution (m/Δm) was higher than 3800. All measurements were automatically performed using an auto-sampling system (Gerstel GmbH, Mulheim am Ruhr, Germany) connected to the PTR-MS inlet, namely a PEEK capillary tube (inner diameter, 0.40 mm), heated at 110°C. Vials containing the milk samples were incubated at 50°C for 30 minutes prior to analysis. Three instrumental replicates were measured for each HLM UHT sample. Each vial was measured for 60 seconds (flow rate of 35 sccm) with an acquisition rate of one spectrum per second (m/z range: 21 – 300).

### 4.2.5 | Peak selection and data handling

Results of the extracted m/z were expressed with three decimal places. The procedure of dead time correction, peak extraction and internal calibration of the data was based on a previous article published by Cappellin and co-workers (22). The following mass peaks were used for internal calibration: 21.0221 ($H_3O^+$), 29.9974 ($NO^+$), and 203.9430 (1,3-diiodobenzene fragment). The latter was continuously injected as internal reference throughout the PTR-TOF-MS analysis using the PerMaSCal device (Ionicon, Innsbruck, Austria). The formula proposed by Lindinger et al. (1998) was applied by assuming a constant reaction rate coefficient of kR = 2 x $10^{-9}$ $cm^3/s$ to express the results in absolute concentrations (ppbV, part per billion by volume) (23). This approximation lead to a systematic error that in most cases was below 30% for all the compounds (24). Mass spectra signals were averaged over 30 spectra.

### 4.2.6 | Statistical analysis of the results

$^{13}C$ isotopologues and interference masses were removed from the dataset. Mass peaks detected in the samples were compared with the blanks via Student t-test applying Bonferroni correction to select those mass peaks significantly higher than blank. On this reduced dataset, principal component analysis (PCA) was performed after log-transformation and Pareto scaling to explore pattern in the data. For data exploration, replicates of production were kept separated to visualize the different milk batches. Loadings considered relevant in defining the trends of the PCA, namely the ones with values ≥ 0.15 and ≤ -0.15 on the 1st component and those ≥ 0.10 and ≤ -0.10 on the 2nd component, were further considered to reduce the dimensionality of the dataset. Selected mass peaks on which a tentative identification was attempted were reported in a summary table (Table 4.2). The table was also implemented with those compounds discarded by the described dataset reduction procedure but revealed by the GC analysis. Mean ± standard deviation of the 3 instrumental replicates coming from the 3 different batches of production (n=9) was further considered for each selected peak mass. 2-ways ANOVA with Tukey post-hoc was applied when necessary to investigate the evolution of the selected VOCs in UHT LFM samples. A $\alpha \leq .05$ was chosen as threshold for

significant differences. Statistical analysis was performed using the software package STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK, USA) and the R packages FactoMineR and factoextra.

## 4.3. Results and Discussions

### 4.3.1 | VOCs fingerprinting by PTR-TOF-MS

In total 368 mass peaks were extracted from the PTR-ToF-MS spectra and 268 mass peaks were found significantly higher than blanks. Principal component analysis was performed to explore the spatial distribution of the UHT LFM samples as function of the considered mass peaks. Pareto scaling was applied to adjust different fold changes among the variables and penalize the ones with large variation in the measurements, most likely associated to noise (25). Figure 4.2 shows the score plot of the 1st and 2nd principal component (PC1 and PC2), explaining respectively 43.81% and 10.90% of the total variance. The analysis distinguished the UHT LFM as function of the batch of production. Moreover, the effect of storage time was also highlighted. At 0 days of storage, the three different replicates (weeks) of production were somehow separated. The effect seemed to be explained by the combination of PC1 and PC2. Batch-to-batch milk variability appeared not relevant at intermediate and final stages of shelf life. Batch-to-batch variability in UHT lactose-free milk was already reported by Jansson and co-workers (2014) (26). In that case, variations emerged due to initial differences in ketones concentration. Many factors can render the VOCs profile of milk variable among different batches of production. For example, changes in composition of the pasture fed to cows can convey different aromas to the milk (27). From the results, we can conclude that the applied PTR-ToF-MS methodology was appropriate to discriminate UHT LFMs coming from different batches of milk. Nevertheless, from the explorative analysis the three commercial lactases (Lac1, Lac2 and Lac3) did not lead to a clear distinction of the samples, suggesting a similar VOCs profiling among the products during shelf life.

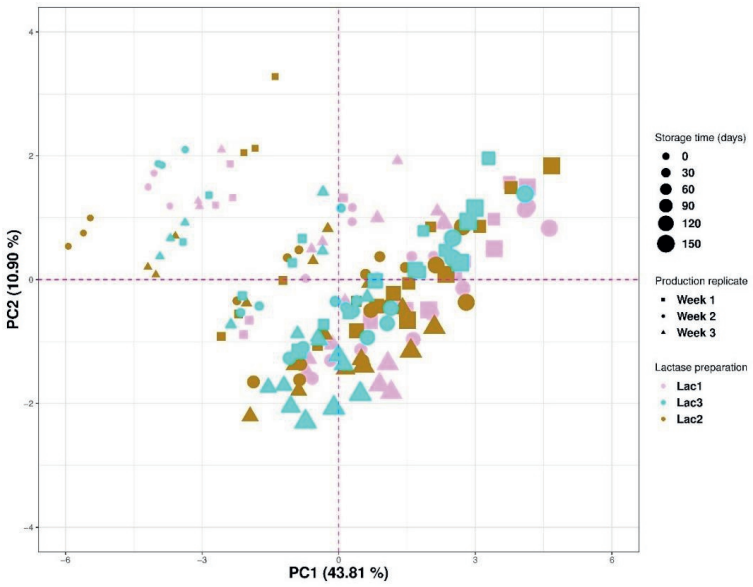



***Figure 4.2:*** *PCA score plot of the 1st (PC1) and 2nd (PC2) component, which describe respectively the 43.81% and 10.90% of the total variance. Analysis was performed on the Pareto scaled and mean-centered data including all the time points.*

### 4.3.2 | Mass peak identification by HS-SPME GC-MS

HS-SPME GC-MS analysis was carried out in support to the tentative identification of the mass peaks detected by PTR-ToF-MS. A total of 24 VOCs was identified in the headspace of the UHT LFMs (Table 4.1). The profile was in line with the literature available on the topic (7,26). The compounds detected by HS-SPME GC-MS with higher frequency were ketones. The class is considered a good indicator of deterioration of the milk upon heating, as their formation goes hand to hand with the severity of the treatment(28). Ketones were followed by aldehydes in terms of incidence. Even in this case, all the 4 identified aldehydes were reported in UHT lactose-free milk by other authors (26). Besides the detection in UHT milk straight after production, aldehydes and methyl-ketones are important VOCs to monitor because of their contribution to the stale and oxidize flavour of the milk during storage (29).

**Table 4.1:** Volatiles compounds identified by HS-SPME GC-MS in the headspace on UHT LFM produced along the first replicate (week) of production both at the beginning and the end of the shelf life study (namely at 0 and 150 days respectively).

| Volatile compounds | Rt[a] | RI[b] |
|---|---|---|
| 2-butanone[c] | 2.67 | 909 |
| 2-methylbutanal | 2.86 | 920 |
| 2-pentanone | 4.02 | 985 |
| Toluene | 5.47 | 1050 |
| Dimethyldisulfide | 6.35 | 1087 |
| Hexanal | 6.62 | 1099 |
| Ethyll-benzene | 7.91 | 1138 |
| 2-heptanone | 9.66 | 1190 |
| Xylene | 9.73 | 1192 |
| 2-nonanone | 16.56 | 1394 |
| Nonanal | 16.69 | 1398 |
| Benzaldehyde | 20.91 | 1531 |
| 1-octanol | 22.23 | 1574 |
| 2-undecanone | 23.13 | 1604 |
| γ-Butyrolactone | 24.05 | 1636 |
| Aceto-phenone | 24.77 | 1660 |
| 2-tridecanone | 29.12 | 1816 |
| Dimethyl-sulfone | 31.55 | 1908 |
| Hexanoic acid | 31.10 | 1891 |
| p-cresol | 36.18 | 2095 |
| m-cresol | 36.36 | 2102 |
| Octanoic acid | 36.42 | 2105 |
| δ-decalactone | 38.64 | 2199 |
| Decanoic acid | 42.24 | 2295 |

[a] Retention time (min)
[b] Linear retention index
[c] Compounds tentatively identified matching the NIST-2014/Wiley 7.0 libraries

Mass peaks considered relevant for the trends showed in the PCA (Figure 4.2) were investigated in more details and those tentatively identified are summarized in Table 4.2. The identity of 11 VOCs was confirmed by the results of HS-SPME GC-MS analysis. Additionally, the table was also implemented with the mass peaks whose identification was attempted based on relevant literature, the chemical formula and the fragmentation pattern (21,30,31). This led to a total 18 VOCs associated to a mass peak identified in the UHT LFMs headspace. Mass peak *m/z* = 73.064 (associated to 2-butanone/butanal) was the reported compound which registered the highest abundancy (ppbV) at the end of the shelf life period. It was followed by *m/z* = 69.070, *m/z* = 63.026 and *m/z* = 49.011. These masses were associated respectively to isoprene, dimethyl sulphide (DMS) and methanethiol (MeSH). Some compounds were present at higher concentration but, as the trend shown by the PCA was not considered relevant and a match with the GC-MS results was not found, it was chosen to leave them out from the summary table. Mass peak *m/z* = 45.033 is a clear example. It was associated to acetaldehyde, a well-known index of light oxidation in dairy products

(32). Its concentration in the headspace ranged between 3.58 ppbV and 77.08 ppbV throughout the storage of the UHT LFMs. However, the impact on the PCA (Figure 4.2) was tiny, so it was decide to exclude it from further statistical considerations.

### 4.3.3 | Effect of storage time on the VOCs profile

Two-ways ANOVA and Tukey HSD were performed to investigate the effect of storage time and commercial lactases on the reported mass peaks. The analysis highlighted the presence of significant differences among UHT LFMs due to storage time. In fact, most of the reported mass peaks were found at significantly higher concentrations at the end of the storage (150 days of storage) compared to the freshly produced samples (0 days of storage). Mass peaks tentatively associated to methyl ketones registered a significant increase during storage for all the UHT LFMs produced with the three commercial lactases. All the methyl-ketones detected by PTR-ToF-MS were also confirmed by the HS-SPME GC-MS analysis, except for mass peaks *m/z* = 129.128, associated to 2-octanone/octanal. It is not the first time that this VOC is reported in UHT milk (33). However, in our study the compound was detected at very low levels (< 0.05 ppbV). 2-pentanone (*m/z* = 87.081) and 2-heptanone (*m/z* = 115.112) were the methyl-ketones that experienced the most remarkable increase during shelf life. In the freshly produced samples, 2-pentanone was present in the range of 0.40-0.48 ppbV and increased up to 1.23-1.40 ppbV depending of the lactase preparation employed (Lac1, Lac2, Lac3). 2-heptanone followed a similar trend: it increased progressively from 0.55-0.60 ppbV and it reached 1.22-1.35 ppbV after 150 days of storage. Significant increase of methyl ketones during shelf life was already described both for conventional and UHT lactose-free milk. Jansson et al. (2014) reported higher rates of increase of methyl ketones in the latter (19). Methyl ketones are formed upon heating either by the decarboxylation of β-keto fatty acids or the β-oxidation and decarboxylation of free saturated fatty acids (34). Jensen et al. (2015) estimated the class as good predictor for the development of "stale flavour" during shelf life of lactose-free milk (10).

Even though an overall increase in VOCs in UHT LFM was registered during storage, some mass peaks followed a different trend. Mass peak *m/z* = 49.011, tentatively identified as methanethiol (MeSH) decrease during storage, although the drop was not statistically significant when biological variability of the milk was considered. Methanethiol is formed from methional and contributes, together with other volatiles sulphur compounds, to the "cooked flavour" of pasteurized and UHT milk (35). Presence of free methional in the milk could therefore justify detection of MeSH in our experiment. Temporal decrease of MeSH is reasonable because it reflects the proceeding of oxidation, which might lead to the formation of DMS and $H_2S$ (35). Protein-bound methionine is also the precursor of DMS (*m/z* = 63.026) so its significant increase might be also associated with the decrease of MeSH during storage. Thus, the result indicated possible degradation of methionine during storage of UHT LFM, already reported in the literature and potentially imputable to the proteolytic side activity originally present in the commercial lactase preparations (7).

### 4.3.4 | Effect of the different types of commercial lactase preparations on VOCs profile

Quality losses in UHT lactose-free milk has been associated with presence of proteolytic and arylsulfatase activities in the commercially available lactase preparations. Thus, besides storage time, the effect of different commercial lactase preparations (Lac1, Lac2, Lac3) on the VOCs profiling of UHT LFM was also investigated. Overall, almost all the mass peaks did not change significantly at each storage time based on the different lactases employed. At a first glance, the results might indicate that the tested commercial lactases were similar and led to an almost indistinguishable VOCs profile in the products. Alternatively, the manufacturing process might explain the similarity in VOCs profiling among the samples. The applied manufacturing process, so-called "in batch", relies on the inactivation of the lactase by heating the milk at ultra-high-temperature (UHT) before packaging and storage (7,36). With regards to the proteolytic side activity of lactase, the literature lacks information concerning the relative stability upon heating. A review published by Dekker and co-workers (2019) reported that, when lactose hydrolysis is performed before the thermal treatment, the proteolytic side activity of the lactase is inactivated (36). The finding is in line with our results and the similar VOCs profiles detected by PTR-ToF-MS analysis might be a consequence of the applied process for UHT LFM manufacturing. Nevertheless, the analysis of the variance revealed the presence of significant differences for the mass peak *m/z* = 107.049. Basically, its concentration over time increased for all the UHT LFM samples following different trends (Figure 4.3).

**Table 4.2:** Tentatively identified mass peaks in the UHT LFM samples over a 150-days storage at 20°C. Values are reported in ppbV as mean of three measurements from the three different production replicates (n=9).

| Measured m/z | Tentative Identification | GC-MS | Lac3 | | | | | | Lac2 | | | | | | Lac1 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | - | 30 | 60 | 90 | 120 | 150 | - | 30 | 60 | 90 | 120 | 150 | - | 30 | 60 | 90 | 120 | 150 |
| 43.054 | Alkyl fragment | | 0.36 | 0.36 | 0.38 | 0.37 | 0.36 | 0.39 | 0.34 | 0.34 | 0.37 | 0.33 | 0.34 | 0.37 | 0.39 | 0.40 | 0.42 | 0.39 | 0.41 | 0.43 |
| 49.011 | Methanethiol (MeSH) | | 3.64 | 2.24 | 1.53 | 1.74 | 1.40 | 2.10 | 3.39 | 2.16 | 1.85 | 1.68 | 1.77 | 1.90 | 3.08 | 2.74 | 1.97 | 1.75 | 1.89 | 1.87 |
| 63.026 | Dimethyl sulfide (DMS) | | 0.43 | 0.87 | 1.28 | 1.45 | 1.52 | 2.39 | 0.42 | 0.86 | 1.50 | 1.61 | 1.83 | 2.32 | 0.46 | 1.08 | 1.52 | 1.71 | 2.19 | 2.50 |
| 69.070 | Isoprene | | 0.44 | 1.65 | 2.74 | 2.73 | 2.72 | 3.39 | 0.47 | 1.76 | 3.16 | 2.87 | 2.99 | 3.61 | 0.51 | 1.87 | 3.30 | 3.08 | 3.59 | 3.65 |
| 73.064 | 2-butanone; butanal | X | 10.32 | 8.15 | 8.74 | 10.84 | 9.18 | 9.68 | 9.91 | 8.28 | 8.62 | 9.33 | 7.78 | 8.67 | 6.84 | 5.45 | 6.85 | 8.89 | 7.26 | 7.89 |
| 87.044 | 2,3-butanedione; γ-butyrolactone | X | 0.24 | 0.47 | 0.59 | 0.62 | 0.62 | 0.80 | 0.23 | 0.47 | 0.65 | 0.60 | 0.66 | 0.77 | 0.24 | 0.53 | 0.66 | 0.67 | 0.75 | 0.86 |
| 87.081 | 2-pentanone; 2/3-methylbutanal; pentanal | X | 0.48 | 0.80 | 1.01 | 1.05 | 1.09 | 1.39 | 0.40 | 0.73 | 0.97 | 0.94 | 1.03 | 1.23 | 0.43 | 0.83 | 1.03 | 1.03 | 1.18 | 1.40 |
| 89.060 | Butanoic Acid; Acetoin | X | 0.37 | 0.40 | 0.38 | 0.37 | 0.36 | 0.36 | 0.43 | 0.44 | 0.42 | 0.38 | 0.40 | 0.41 | 0.48 | 0.43 | 0.40 | 0.39 | 0.43 | 0.43 |
| 93.066 | Toluene | X | 0.10 | 0.10 | 0.11 | 0.10 | 0.09 | 0.10 | 0.09 | 0.10 | 0.09 | 0.09 | 0.10 | 0.11 | 0.09 | 0.09 | 0.10 | 0.09 | 0.10 | 0.10 |
| 97.062 | 2-Ethyl furan | | 0.03 | 0.04 | 0.04 | 0.04 | 0.04 | 0.05 | 0.03 | 0.04 | 0.05 | 0.05 | 0.05 | 0.05 | 0.03 | 0.04 | 0.05 | 0.05 | 0.05 | 0.05 |
| 97.101 | Heptanal fragment | | 0.07 | 0.09 | 0.09 | 0.09 | 0.10 | 0.11 | 0.06 | 0.08 | 0.10 | 0.09 | 0.10 | 0.11 | 0.07 | 0.09 | 0.10 | 0.10 | 0.11 | 0.12 |
| 101.096 | Hexanal | X | 0.09 | 0.14 | 0.16 | 0.14 | 0.15 | 0.17 | 0.08 | 0.13 | 0.16 | 0.15 | 0.15 | 0.17 | 0.09 | 0.15 | 0.18 | 0.16 | 0.17 | 0.19 |
| 107.049 | Benzaldehyde | X | 0.06 | 0.11 | 0.13 | 0.15 | 0.14 | 0.18 | 0.05 | 0.11 | 0.15 | 0.16 | 0.18 | 0.21 | 0.06 | 0.12 | 0.15 | 0.18 | 0.20 | 0.24 |
| 115.112 | 2-heptanone | X | 0.60 | 0.77 | 0.93 | 0.96 | 1.00 | 1.22 | 0.55 | 0.77 | 0.98 | 0.96 | 1.07 | 1.22 | 0.58 | 0.87 | 1.01 | 1.04 | 1.18 | 1.35 |
| 117.091 | Hexanoic acid | X | 0.08 | 0.07 | 0.07 | 0.08 | 0.07 | 0.08 | 0.07 | 0.06 | 0.07 | 0.07 | 0.07 | 0.08 | 0.08 | 0.07 | 0.07 | 0.07 | 0.07 | 0.10 |
| 129.128 | 2-octanone; octanal | | 0.03 | 0.03 | 0.04 | 0.03 | 0.04 | 0.04 | 0.02 | 0.03 | 0.04 | 0.03 | 0.04 | 0.04 | 0.03 | 0.03 | 0.04 | 0.04 | 0.04 | 0.04 |
| 143.145 | 2-nonanone; nonanal | X | 0.12 | 0.13 | 0.15 | 0.15 | 0.16 | 0.18 | 0.12 | 0.12 | 0.15 | 0.15 | 0.16 | 0.18 | 0.13 | 0.14 | 0.15 | 0.16 | 0.18 | 0.19 |
| 145.123 | Octanoic acid | X | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 |

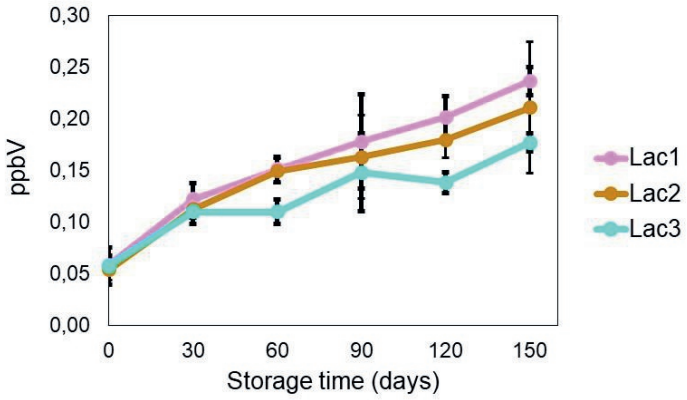



***Figure 4.3:*** *Time evolution (day 0-150) during storage at 20°C of the mass peak m/z 107.048, tentatively identified as benzaldehyde, of UHT LFMs produced with three different commercial lactase preparations (Lac1, Lac2, Lac3).*

This means that for *m/z* = 107.049, tentatively identified as benzaldehyde, the temporal evolution was dependent on the lactase preparations employed. UHT LFM produced with Lac1 and Lac2 were significantly higher in benzaldehyde both after 60 and 120 days of storage. At the end of the shelf life (150 days), Lac1 was the commercial lactase preparation causing the highest release of benzaldehyde in the UHT LFM headspace. The concentration was significantly higher than samples produced with Lac3, but not in comparison to Lac2. Benzaldehyde can be considered a thermally induced compound in milk (37). The formation may occur alongside Maillard reaction starting from phenylalanine (6). The mechanism involves the conversion of the amino acid to phenylacetaldehyde via Strecker degradation and subsequent oxidation to benzaldehyde (38). Following this pathway, our results showed different extent of benzaldehyde formations possibly due to different degrees of proteolytic side activity in the tested lactases. A study published by Troise and co-workers in 2016 already reported changes of phenylalanine and benzaldehyde in a commercial UHT lactose-free milk produced by "in-batch" technology (7). In that case, release of phenylalanine was minimum and seemed not related to an increase of benzaldehyde during shelf life. On the other hand, only one commercial milk was evaluated and the lactase preparation employed by producers was unknown. Thus, further research on the link between phenylalanine and benzaldehyde is required. Eventually, the significant increase of benzaldehyde over time might suggest the heat stability of some proteolytic side activity natively present in the commercial lactase, another aspect which should be further investigated.

## 4.4. Conclusion

In the present study, changes in the VOCs profile of ultra-high-temperature lactose-free milk (UHT LFM) during storage at ambient temperature were evaluated by PTR-ToF-MS coupled with a multipurpose autosampler. Applying PTR-ToF-MS, we significantly diminish the time of analysis. This allowed the design of a more complex experiment, in which several variables were simultaneously assessed. For example, the inclusion of different milk batches gave a realistic interpretation of industrial variability and allowed to experiment the response of PTR-ToF-MS in a complex lifelike situation. Batch-to-batch variability of the milk was highlighted by principal component analysis (PCA), which also denoted a temporal evolution of VOCs profiles of UHT LFMs during storage at 20°C. Different VOCs profiling induced by the different commercial lactases employed did not emerge for most of the identified mass peaks. Possibly, the UHT treatment, which in this case occurred after lactose hydrolysis, inactivated most of the side activity of the lactase preparations. However, the different evolution of *m/z* 107.049 (benzaldehyde) during storage might be associated with the lactase preparations employed: phenylalanine was pointed out as possible precursor of benzaldehyde formation and it can derive from the proteolytic side activity originally present in the lactases. Therefore, the study suggested benzaldehyde as possible marker to monitor in UHT LFM if the aim is to attempt a discrimination based on the lactase preparations used. However, understanding whether the slight variations found in the study can affect the final quality of the products is still uncertain.

## 4.5 References

1] A. Szilagyi, N. Ishayek. Lactose Intolerance, Dairy Avoidance, and Treatment Options. Nutrients. 2018, 10, 1994.

[2] S.D. Nielsen, D. Zhao, T.T. Le, V. Rauh, J. Sørensen, H.J. Andersen, L.B. Larsen. Proteolytic side-activity of lactase preparations. Int. Dairy J. 2018, 78, 159–168.

[3] P. Jelen, O. Tossavainen. Low lactose and lactose-free milk and dairy products - Prospects, technologies and applications. Aust. J. Dairy Technol. 2003, 58, 161–165.

[4] L.L. Francis, D.H. Chambers, S.H. Kong, G.A. Milliken, I.J. Jeon, K.A. Schmidt. Serving Temperature Effects on Milk Flavour, Milk Aftertaste, and Volatile Compound Quantification in Nonfat and Whole Milk. J. Food Sci. 2005, 70, s413–s418.

[5] E. Valero, M. Villamiel, B. Miralles, J. Sanz, I. Martínez-Castro. Changes in flavour and volatile components during storage of whole and skimmed UHT milk. Food Chem. 2001, 72, 51–58.

[6] T. Jansson, V. Rauh, B.P. Danielsen, M.M. Poojary, S.S. Waehrens, W.L.P. Bredie, J. Sørensen, M.A. Petersen, C.A. Ray, M.N. Lund. Green Tea Polyphenols Decrease Strecker Aldehydes and Bind to Proteins in Lactose-Hydrolyzed UHT Milk. J. Agric. Food Chem. 2017, 65, 10550–10561.

[7] A.D. Troise, E. Bandini, R. De Donno, G. Meijer, M. Trezzi, V. Fogliano. The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. Food Res. Int. 2016, 89, 514–525.

[8] T. Stressler, D. Leisibach, S. Lutz-Wahl, A. Kuhn, L. Fischer. Homologous expression and biochemical characterization of the arylsulfatase from Kluyveromyces lactis and its relevance in milk processing. Appl. Microbiol. Biotechnol. 2016, 100, 5401–5414.

[9] M. V Santos, Y. Ma, Z. Caplan, D.M. Barbano. Sensory Threshold of Off-Flavours Caused by Proteolysis and Lipolysis in Milk 1. J. Dairy Sci. 2003, 86, 1601–1607.

[10] S. Jensen, T. Jansson, N. Eggers, M.R. Clausen, L.B. Larsen, H.B. Jensen, C. Ray, A. Sundgren, H.J. Andersen, H.C. Bertram. Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk. Eur. Food Res. Technol. 2015, 240, 1247–1257.

[11] F. Biasioli, F. Gasperi, C. Yeretzian, T.D. Märk. PTR-MS monitoring of VOCs and BVOCs in food science and technology. TrAC - Trends Anal. Chem. 2011, 30, 968–977.

[12] L. Cappellin, F. Loreto, E. Aprea, A. Romano, J. Sánchez del Pulgar, F. Gasperi, F. Biasioli. PTR-MS in Italy: A multipurpose sensor with applications in environmental, agri-food and health science. Sensors (Switzerland). 2013, 13, 11923–11955.

[13] A.M. Ellis, C.A. Mayhew. Proton transfer reaction mass spectrometry: principles and applications, Elsevier Ltd, 2013.

[14] E. Aprea, F. Biasioli, F. Gasperi, D. Mott, F. Marini, T.D. Märk, Assessment of Trentingrana cheese ageing by proton transfer reaction-mass spectrometry and chemometrics. Int. Dairy J. 2007, 17, 226–234.

[15] E. Boscaini, S. Van Ruth, F. Biasioli, F. Gasperi, T.D. Märk. Gas chromatography-olfactometry (GC-O) and proton transfer reaction-mass spectrometry (PTR-MS) analysis of the flavour profile of Grana Padano, Parmigiano Reggiano, and Grana Trentino cheeses. J. Agric. Food Chem. 2003, 51, 1782–1790.

[16] S.M. van Ruth, A. Koot, W. Akkermans, N. Araghipour, M. Rozijn, M. Baltussen, A. Wisthaler, T.D. Märk, R. Frankhuizen. Butter and butter oil classification by PTR-MS. Eur. Food Res. Technol. 2008, 227, 307–317.

[17] C. Soukoulis, E. Aprea, F. Biasioli, L. Cappellin, E. Schuhfried, T.D. Märk, F. Gasperi. Proton transfer reaction time-of-flight mass spectrometry monitoring of the evolution of volatile compounds during lactic acid fermentation of milk. Rapid Commun. Mass Spectrom. 2010, 24, 2127–2134.

[18] A. Fabris, F. Biasioli, P.M. Granitto, E. Aprea, L. Cappellin, E. Schuhfried, C. Soukoulis, T.D. Märk, F. Gasperi, I. Endrizzi. PTR-TOF-MS and data-mining methods for rapid characterisation of agro-industrial samples: Influence of milk storage conditions on the volatile compounds profile of Trentingrana cheese. J. Mass Spectrom. 2010, 45, 1065–1074.

[19] T. Jansson, M.R. Clausen, U.K. Sundekilde, N. Eggers, S. Nyegaard, L.B. Larsen, C. Ray, A. Sundgren, H.J. Andersen, H.C. Bertram. Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. J. Agric. Food Chem. 2014, 62, 7886–7896.

[20] M. Bergamaschi, G. Bittante, F. Gasperi, A. Cecchinato, C. Cipolat-Gotet, E. Betta, F. Biasioli, E. Aprea. Effects of dairy system, herd within dairy system, and individual cow characteristics on the volatile organic compound profile of ripened model cheeses. J. Dairy Sci. 2015, 98, 2183–2196.

[21] M. Pedrotti, I. Khomenko, L. Cappellin, M. Fontana, M. Somenzi, L. Falchero, M. Arveda, V. Fogliano, F. Biasioli. Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. J. Mass Spectrom. 2018, 53, 753–762.

[22] L. Cappellin, F. Biasioli, A. Fabris, E. Schuhfried, C. Soukoulis, T.D. Märk, F. Gasperi. Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS. Int. J. Mass Spectrom. 2010, 290, 60–63.

[23] W. Lindinger, A. Hansel, A. Jordan. On-line monitoring of volatile organic compounds at pptv levels by means of Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) Medical applications, food control and environmental research. Int. J. Mass Spectrom. Ion Process. 1998, 173, 191–241.

[24] L. Cappellin, T. Karl, M. Probst, O. Ismailova, P.M. Winkler, C. Soukoulis, E. Aprea, T.D. Märk, F. Gasperi, F. Biasioli. On quantitative determination of volatile organic compound concentrations using proton transfer reaction time-of-flight mass spectrometry. Environ. Sci. Technol. 2012, 46, 2283–2290.

[25] M.J. van der W. Robert a van den Berg, Huub C J Hoefsloot, Johana Westerhuis, Age K Smilde. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics. 2006, 7, 1–15.

[26] T. Jansson, S. Jensen, N. Eggers, M.R. Clausen, L.B. Larsen, C. Ray, A. Sundgren, H.J. Andersen, H.C. Bertram. Volatile component profiles of conventional and lactose-hydrolyzed UHT milk - A dynamic headspace gas chromatography-mass spectrometry study. Dairy Sci. Technol. 2014, 94, 311–325.

[27] T. Rapisarda, C. Pasta, G. Belvedere, I. Schadt, F. La Terra, G. Licitra, S. Carpino. Variability of volatile profiles in milk from the PDO Ragusano cheese production zone. Dairy Sci. Technol. 2013, 93, 117–134.

[28] G. Contarini, M. Povolo. Volatile fraction of milk: Comparison between, purge and trap and solid phase microextraction techniques. J. Agric. Food Chem. 2002, 50, 7350–7355.

[29] M.L. Perkins, B.R. D'Arcy, A.T. Lisle, H.C. Deeth. Solid phase microextraction of stale flavour volatiles from the headspace of UHT milk. J. Sci. Food Agric. 2005, 85, 2421–2428.

[30] E. Zardin, O. Tyapkova, A. Buettner, J. Beauchamp. Performance assessment of proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) for analysis of isobaric compounds in food-flavour applications. LWT - Food Sci. Technol. 2014, 56, 153–160.

[31] E. Aprea, A. Romano, E. Betta, F. Biasioli, L. Cappellin, M. Fanti, F. Gasperi. Volatile compound changes during shelf life of dried Boletus edulis: Comparison between SPME-GC-MS and PTR-ToF-MS analysis. J. Mass Spectrom. 2015, 50, 56–64.

[32] M. Van Aardt, S.E. Duncan, D. Bourne, J.E. Marcy, T.E. Long, C.R. Hackney, C. Heisey. Flavour threshold for acetaldehyde in milk, chocolate milk, and spring water using solid phase microextraction gas chromatography for quantification. J. Agric. Food Chem. 2001, 49, 1377–1381.

[33] M.M. Calvo, L. de La Hoz. Flavour of Heated Milks. A review. Int. Dairy J. 1992, 2, 69–81.

[34] H.C. Deeth, M.J. Lewis. High temperature processing of milk and milk products, 2017.

[35] R. de Wit, H. Nieuwenhuijse. Kinetic modelling of the formation of sulphur-containing flavour components during heat-treatment of milk. Int. Dairy J. 2008, 18, 539–547.

[36] P.J.T. Dekker, D. Koenders, M.J. Bruins. Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. Nutrients. 2019, 11, 551.

[37] R.V. Potineni, D.G. Peterson. Influence of Thermal Processing Conditions on Flavour Stability in Fluid Milk: Benzaldehyde. J. Dairy Sci. 2010, 88, 1–6.

[38] F.L. Chu, V.A. Yaylayan. Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. J. Agric. Food Chem. 2008, 56, 10697–10704.

5

# Quality control of raw hazelnuts by rapid and non-invasive fingerprinting of volatile compound release

Pedrotti M., Khomenko I., Genova G., Castello G., Spigolon N., Fogliano V., Biasioli F.

*Submitted for publication at LWT – Food Science and Technology*

## Abstract

While most of the hazelnuts (*Corylus avellana* L.) are consumed after being toasted and mixed with other ingredients, for manufactures it would be important to have quality control tests on the raw product that they purchase from farmers and suppliers. In this study, the possibility to assess and predict sensory quality defects of raw hazelnuts based on volatilome analysis by proton transfer reaction mass spectrometry (PTR-MS) was explored. PTR-ToF-MS coupled with a multipurpose autosampler, was applied on three sample sets of raw hazelnuts classified according to industrial sensory evaluation. Firstly, the link between volatile markers for different visual and sensory defects was investigated. Uncompliant hazelnuts showed higher concentrations for a higher number of volatile compounds than good quality samples, included some key aroma compounds of hazelnuts. Secondly, by measuring samples constituted of different percentage of good quality and unacceptable quality hazelnuts, the method sensitivity in recognizing defects percentage was determined. For some VOCs a significant concentration difference was detected between samples containing 10 and 20% of unacceptable quality samples. Thirdly, data clustering was applied to set a sample classification model based on VOCs fingerprints obtained by different precursor ions ($H_3O^+$, $NO^+$ and $O_2^+$). The classification accuracy of unsupervised clustering was higher than 90% for all the ionization modes. The results suggest that the applied methodology is suitable to support sensory quality control programs of raw hazelnuts in confectionary industries.

**Keywords:** Proton transfer reaction mass spectrometry, rotten hazelnuts, *Corylus avellana* L., quality control, volatile metabolome.

## 5.1 Introduction

Hazelnuts *(Corylus avellana L.)* have a relevant role in agricultural market due to their nutritional value and their unique and distinctive flavour (1,2) which makes them appreciated as ingredient in a variety of food products. More than one million tons of hazelnuts were produced worldwide in 2017, being Turkey the main producer (67.1%) followed by Italy (13.1 %). The global hazelnut consumption has been evaluated of 0.52 billion USD and it has been estimated that in the next five years it will have a compound annual growth rate of 10.1% (3). Only 5% of hazelnuts production is intended for direct consumption while about 95% is used and processed by confectionary, chocolate and bakery industries (4).

Hazelnuts agroindustry market standards imply severe quality control: cultivar, cultural techniques, geographical origin, harvesting time, post-harvest management and processing, morphological and physio-chemical characteristics and aroma are the main parameters monitored to assess the final quality of hazelnuts (5–7).

The "rotten hazelnut" is one of the major defects affecting commercial quality, yield losses and market values being associated with negative sensory attributes, such as mold, old, bitter and earthy tastes (8). In commercial evaluation, rotten includes different kinds of defects like brown spotted or moldy nut kernels and can originate along the supply chain, especially during harvesting and post-harvesting stages. Sorting technologies able to recognize in a fast and non-invasive way defected nuts were tested in agroindustry and different researches tried to improve their sorting performance (9). However, most of the sorting technologies check only for visible defects which represent less than 50% of uncompliant products (8). Investigation of raw hazelnuts "volatilome" - the main responsible of hazelnuts flavour perception – could then be a valid alternative for quality control evaluation. Surprisingly, it has been evaluated only marginally as most of the studies have been focusing on volatile organic compounds (VOCs) and aroma produced after the roasting processes. Burdack-Freitag and Schieberle (2010) characterized and quantify 37 odour-active compounds in raw nuts, calculated the odour activity values of 19 odorants and tested them through aroma recombination experiments for Tonda Romana hazelnuts (11). In a recent study, Rosso *et al.* (12) presented a GCxGC-MS approach to evaluate high-quality hazelnuts volatilome evolution during the production chain. The approach showed that drying temperatures and storage have a relevant effect in affecting aroma potential.

Due to the relatively low VOCs abundancy in raw hazelnuts it is necessary to develop sensitive methodologies for industrial quality control purposes: proton transfer reaction mass spectrometry (PTR-MS) is a rapid and robust techniques that has been implemented in different food applications (13–15), also for raw material quality control (16–18).

In this paper, we coupled industrial sensory evaluation to VOC emission fingerprinting obtained by PTR-ToF-MS in combination with a multipurpose autosampler (19), a Switching Reagent Ionization (SRI)

system (20), tailored data analysis and data mining tools to build predictive models for raw hazelnuts quality. This research includes three experiments realized on different sample sets aiming at (i) identifying possible VOCs markers linked to different levels of visual defects (light, dark and moldy rotten), (ii) determining method sensitivity in detecting defects when mixing different percentages of good quality and uncompliant products to simulate industrial application and (iii) setting efficient models to predict the sensory quality of raw hazelnuts based on non-invasive and rapid PTR-MS fingerprint.

## 5.2 Material and Methods

### 5.2.1 | Hazelnut samples

For the first experiment (visual defects) two categories of raw hazelnuts samples (*Corylus avellana L.)* were considered: the good quality samples (YES), and the rotten samples. These samples were divided by the industrial evaluation in three different classes according to the degree and type of defect (8,21): samples characterized by internal discoloration of kernels which tends to opaque white to translucent, buttery yellow color (LIGHT), samples characterized by a darker color and by black spots (DARK) and samples characterized by white and green molds on the surface or inside (MOLD). These samples resulted from a visual inspection of different lots of Turkish hazelnuts (Akçakoca region) from 2017 harvest with a high percentage of rotten nuts. The visual inspection was conducted by the industrial partner on chopped hazelnuts according to the industry quality standards (see Figure 5.1). Each class of defected samples was measured as pure (100%) or as mixed with different quantities of "YES" sample (90%, 50%, 20% "YES") after being ground.

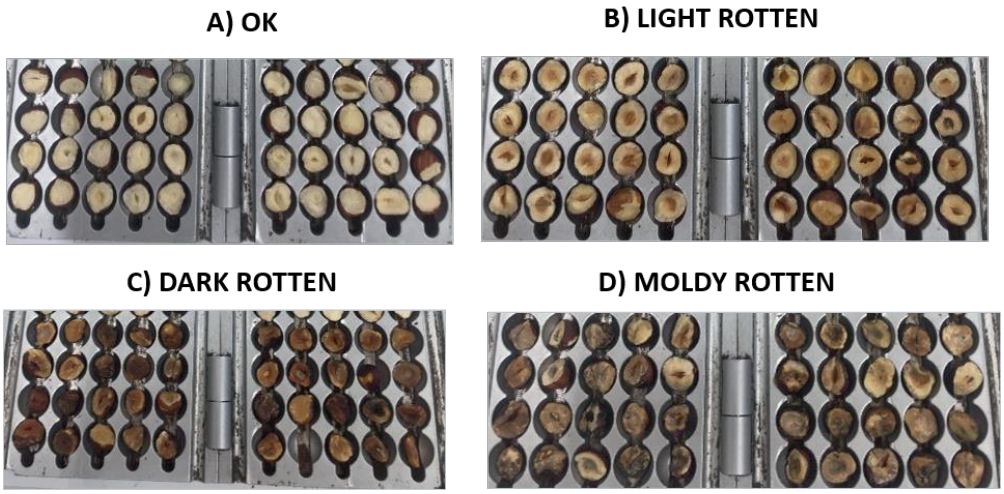



***Figure 5.2:*** *Example of raw hazelnuts samples analysed in the visual defects experiment. The rotten samples were divided in three classes of defects.*

For the second experiment (sensory defects) two classes of ground raw hazelnuts samples (*Corylus avellana L.*) were obtained: good quality samples (YES) and bad quality samples (NO). The sampling was based on industrial sensory evaluation and originated from Turkish hazelnuts (Akçakoca region) from 2016 harvest and all samples had a selected caliber of 13-14 mm. The grains were then mixed to create different levels of defects to simulate real industrial applications (Table 5.1).

**Table 5.1:** Sample description for the three experiments. For each sample the grained percentage that has been used, the number of replicates and PTR-MS ionization mode is indicated.

| Code | Number of samples (percentages) | Replicates (biologic) | PTR-MS ionization mode |
|---|---|---|---|
| | *Experiment 1: visual defects* | | |
| **YES** | 1 (100%) | 4 | $H_3O^+$ |
| **PALE** | 4 (10, 50, 80, 100%) | 4 | $H_3O^+$ |
| **DARK** | 4 (10, 50, 80, 100%) | 4 | $H_3O^+$ |
| **MOLD** | 4 (10, 50, 80, 100%) | 4 | $H_3O^+$ |
| | *Experiment 2: sensory defects:* | | |
| **YES** | 1 (100%) | 5 | $H_3O^+$ |
| **NO** | 6 (5, 10, 20, 50, 80, 100%) | 5 | $H_3O^+$ |
| | *Experiment 3: blind classification (for details see Table 2)* | | |
| **YES** | 5 x 4 (blind replicates) | 3 x 2 technical | $H_3O^+$, $NO^+$, $O_2^+$ |
| **NO** | 5 x 4 (blind replicates) | 3 x 2 technical | $H_3O^+$, $NO^+$, $O_2^+$ |
| **REF** | 1 (Tonda Romana Gentile) x 4 | 3 x 2 technical | $H_3O^+$, $NO^+$, $O_2^+$ |



For the third experiment (blind classifications) 44 samples were provided by the industrial partner in blind. These samples were four biological replicates of 11 different samples divided in classes according to industrial evaluation: good quality samples (YES), bad quality samples (NO) and some samples that represent industrial best reference for quality (REF). All samples had a selected caliber of 13-14 mm and were stored at 5 ±0.1°C with a controlled atmosphere (78% $N_2$–21%$O_2$) and with 65% of ERH (equilibrium relative humidity) except for one "YES" sample ("I") that was stored at 5°C but in a modified atmosphere (99% $N_2$–1% $O_2$). REF samples resulted from 2017 harvest and were from mono-cultivar *Tonda Gentile Trilobata*. "NO" hazelnut samples resulted from 2015 and 2016 harvests while "YES" samples from 2016 and 2017 harvests. Both "YES" and "NO" were a Turkish blend harvested in the *Ordu* and *Akçakoca* (AKC) regions. Additional details can be found in Table 5.2. Each sample was shipped as four blind replicates resulting in a total of 44 samples.

All raw hazelnuts from the three experiments, once collected from the industrial partner were stored at -20°C ± 1°C. An overview of the samples measured is presented in Table 5.1. For the sake of clarity, in the paper we will refer to the experiments as: visual defects, sensory defects and blind classification experiments.

**Table 5.2:** Additional details on the samples of the third experiment. Different hazelnuts from different regions, different harvest years and with a different storage (controlled atmosphere) were selected. All the unshelled hazelnuts were stored at a 5°C temperature.

| Code | Origin | Year | Storage atmosphere | Tag replicates | | | | Sensory | Sensory description |
|---|---|---|---|---|---|---|---|---|---|
| A | AKC | 2016 | 78% $N_2$–21%$O_2$ | 1 | 2 | 3 | 4 | NO | Rancid |
| B | ORDU | 2016 | 78% $N_2$–21%$O_2$ | 5 | 6 | 7 | 8 | NO | Weak old |
| C | AKC | 2016 | 78% $N_2$–21%$O_2$ | 9 | 10 | 11 | 12 | NO | Old |
| D | ORDU | 2015 | 78% $N_2$–21%$O_2$ | 13 | 14 | 15 | 16 | NO | Mold, rancid |
| E | AKC | 2015 | 78% $N_2$–21%$O_2$ | 17 | 18 | 19 | 20 | NO | Mold, rancid |
| F | AKC | 2016 | 78% $N_2$–21%$O_2$ | 21 | 22 | 23 | 24 | YES | |
| G | AKC | 2016 | 78% $N_2$–21%$O_2$ | 25 | 26 | 27 | 28 | YES | |
| H | ORDU | 2016 | 78% $N_2$–21%$O_2$ | 29 | 30 | 31 | 32 | YES | |
| I | AKC | 2016 | 99% $N_2$–1% $O_2$ | 33 | 34 | 35 | 36 | YES | |
| L | AKC | 2017 | 78% $N_2$–21%$O_2$ | 37 | 38 | 39 | 40 | YES | |
| REF | Piedmont | 2017 | 78% $N_2$–21%$O_2$ | R1 | R2 | R3 | R4 | REFERENCE | Tonda Gentile Trilobata |

### 5.2.2 | Sample preparation

Raw hazelnuts for the visual defects and blind classification experiments were ground by a IKA® A11 basic analytical mill (IKA, China) under liquid nitrogen to keep samples frozen and to ensure uniform particle size distribution. This was done to include intra-batch variability and to estimate biologic variability. Approximately 15 grams of raw hazelnuts were grinded each time. 3.00±0.05 g grain hazelnuts were then transferred into 20 mL vials, which were previously conditioned for 1 day at 65°C in vials. For the visual defects experiment, measurements were performed in four replicates by preparing four different grains for each sample. For each lot of the blind classification experiment, three hazelnuts grains were prepared and measured in duplicate. For the sensory defects experiments, hazelnuts grains were already available and five replicates of 3.00 ± 0.05 g grain for each sample were prepared. All samples were kept at 6°C until PTR-MS analysis.

### 5.2.3 | Hazelnut sensory analysis

For both the sensory defects and the blind classification experiment, sensory evaluation was carried out by 30 internal judges according to the "A – not A" test (ISO 8588:2017) where the sample under evaluation is compared to an industrial reference standard. First, panellists were instructed to evaluate odour intensity of the samples. Then, judges were asked to assess the flavour intensity by tasting the sample. After the evaluation, judges were asked to rinse the mouth with water. For the blind classification

experiment a flash profiling (22,23) with 15 panellists (aged between 30-50 years, 6 women) was also conducted to give an indication of the aroma defect for the "NO" samples.

### 5.2.4 | PTR\SRI-ToF -MS analysis

All measurements were performed by using a multipurpose GC sampler (Gerstel GmbH, Mulheim am Ruhr, Germany) connected to PTR-ToF-MS through an heated PEEK capillary tube (D= 1 mm, T= 110°C) as previously described (24). All samples were incubated at 50°C for 25 minutes for headspace equilibration and then measured for 60 seconds with an acquisition rate of one spectrum per second and a flow rate of 35 sccm. The measurement order was randomized, and, after each measurement, a waiting time of 3 minutes was set to prevent memory effects. Empty vials were used as blanks.

A commercial PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) in its standard configuration (V mode) was used. The instrument was equipped with a SRI system that allowed operation in $H_3O^+$, $NO^+$ or $O_2^+$ modes as described elsewhere (25,26). SRI was used only for the blind classification experiment. The instrumental conditions were as following: drift pressure 2.80 mbar, drift temperature 110°C, ion source current and drift voltages were adjusted according to the ion mode to get the optimal instrument conditions. Ion source current was set at 3.5 mA for $H_3O^+$ mode, 5.0 mA for $NO^+$ and $O_2^+$ ones. A drift voltage of 537, 548 and 458 V was used for $H_3O^+$, $NO^+$ and $O_2^+$, resulting in an E/N value of 128, 132 and 105 Td respectively. For the first and second experiment a radio frequency ion funnel to improve sensitivity (27) was used which resulted in a different drift voltage (628 V) and a ion funnel voltage of 18.2 V. In all cases, the mass resolution (m/Δm) was at least 3800, and data were collected for the mass range m/z from 20 to 250.



### 5.2.5 | Data processing

PTR\SRI-ToF-MS spectra were processed according to the procedure described elsewhere (28,29). In brief, internal calibration of the mass axis by using mass peaks 21.0221 ($H_3{}^{18}O^+$), 29.9974 ($NO^{+)}$, and 203.9430 (fragment of 1,3-Diodobenzene used as internal gas standard), dead time correction and peak extraction were performed to reach a mass accuracy of ~0.001, sufficient for determining sum formula of volatile compounds. Peak intensities from the mass spectra were converted in concentrations in ppbV (parts per billion by volume) according to the procedure reported in Lindinger *et al.* (30), assuming a constant reaction rate coefficient ($k = 2 \times 10^{-9}$ $cm^3$ $s^{-1}$) which leads to a systematic error in the concentration estimation below 30% (31,32). For each sample, the average of the first 40 spectra of the headspace measurement was used for further data analysis. The experimental *m/z* values are reported up to the third decimal digits.

### 5.2.6 | Data analysis

For the first and the second experiment the data extraction of PTR-ToF-MS spectra provided 292 mass peaks for $H_3O^+$ mode. For the third experiment the data extraction of PTR/SRI-ToF-MS spectra provided 232 mass peaks for $H_3O^+$ and $NO^+$ while 304 mass peaks were extracted for $O_2^+$ mode. For all the data sets, a mass peaks selection procedure was applied to extract relevant information and reduce the noise signals associated to PTR-ToF-MS measurements by excluding: i) all peaks with a concentration not significantly higher than the blanks (t.test with $p < .01$ after Bonferroni correction for multiple tests), ii) $^{13}C$ isotopologues with an exception when the parent ion had a saturated signal (*e.g. m/z* 43.021, 69.07 and 73.065) and iii) signals related to interfering ions at *m/z* 29.997 ($NO^+$), 32.998 ($O_2^+$ isotope), 37.032, 55.039, 73.050 (water clusters), 44.997 ($CO_2^+$). This procedure allowed to select, in case of the first and second experiment, 179 and 212 mass peaks. For the third experiment 120 mass peaks for $H_3O^+$, 104 for $O_2^+$ and 105 for $NO^+$ were selected which were used for further statistical analysis.

For all experiments, preliminary principal components analysis (PCA) was performed after logarithmic transformation and mean centering of the mass peaks of the reduced data frames, for data visual inspection. One-way ANOVA and post-hoc test (Tukey honest significant difference) with Bonferroni corrections ($P < .005$) was performed to find the mass peaks that were significantly different for the hazelnuts classes. A further noise reduction was obtained by considering only mass peaks above a threshold of 0.5 ppbV for at least one of the classes. For the visual defects experiment, the classes with 100% of defects were used. The results of the analysis for the visual and sensory defects experiments were summarized in a table. Tentative peak identification was performed by using the in-house library developed by the authors and through literature review (10,12,33–36).

Finally, the reduced data sets obtained with the different ionization modes from the blind classification experiment, were scaled (mean centering and unit variance) and represented as heat maps. Firstly, a, K-mean clustering (with $k = 2$) was performed on the samples (columns) to divide between "YES" and "NO" classes. A hierarchical clustering on Manhattan distances with a Ward clustering method (37) was then applied on each of the two groups. For the mass peaks (rows) only hierarchical clustering was applied. The same was applied also to the data from the other ionization modes ($NO^+$ and $O_2^+$).

Data analysis was performed with core functions of R programming language and its external packages (38,39) (ChemometricsWithR, mixOmics, multcomp, vegan, matrixStats, ComplexHeatmap, ggplot2) and Excel (version 14.0.7224.5000).

## 5.3 Results

### 5.3.1 | The first experiment: VOCs linked to visual defects

As presented in Figure 5.2, the first two components of PCA analysis explain about 79% of the total variability and the different hazelnuts classes (YES, LIGHT, DARK, MOLD) are distinguishable. The different hazelnuts are distributed in the space as clusters: particularly the PC1 separates the "YES" samples from the "DARK" and "LIGHT" while the PC2 separates the "YES" class from the "MOLD" class. According to the loadings plot, most of the mass peaks contributes to the PC1 and have a higher concentration for the "DARK" and "LIGHT" classes. Research on specific causes of mold in hazelnuts is still scarce but most probably these two types of visual defects are originated by different type of microorganisms. While *Mycospharella punctiformis* has been associated to necrosis of kernel tips (black spots) and *Nematospera coryli* to kernel dark spots, *Diaphorte* genus and *Septoria ostryae* are commonly associated to the internal discoloration of kernels (8). The higher the percentage of these defects in the samples, the higher is the concentration of the mass peaks characterizing the PC1 and is possible to separate the "DARK" and "LIGHT" classes, indicating that different microorganisms produce different aroma compounds. The "MOLD" class is characterized by few specific mass discussed more in details in the next section while the "YES" class has a lower concentration of all VOCs.

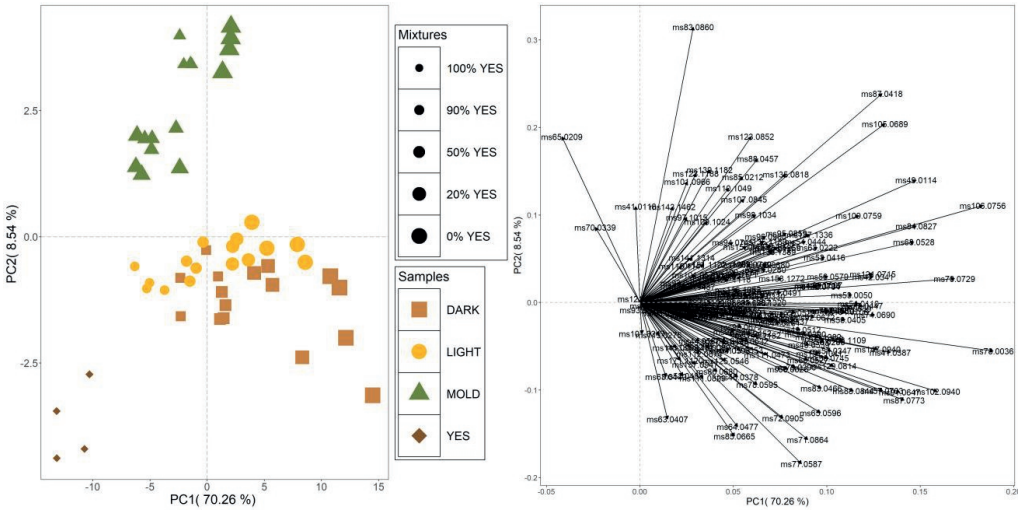



***Figure 5.2:*** *Score and loading plots of principal component analysis (PCA) for the first experiment (visual defects). The first two principal components are shown. The different colors indicate the different sample classes while the different sizes represent the different percentage of mixture of the samples. For each sample the four replicates are shown.*

The distinction of the defected classes observed with the PCA was confirmed by univariate data analysis. Univariate data analysis found 105 mass peaks significantly different for at least one of the classes ($P < .005$) and are reported in Table S5.1 (supplementary materials). Table 5.3 is a reduced version of Table S5.1 where is reported a selection of the key compounds of hazelnuts (10,12,33–36) characteristic for each class.

No peak was significantly higher for "YES" samples. This result is in line with the previous observation from the PCA where the "YES" samples were characterized by low intensities for most of the measured VOCs. The "DARK" class showed the highest portion of mass peaks significantly higher than the other classes (73%). The mass peaks 41.039, 42.034 and 87.077 tentatively identified respectively as an alkyl fragment, acetonitrile and 2/3-methylbutanal/2-pentanone/pentanal were the most concentrated compounds. The "LIGHT" class had 18 mass peaks with the highest concentration. Among these *m/z* 81.070, 89.061, 99.083, 101.058 and 155.148 are associated to linalool/monoterpenes fragment, butanoic acid/ethyl acetate/acetoin, 2,3-pentanedione and 2-decenal/linalool. The "LIGHT" class, together with the "MOLD" class had also high levels of *m/z* 115.114 tentatively identified as a mixture of different compounds, including prenyl ethyl ether. This compound has been identified as a key component for a metallic, solvent like off-flavour in hazelnuts when present together with significant concentrations of hazelnuts terpenes (40,41). The "MOLD" class had as well other 6 mass peaks that showed higher concentrations then the other classes: *m/z* 83.086, 87.042, 101.097, 109.102, 139.118 and 143.146. Most of these mass peaks were tentatively identified as aldehydes and ketones like the *m/z* 101.097 which could be hexanal, 3-methyl-2-pentanone, its isomer 2-hexanone or a contribution of all the three different molecules. The measurements performed on the third experiment with $NO^+$ as primary ion - able to separate aldehydes and ketones (42) - can give further indications to disentangle the contributions of each compound. The *m/z* 99.082 resulting from the hydride ion transfer reaction of the aldehyde with $NO^+$ presented a comparable concentration to the *m/z* 101.097 obtained from the $H_3O^+$ dataset and associated to hexanal. On the other hand, the *m/z* 100.086 and 130.087 in the $NO^+$ dataset, that could correspond respectively to the 3-methyl-2-pentanone deriving from the charge-transfer reaction and to the 2-hexanone deriving from the ion-molecule association reaction with $NO^+$ (depending on their different ionization energy), had lower concentrations (43) than the aldehyde. Hexanal and its fragment (*m/z* 83.086) originates from oxidation of unsaturated fatty acids, in hazelnuts mostly oleic and linoleic acid (44–46). Fungal growth, together with other factors, is known to be related to occurrence of oxidative processes and changes in the activity of hydrolytic enzymes (9,47). Therefore, the higher concentration of hexanal in the "MOLD" class possibly derives from an augmented hydrolysis of fatty acids due to fungal activity.

**Table 5.3:** In the table is presented a selection of tentatively identified mass peaks from the first and second experiment. These mass peaks were found significantly different among the classes ($P. <.005$, Bonferroni correction), with a concentration higher than 0.5 ppbV.

| Mass peak | Chemical formula | Tentative ID | 100% YES | 95% YES | 90% YES | 80% YES | 50% YES | 20% YES | 0% YES | YES | LIGHT | DARK | MOLD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ms41.039** | C3H5+ | Alkyl fragment | 34.1±8.2a | 36.8±4.9a | 41.6±6.8a | 48.4±6.4a | 81.9±16.3b | 91.3±13.7b | 148.9±29.2c | 47.7±6.7a | 517±141.2b | 876.2±214.5c | 197.5±4.3a |
| **ms42.02** | | | 31.8±9.4ab | 27.3±8.6a | 26.7±9.4ab | 36±11.8ab | 47.5±14.6bc | 53.6±16.8c | 60.9±19.6c | 14.6±1.6a | 33.3±4.2c | 38.4±1.9c | 24±0.7b |
| **ms49.011** | CH4SH+ | Methanethiol | 0.1±0a | 0.2±0ab | 0.3±0b | 0.5±0c | 1.1±0.1d | 1.6±0.2e | 1.7±0.3e | 1±0.1a | 14.9±1.9b | 52.1±10.8c | 23.5±4.2b |
| **ms74.069** | C[13]C3H9O | 2-butanone isotope / 2-methylpropanal isotope | 1.9±0.5a | 1.8±0.3a | 1.9±0.3a | 2±0.3a | 2.3±0.4ab | 2.2±0.3a | 2.7±0.4b | 2.8±1.6a | 38.7±9.8b | 39±12.5b | 17.6±0.7a |
| **ms81.07** | C6H8H+ | Linalool fragment / monoterpenes fragment | 4.8±1.2a | 6.6±1.6a | 9.2±2.4a | 20.6±3.6b | 58.6±21.8c | 86.9±10.8d | 94.8±6.1d | 33.2±5.8a | 131±34.3b | 122.5±60.7b | 77.4±8.7ab |
| **ms83.086** | C6H11+ | Hexanal fragment | 6±2.4a | 9.5±1.6ab | 14.4±4.5ab | 20.4±6b | 91±22.3c | 103.5±13.4c | 133.8±11.3d | 6.8±1.9a | 58.7±17.8b | 18.9±5.3a | 88.2±14.3c |
| **ms85.067** | C5H8OH+ | 3-penten-2-one / (E)-2-pentenal | | | | | | | | 16.9±5.1a | 17.6±4.5a | 46±5.1b | 11.4±0.7a |
| **ms85.099** | C6H13+ | 1-Hexanol fragment (dehydratation) | 0.5±0.2a | 0.6±0.2a | 0.8±0.3a | 1.5±0.3a | 4.5±1.6b | 4.6±0.9b | 9.9±1.7c | 0.9±0.2a | 2.4±0.1b | 3.6±0.8c | 1.6±0.3ab |
| **ms87.042** | C4H6O2H+ | 2,3-butanedione | 2.3±0.7a | 2.6±0.5a | 2.4±0.3a | 2.8±0.4a | 2.9±1ab | 4.3±1.3bc | 4.4±1.6c | 4.7±3a | 67.3±19.5b | 114.8±42.5c | 120.9±9.3c |
| **ms87.077** | C5H10OH+ | 2/3-methylbutanal/ 2-pentanone / pentanal | 38.7±10.2a | 38.5±6a | 41.7±8.3a | 42.9±6.5a | 54.3±10.7b | 49.3±8.6ab | 67.3±17.6c | 88±21a | 567.3±152.3b | 1841±156.6c | 264.1±6.7a |
| **ms89.061** | C4H8O2H+ | butanoic acid / ethyl acetate / acetoin | 5±1.4ab | 4.6±0.6a | 4.5±0.9a | 5.5±0.6ab | 5.8±1.1bc | 6.6±1.4cd | 7.7±1.6d | 6.2±1.1a | 54.4±9.3c | 50.1±2.5c | 31.6±1.4b |
| **ms91.065** | C4H10O2H+ | 2,3-butanediol | 1±0.4a | 1.1±0.2a | 1.1±0.2a | 1.2±0.2ab | 1.5±0.3bc | 1.6±0.3c | 2.2±0.4d | 1.7±0.7a | 14±5.4a | 41.8±15.4b | 5.1±0.2a |
| **ms99.083** | C6H10OH+ | 2-hexenal | 0.5±0.1a | 0.6±0.1a | 0.6±0.1a | 0.7±0.1a | 0.9±0.2b | 1±0.1b | 1.4±0.2c | 0.9±0.1a | 1.8±0.4c | 1.5±0.1bc | 1.2±0.2ab |
| **ms101.058** | C5H8O2H+ | 2,3-pentanedione | 1.2±0.3b | 1.1±0.2b | 1.1±0.2b | 1±0.1b | 1±0.3b | 0.9±0.4b | 0.5±0.2a | 1.7±0.2a | 4.6±1.8b | 2.9±0.3ab | 4.2±0.1b |
| **ms101.097** | C6H12OH+ | Hexanal, 3-methyl-2-pentanone, 2-hexanone | 2.3±0.8a | 3.2±0.5a | 3.9±0.9a | 5±1.1a | 16.1±4.5b | 16±3b | 34±7.9c | 4.1±0.7a | 11±2.6bc | 7.6±1.2b | 14.6±2.4c |
| **ms109.102** | C8H13+ | Monoterpene fragment | 0.2±0.1a | 0.3±0a | 0.2±0a | 0.4±0.1a | 0.8±0.3b | 0.9±0.1b | 1.4±0.2c | 0.5±0.1a | 1.2±0.2ab | 1.6±0.1b | 1.9±0.7b |
| **ms115.114** | C7H14OH+ | 2/4-heptanone / heptanal/ prenyl ethyl ether | 1.1±0.3a | 1.4±0.2a | 1.4±0.2a | 1.8±0.3a | 3.4±0.8b | 3.9±0.6b | 6.4±1.2c | 2.1±0.3a | 4.1±0.5b | 3.2±0.7b | 4.3±0.4b |
| **ms127.112** | C8H14OH+ | 5-methyl-(E)-2-hepten-4-one / 2-octenal / 2-ethyl-2-hexenal | 0.3±0.1a | 0.3±0a | 0.3±0a | 0.4±0.1a | 0.7±0.2b | 0.8±0.1b | 1.2±0.2c | 0.5±0.1a | 1.2±0.2b | 2±0.1c | 1.1±0.2b |
| **ms129.081** | C7H12O2H+ | 5-propyldihydro-2(3H)-furanone | 0.11±0.03c | 0.12±0.02c | 0.12±0.01c | 0.11±0.02bc | 0.09±0.03b | 0.08±0.02b | 0.04±0.03a | 0.2±0a | 1.1±0.1b | 2.1±0.3c | 0.4±0a |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ms129.128** | C8H16OH+ | 3/5-methyl-4-heptanone / octanal / 2-octanone | 0.6±0.2a | 0.8±0.1a | 0.9±0.1a | 1.2±0.2a | 3±0.9b | 2.9±0.5b | 5±1c | 0.9±0.6a | 3.4±0.5b | 3.9±1b | 3.1±0.2b |
| **ms131.104** | C7H14O2H+ | Ethyl 2-methylbutanoate / heptanoic acid | 0.2±0a | 0.2±0a | 0.2±0a | 0.2±0.1a | 0.4±0.1b | 0.5±0.1b | 0.6±0.1c | 0.4±0.1a | 2.6±0.5b | 2.9±0.7b | 0.8±0.1a |
| **ms135.119** | C10H15+ | p-cymene | 0.1±0a | 0.1±0a | 0.1±0ab | 0.1±0b | 0.2±0.1c | 0.3±0d | 0.4±0.1e | 0.2±0a | 0.9±0.1c | 1.3±0.1d | 0.6±0b |
| **ms137.134** | C10H17+ | Monoterpenes, linalool fragment, 2-decenal fragment | 1.7±0.5a | 2.3±0.7a | 3.4±1a | 8.3±1.7a | 26.3±10.9b | 44±7.8c | 73.8±17.6d | 14.9±2.7a | 85±9.5b | 168.9±40.7c | 93.3±25.8b |
| **ms139.118** | C9H14OH+ | 2,4-nonadienal / 2-pentylfuran | 0.2±0a | 0.2±0ab | 0.2±0ab | 0.3±0b | 0.5±0.1c | 0.6±0.1d | 0.8±0.1e | 0.3±0a | 0.8±0.1b | 1.2±0c | 1.5±0.2d |
| **ms143.146** | C9H18OH+ | 3,5-dimethyl-4-heptanone / 2(3H)-furanone,5-butyldihydro / nonanal / 2-nonanone | 0.2±0.1a | 0.2±0a | 0.2±0a | 0.3±0.1a | 0.7±0.2b | 0.6±0.1b | 1±0.2c | 0.3±0a | 0.6±0.1b | 0.6±0.1b | 0.9±0.1c |
| **ms153.127** | C10H16OH+ | 2,4-decadienal | | | | | | | | 0.1±0a | 0.5±0b | 0.7±0.1c | 0.4±0b |
| **ms155.148** | C10H18OH+ | 2-decenal / linalool | 0.02±0.01a | 0.02±0.00a | 0.02±0a | 0.03±0a | 0.1±0.01b | 0.1±0b | 0.1±0c | 0.02±0.01a | 0.17±0.03c | 0.16±0.02c | 0.11±0.02b |

This leads to an increased production of free fatty acids that then, through auto-oxidation reactions, could lead to develop of rancidity, off-flavours and bitterness (48). The presence of specific volatile markers for the "DARK" and the "LIGHT" classes corroborates the hypothesis that the two classes of rotten defects originates from different type of microorganisms which affects the fruit metabolism differently.

### 5.3.2 | The second experiment: sensory defects linked to VOCs threshold

In the sensory defects experiment different mixtures composed by "YES" and "NO" samples were analysed. The results from the univariate data analysis, where 69 mass peaks were found to be significantly different in at least one of the mixtures ($P < 0.005$), are also shown in Table 5.3 and S5.1. These results confirmed the trend found for the visual defects experiment: samples with higher percentage of "NO" (defected) sample had higher VOCs emission. When comparing the 100% "YES" *vs* 0% "YES" all the mass peaks but two - *m/z* 101.058 and 129.081 tentatively identified as 2,3-pentanedione and 5-propyldihydro-2(3H)-furanone - resulted to have a significantly higher concentration in the 0% "YES" class. It may be that when these compounds are over a certain concentration, the samples are penalized by industrial sensory evaluation and indicated as non-compliant.

Some mass peaks were tentatively identified as key aroma compounds of raw hazelnuts aroma (11,35) as. *e.g. m/z* 129.128 which was tentatively associated to 3/5-methyl-4-heptanone, octanal or 2-octanone (Figure 5.3A). The 5-methyl-4-heptanone is known for its *fruity and hazelnuts-like* aroma, it has a very low odour threshold (0.2 µg/kg in oil) and the highest odour active value after linalool in raw hazelnuts (10,11). The same authors hypothesized that this compound is biochemically formed in the raw nut since the compound has been observed to decrease during roasting. This mass peak could be associated also to octanal (11,12). Again, to better determine the contribution of the aldehyde and the ketones for *m/z* 129.128 it is useful to examine the $NO^+$ data in the blind classification experiment. In this case, m/z 158.118 corresponding to the compound obtained by the ion-ketone association reaction ($M*NO^+$), has a concentration corresponding to the 10% (on average 0.1 ppbV) of *m/z* 127.114 resulting from the charge-transfer reaction of the octanal with $NO^+$. Based on these evidences, the authors believe that the concentration of *m/z* 129.128 showed in Figure 5.3A is mainly due to the contribution from octanal, even if, the contribution of the ketones mix (3/5-methyl-4-heptanone and 2-octanone) to the aroma, is still relevant due to their low odour threshold.

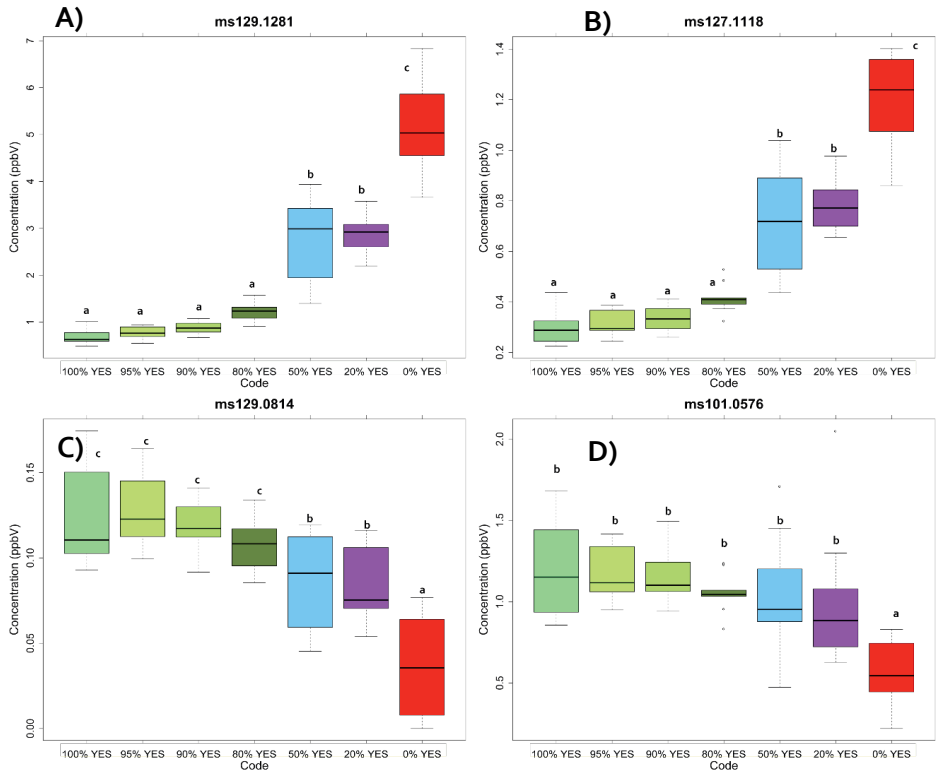



***Figure 5.3:*** *Boxplots of four relevant mass peaks for the samples of the sensory defects experiment. A) m/z 129.128 ($C_8H_{16}OH^+$) tentatively identified as 3/5-methyl-4-heptanone, octanal or 2-octanone. B) m/z 127.118 ($C_8H_{14}OH^+$) tentatively identified as 5-methyl-(E)-2-hepten-4-one, 2-octenal and/or 2-ethyl-2-hexenal. C) m/z 129.081 ($C_7H_{12}O_2H^+$) tentatively identified as 5-propyldihydro-2(3H)-furanone. D) m/z 101.058 ($C_5H_8O_2H^+$) tentatively identified as 2,3-pentanedione.*

*m/z* 127.112 (Figure 5.3B) was tentatively identified as 5-methyl-(E)-2-hepten-4-one, 2-octenal and/or 2-ethyl-2-hexenal. The 5-methyl-(E)-2-hepten-4-one, also known as "filbertone", is a key flavour compound in both raw and roasted hazelnuts. It occurs in two enantiomeric forms but the (S)-enantiomer was shown to be the prevalent form in hazelnuts (49). This compound has been evaluated as quality marker for determining hazelnut content of different hazelnut pastes (50,51) due to the fact that its content increases during roasting. Filbertone aroma has been described has *fruity*, *hazelnut* and *dried fruit* at low threshold (0.05 µg/L in water at 25°C) (52) while at higher concentrations (> 25 ppbV in water) the compound tends to smell metallic (53).

When looking at the technique sensitivity in discriminating percentage of defected sample, the most common trend is the one showed by m/z 129.128 (Figure 5.3A) where a significant difference was found between the 80% and 50% "YES" samples. About 58% of the mass peaks reported in Table S5.1 had the

same trend, indicating that the technique can discriminate between samples contaminated with 20% and 50% ground hazelnuts of poor quality. However, about 15% of the mass peaks in Table S5.1 had a significant difference between the samples made with 90% and 80% levels of "YES" samples like for example, *m/z* 81.070 - tentatively identified as a fragment of linalool and as a fragment of different monoterpenes. These markers have a more stringent cut-off value for discriminating samples quality and could then be considered for potential applications for quality control.

Figure 5.3C shows *m/z* 129.081 tentatively identified as 5-propyldihydro-2(3H)-furanone (or γ-heptalactone). This lactone, used as food additive to deepen fatty notes of nut flavours, has been identified in many different fruits as well as in hazelnuts (12). In this case, even if at low concentrations, the trend is inverse: increasing the quantity of "NO" sample decreased the compound concentration. A similar trend is highlighted as well for *m/z* 101.058, tentatively identified as 2,3-pentanedione (Figure 5.3D). This molecule, a sugar degradation product which gives a *sweet, buttery* and *caramel-like* odour (54), was found significantly lower for the 0% "YES" sample (Table 5.3). This indicates that good quality hazelnuts need to have a minimal concentration of 2,3-pentanedione. These results indicate that not only the presence or the absence of a key odorant is fundamental, but as well the concentration levels plays a role in determining the final raw hazelnuts quality.

### 5.3.3 | Blind classification experiment: hierarchical clustering and heat map

In Figure 5.4 is presented the heat map for the selected mass peaks ($H_3O^+$ mode) of the blind classification experiment samples. Colors in the figure indicate concentration for each mass peak from blue (low) to red (high). The K-means clustering (k=2), separated the 44 samples into two main clusters corresponding to "NO" and "YES" (including the REF) classes. Only two samples (23 and 26), corresponding to two different lots from the same harvest year (2016) and the same location (OLT, see Table S5.2), were misclassified leading to a classification accuracy of about 95%. The misclassification was overcome when supervised classification model was applied (PLS-DA, data not shown) due to the possibility to use the samples classification information to build, train and test the model. The blind replicates were classified correctly, highlighting the good reproducibility of the method.

When looking at the mass peaks, two main clusters are visible (C1 and C2 in Figure 5.4). The C1 cluster contains most of the selected mass peaks that were also found in the previous two experiments. The heatmap shows that most of them had higher concentrations for the "NO" samples, confirming that good quality samples are characterized by low volatiles emissions. An exception can be noticed for the replicates 33-36 coming from the sample "I", the one stored at controlled atmosphere (99% $N_2$–1% $O_2$), which have the highest concentrations for mass peaks tentatively associated to fragment of 2-methyl-1H-pyrrole, linalool, 2-decenal and as monoterpenes. Previous studies not only showed the presence of α-pinene and limonene in raw hazelnuts (10,12,54), but they proofed that reducing oxygen content in the storage atmosphere had a significant effect in limiting oxidation phenomena and maintaining fruits quality (55–57). Moreover, Rosso

*et al*. (2018) reported lower levels of VOCs known to be secondary products of lipid oxidation like hexanal, octanal and 2-heptenal, in hazelnuts stored in protective modified atmosphere (99%$N_2$ -1%$O_2$). In our case, we observed an increase of potent odorants such as linalool, responsible for *flowery* notes (58), meaning that the controlled atmosphere prevented autoxidation of this compound.

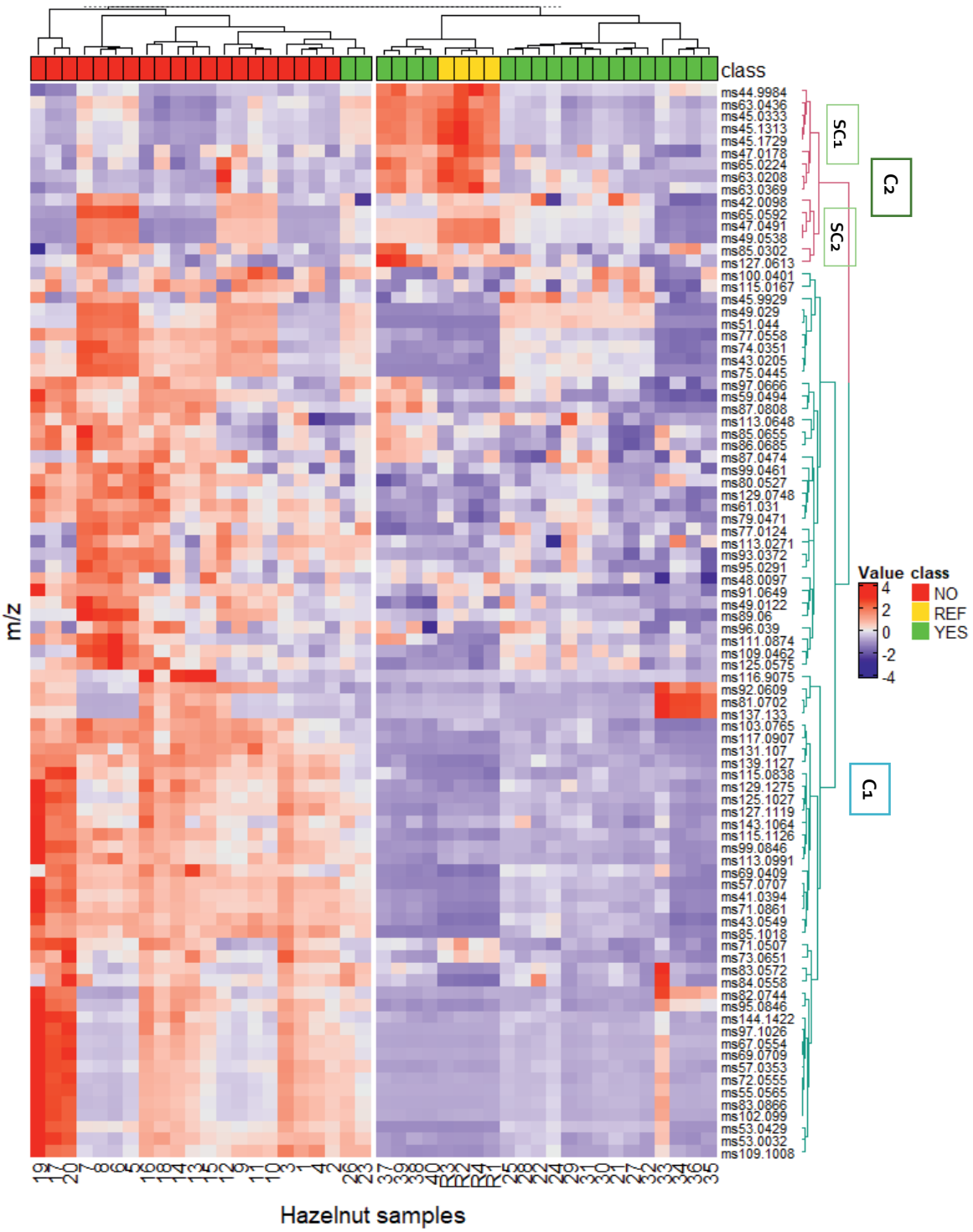

class
m/z
Hazelnut samples
SC1
SC2
C2
C1
Value class
4
2
0
-2
-4
NO
REF
YES
ms44.9984
ms63.0436
ms45.0333
ms45.1313
ms45.1729
ms47.0178
ms65.0224
ms63.0208
ms63.0369
ms42.0098
ms65.0592
ms47.0491
ms49.0538
ms85.0302
ms127.0613
ms100.0401
ms115.0167
ms45.9929
ms49.029
ms51.044
ms77.0558
ms74.0351
ms43.0205
ms75.0445
ms97.0666
ms59.0494
ms87.0808
ms113.0648
ms85.0655
ms86.0685
ms87.0474
ms99.0461
ms80.0527
ms129.0748
ms61.031
ms79.0471
ms77.0124
ms113.0271
ms93.0372
ms95.0291
ms48.0097
ms91.0649
ms49.0122
ms89.06
ms96.039
ms111.0874
ms109.0462
ms125.0575
ms116.9075
ms92.0609
ms81.0702
ms137.133
ms103.0765
ms117.0907
ms131.107
ms139.1127
ms115.0838
ms129.1275
ms125.1027
ms127.1119
ms143.1064
ms115.1126
ms99.0846
ms113.0991
ms69.0409
ms57.0707
ms41.0394
ms71.0861
ms43.0549
ms85.1018
ms71.0507
ms73.0651
ms83.0572
ms84.0558
ms82.0744
ms95.0846
ms144.1422
ms97.1026
ms67.0554
ms69.0709
ms57.0353
ms72.0555
ms55.0565
ms83.0866
ms102.099
ms53.0429
ms53.0032
ms109.1008

***Figure 5.4:*** *Heat map-illustrating, from blue (low values) to red (high values), the relative concentration (ppbV) of 88 mass peaks from the 44 raw hazelnuts samples of the blind classification experiment. The color annotation at the top of the heatmap indicates samples classification from industry. The data for each hazelnut sample were averaged on its replicates and the mass peaks were scaled (mean centering and unit variance). For the samples (columns) firstly, K-mean clustering (k = 2) was applied to split the samples in two groups followed by a hierarchical clustering on the Manhattan distances with a Ward method. The dashed line at the top of the samples' dendrogram indicates the K-mean clustering. For the mass peaks (rows) only hierarchical clustering was applied. Mass peaks were divided in two main clusters highlighted by different colors (C1 and C2) with the latter divided in two additional sub-clusters (SC1 and SC2).*

For most of the mass peaks contained in the cluster C1, the samples 13-20 coming from the replicates of sample "D" and "E", had the highest emissions. Both samples "D" and "E" belonged to 2015 harvest from the *Akçakoca* region and were evaluated as "NO" samples (Table 5.2). This information highlights the aging effect on VOCs emissions. The cluster C2 correspond to mass peaks mostly related to acetaldehyde, ethanol and methanol clusters, ethanethiol/dimethyl sulfide, methanethiol and m/z 42.001, 44.998, 45.033, 45.131, 45.173, 47.0178, 47.0491. In the cluster are also present *m/z* 127.061, 85.03, 89.06, 74.035 and 77.012. These mass peaks were divided into two different sub-clusters (SC1 and SC2). The samples R1-R4 and 37-40 corresponding to the replicates of the reference samples and the sample "L" were characterized by higher content of mass peaks especially from SC2. Although from different varieties and geographical origin, all these samples were coming from the most recent harvest in 2017. The reference samples were Piedmont hazelnuts know as *Tonda Gentile Trilobata* , a PDO product chosen by the industry as its reference for excellent quality. The mass peaks highlighted by the SC2 are then good candidates for discriminating sample freshness and infer about sample age.

Similar results were found for heatmaps built for PTR-ToF-MS data when SRI system was used (see Figure S5.1 and S5.2 in supplementary materials). Sample 23, 9, 2 and sample 9 were misclassified when $NO^+$ was used as precursor ion leading to a classification accuracy (about 91%). Also in this case, "NO" samples showed higher values for most of the mass peaks except for samples 33-36 that had higher levels for *m/z* 137.132 and its fragments and samples 37-40 and R1-R4 with mass peaks related to the SC2 described before. For data generated by using $O_2^+$ the same trend was highlighted: "NO" samples presented higher concentrations in more VOCs then "YES" samples and a similar classification accuracy (about 93%) was found. Samples 23 and 26 were put it in the "NO" cluster while sample 2 was put in the "YES" cluster.

## 5.4 Conclusion

PTR-ToF-MS method was successfully applied to screen the volatilome of different raw hazelnuts (*Corylus avellana* L). The approach described in this paper represents a great advantage for manufactures who need to control raw materials, especially because it is fast and can be performed on few grams of the product. The analysis identified specific volatile makers for different types of visual defects (molds, dark and light hazelnuts) and sensory defects related to taste and aroma. In general, defected hazelnuts showed higher levels of most of detected VOCs: this is true also for compounds identified by previous studies as key hazelnuts aroma compounds like 3/5-methyl-4-heptanone, prenyl ethyl ether, hexanal and linalool. Our analytical strategy was able to discriminate between samples with 20% and 50% grinded defected hazelnuts but for some mass peaks, a significant difference was observed as well between samples made with 10% and 20% of bad quality hazelnuts. Finally, the possibility to predict sensory classification based on unsupervised clustering upon PTR-ToF-MS fingerprints was also demonstrated by, at the same time, extrapolating information about harvest year and storage from VOCs fingerprints.

## 5.5 References


1. Ciarmiello LF, Mazzeo MF, Minasi P, Peluso A, De Luca A, Piccirillo P, et al. Analysis of different European hazelnut (corylus avellana L.) cultivars: Authentication, phenotypic features, and phenolic profiles. J Agric Food Chem. 2014;
2. Wang W, Jung J, McGorrin RJ, Traber MG, Leonard SW, Cherian G, et al. Investigation of drying conditions on bioactive compounds, lipid oxidation, and enzyme activity of Oregon hazelnuts (Corylus avellana L.). LWT - Food Sci Technol. 2018;
3. Intelligence M. Global Hazelnuts Market - Growth, trends and forecast (2020 - 2025) [Internet]. 2019. Available from: https://www.mordorintelligence.com/industry-reports/global-hazelnut-market
4. Eskandari J, Kermani AM, Kouravand S, Zarafshan P. Design, fabrication, and evaluation a laboratory dry-peeling system for hazelnut using infrared radiation. LWT. 2018;
5. Locatelli M, Coïsson JD, Travaglia F, Cereti E, Garino C, D'Andrea M, et al. Chemotype and genotype chemometrical evaluation applied to authentication and traceability of "tonda Gentile Trilobata" hazelnuts from Piedmont (Italy). Food Chem. 2011;
6. Klockmann S, Reiner E, Cain N, Fischer M. Food Targeting: Geographical Origin Determination of Hazelnuts (Corylus avellana) by LC-QqQ-MS/MS-Based Targeted Metabolomics Application. J Agric Food Chem. 2017;
7. Cubero-Leon E, Peñalver R, Maquet A. Review on metabolomics for food authentication. Food Res Int. 2014;
8. Battilani P, Chiusa G, Arciuolo R, Somenzi M, Fontana M, Castello G, et al. Diaporthe as the main cause of hazelnut defects in the Caucasus region. Phytopathol Mediterr. 2018;
9. Moscetti R, Saeys W, Keresztes JC, Goodarzi M, Cecchini M, Danilo M, et al. Hazelnut Quality Sorting Using High Dynamic Range Short-Wave Infrared Hyperspectral Imaging. Food Bioprocess Technol. 2015;
10. Burdack-Freitag A, Schieberle P. Changes in the key odorants of Italian hazelnuts (Coryllus avellana L. Var. Tonda Romana) induced by roasting. J Agric Food Chem. 2010;58(10):6351–9.
11. Burdack-Freitag A, Schieberle P. Characterization of the key odorants in raw italian hazelnuts (Corylus avellana L. var. Tonda Romana) and roasted hazelnut paste by means of molecular sensory science. J Agric Food Chem. 2012;60(20):5057–64.
12. Cialiè Rosso M, Liberto E, Spigolon N, Fontana M, Somenzi M, Bicchi C, et al. Evolution of potent odorants within the volatile metabolome of high-quality hazelnuts (Corylus avellana L.): evaluation by comprehensive two-dimensional gas chromatography coupled with mass spectrometry. Anal Bioanal Chem. 2018;
13. Galle SA, Koot A, Soukoulis C, Cappellin L, Biasioli F, Alewijn M, et al. Typicality and Geographical Origin Markers of Protected Origin Cheese from The Netherlands Revealed by PTR-MS. J Agric Food Chem [Internet]. 2011;59(6):2554–63. Available from: http://pubs.acs.org/doi/pdfplus/10.1021/jf104170r
14. Biasioli F, Yeretzian C, Gasperi F, Mark TD. PTR-MS monitoring of VOCs and BVOCs in food science and technology. Trac-Trends Anal Chem. 2011;30(7):968–77.
15. Pedrotti M, Spaccasassi A, Biasioli F, Fogliano V. Ethnicity, gender and physiological parameters: Their effect on in vivo flavour release and perception during chewing gum consumption. Food Res Int. 2019;
16. Pedrotti M, Khomenko I, Cappellin L, Fontana M, Somenzi M, Falchero L, et al. Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. J Mass Spectrom. 2018;53(9):753–62.
17. Makhoul S, Yener S, Khomenko I, Capozzi V, Cappellin L, Aprea E, et al. Rapid non-invasive quality control of semi-finished products for the food industry by direct injection mass spectrometry headspace analysis: the case of milk powder, whey powder and anhydrous milk fat. J Mass Spectrom [Internet]. 2016;51(9):782–91. Available from:

http://onlinelibrary.wiley.com/store/10.1002/jms.3801/asset/jms3801.pdf?v=1&t=j5v4huhd&s=220736ofa980d515ec2f3cbacb19076da7726 21e

18. Soukoulis C, Cappellin L, Aprea E, Costa F, Viola R, Mark TD, et al. PTR-ToF-MS, A Novel, Rapid, High Sensitivity and Non-Invasive Tool to Monitor Volatile Compound Release During Fruit Post-Harvest Storage: The Case Study of Apple Ripening. Food Bioprocess Technol. 2013;6(10):2831–43.
19. Capozzi V, Yener S, Khomenko I, Farneti B, Cappellin L, Gasperi F, et al. PTR-ToF-MS coupled with an automated sampling system and tailored data analysis for food studies: Bioprocess monitoring, screening and nose-space analysis. J Vis Exp. 2017;
20. Lanza M, Acton WJ, Sulzer P, Breiev K, Jürschik S, Jordan A, et al. Selective reagent ionisation-time of flight-mass spectrometry: A rapid technology for the novel analysis of blends of new psychoactive substances. J Mass Spectrom. 2015;50(2):427–31.
21. Pscheidt JW, Heckert S, Wiseman M, Jones L. Fungi associated with and influence of moisture on development of kernel mold of hazelnut. Plant Dis. 2019;
22. Dairou V, Sieffermann JM. A comparison of 14 jams characterized by conventional profile and a quick original method, the Flash Profile. J Food Sci. 2002;
23. Delarue J, Sieffermann JM. Sensory mapping using Flash profile. Comparison with a conventional descriptive method for the evaluation of the flavour of fruit dairy products. Food Qual Prefer. 2004;
24. Yener S, Romano A, Cappellin L, Mark TD, del Pulgar JS, Gasperi F, et al. PTR-ToF-MS characterisation of roasted coffees (C-arabica) from different geographic origins. J Mass Spectrom [Internet]. 2014;49(9):929–35. Available from: http://onlinelibrary.wiley.com/store/10.1002/jms.3455/asset/jms3455.pdf?v=1&t=j488vtqk&s=5e5f068e2b46bbd362bfc98556585ea01039f6a8
25. Cappellin L, Makhoul S, Schuhfried E, Romano A, Sanchez Del Pulgar J, Aprea E, et al. Ethylene: Absolute real-time high-sensitivity detection with PTR/SRI-MS. the example of fruits, leaves and bacteria. Int J Mass Spectrom. 2014;365–366:33–41.
26. Sánchez del Pulgar J, Soukoulis C, Carrapiso AI, Cappellin L, Granitto P, Aprea E, et al. Effect of the pig rearing system on the final volatile profile of Iberian dry-cured ham as detected by PTR-ToF-MS. Meat Sci. 2013;93(3):420–8.
27. Brown PA, Cristescu SM, Mullock SJ, Reich DF, Lamont-Smith CS, Harren FJM. Implementation and characterization of an RF ion funnel ion guide as a proton transfer reaction chamber. Int J Mass Spectrom. 2017;
28. Cappellin L, Biasioli F, Fabris A, Schuhfried E, Soukoulis C, Mark TD, et al. Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS. Int J Mass Spectrom. 2010;290(1):60–3.
29. Cappellin L, Biasioli F, Granitto PM, Schuhfried E, Soukoulis C, Costa F, et al. On data analysis in PTR-TOF-MS: From raw spectra to data mining. Sensors and Actuators B-Chemical. 2011;155(1):183–90.
30. Lindinger W, Hansel A, Jordan A. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) - Medical applications, food control and environmental research. Int J Mass Spectrom. 1998;173(3):191–241.
31. Biasioli F, Gasperi F, Aprea E, Endrizzi I, Framondino V, Marini F, et al. Correlation of PTR-MS spectral fingerprints with sensory characterisation of flavour and odour profile of "Trentingrana" cheese. Food Qual Prefer. 2006;17(1–2):63–75.
32. Cappellin L, Karl T, Probst M, Ismailova O, Winkler PM, Soukoulis C, et al. On Quantitative Determination of Volatile Organic Compound Concentrations Using Proton Transfer Reaction Time-of-Flight Mass Spectrometry. Environ Sci Technol. 2012;46(4):2283–90.
33. Burdack-freitag A, Schieberle P. Characterization of the Key Odorants in Raw Italian Hazelnuts. J Agric Food Chem. 2012;60:5057–64.

34. Nicolotti L, Cordero C, Bicchi C, Rubiolo P, Sgorbini B, Liberto E. Volatile profiling of high quality hazelnuts (Corylus avellana L.): Chemical indices of roasting. Food Chem. 2013;138(2–3):1723–33.
35. Kiefl J, Schieberle P. Evaluation of process parameters governing the aroma generation in three hazelnut cultivars (Corylus avellana L.) by correlating quantitative key odorant profiling with sensory evaluation. J Agric Food Chem. 2013;61(22):5236–44.
36. Deuscher Z, Andriot I, Sémon E, Repoux M, Preys S, Roger JM, et al. Volatile compounds profiling by using proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS). The case study of dark chocolates organoleptic differences. Journal of Mass Spectrometry. 2019.
37. Murtagh F, Legendre P. Ward's Hierarchical Agglomerative Clustering Method: Which Algorithms Implement Ward's Criterion? J Classif. 2014;
38. R Core Team. R Development Core Team [Internet]. Vol. 55, R: A Language and Environment for Statistical Computing. 2016. p. 275–86. Available from: https://www.r-project.org/
39. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 2016;
40. Amrein TM, Schwager H, Meier R, Frey P, Gassenmeier K. A Metallic, Solvent-like Off-Flavor in Hazelnuts: Identification of Prenyl Ethyl Ether as a Key Flavor Compound and Formation Studies. In: Flavour Science. 2014.
41. Amrein TM, Schwager H, Meier R, Frey P, Gassenmeier KF. Identification of prenyl ethyl ether as a source of metallic, solvent-like off-flavor in Hazelnut. J Agric Food Chem. 2010;
42. Yener S, Romano A, Cappellin L, Granitto PM, Aprea E, Navarini L, et al. Tracing coffee origin by direct injection headspace analysis with PTR/SRI-MS. Food Res Int. 2015;69:235–43.
43. Smith D, Španěl P. Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis. Mass Spectrom Rev. 2005;24(5):661–700.
44. Koyuncu MA, Islam A, Küçük M. Fat and fatty acid composition of hazelnut kernels in vacuum packages during storage. Grasas y Aceites. 2005.
45. Amaral JS, Casal S, Citová I, Santos A, Seabra RM, Oliveira BPP. Characterization of several hazelnut (Corylus avellana L.) cultivars based in chemical, fatty acid and sterol composition. Eur Food Res Technol. 2006;
46. Xu L, Yu X, Li M, Chen J, Wang X. Monitoring oxidative stability and changes in key volatile compounds in edible oils during ambient storage through HS-SPME/GC–MS. Int J Food Prop. 2018;
47. Amaike S, Keller NP. Aspergillus flavus . Annu Rev Phytopathol. 2011;
48. Köksal AI, Artik N, Şimşek A, Güneş N. Nutrient composition of hazelnut (Corylus avellana L.) varieties cultivated in Turkey. Food Chem. 2006;
49. Ruiz del Castillo ML, Gómez Caballero E, Blanch GP, Herraiz M. Enantiomeric composition of filbertone in hazelnuts and hazelnut oils from different geographical origins. JAOCS, J Am Oil Chem Soc. 2002;
50. Čížková H, Rajchl A, Šnebergrová J, Voldřich M. Filbertone as a marker for the assessment of hazelnut spread quality. Czech J Food Sci. 2013;31(1):81–7.
51. Puchl'ová E, Szolcsányi P. Filbertone: A Review. J Agric Food Chem. 2018;
52. Belitz HD, Grosch W, Schieberle P. Food chemistry. Food Chemistry. 2009.
53. Guntert M, Emberger R, Hopp R, Kopsel M, Silberzahn W, Werkhoff P. Chirospecific analysis in flavor and essential oil chemistry. Zeitschrift fur Leb Und-forsch. 1991;
54. Alasalvar C, Shahidi F, Cadwallader KR. Comparison of natural and roasted Turkish Tombul hazelnut (Corylus avellana L.) volatiles and flavor by DHA/GC/MS and descriptive sensory analysis. J Agric Food Chem. 2003;
55. Johnson JA, Yahia EM, Brandl DG. Dried fruits and tree nuts. In: Modified and Controlled Atmospheres for the Storage, Transportation, and Packaging of Horticultural Commodities. 2009.
56. Martin MBS, Fernàndez-Garcìa, Teresa Romero A, Lòpez. Effect of modiefied atmosphere storage on hazelnut quality. J Food Process Preserv. 2001;

57. Ghirardello D, Contessa C, Valentini N, Zeppa G, Rolle L, Gerbi V, et al. Effect of storage conditions on chemical and physical characteristics of hazelnut (Corylus avellana L.). Postharvest Biol Technol. 2013;
58. Kiefl J, Pollner G, Schieberle P. Sensomics analysis of key hazelnut odorants (Corylus avellana L. 'Tonda Gentile') using comprehensive two-dimensional gas chromatography in combination with time-of-flight mass spectrometry (GC×GC-TOF-MS). J Agric Food Chem. 2013;

## 5.6 Supplementary materials

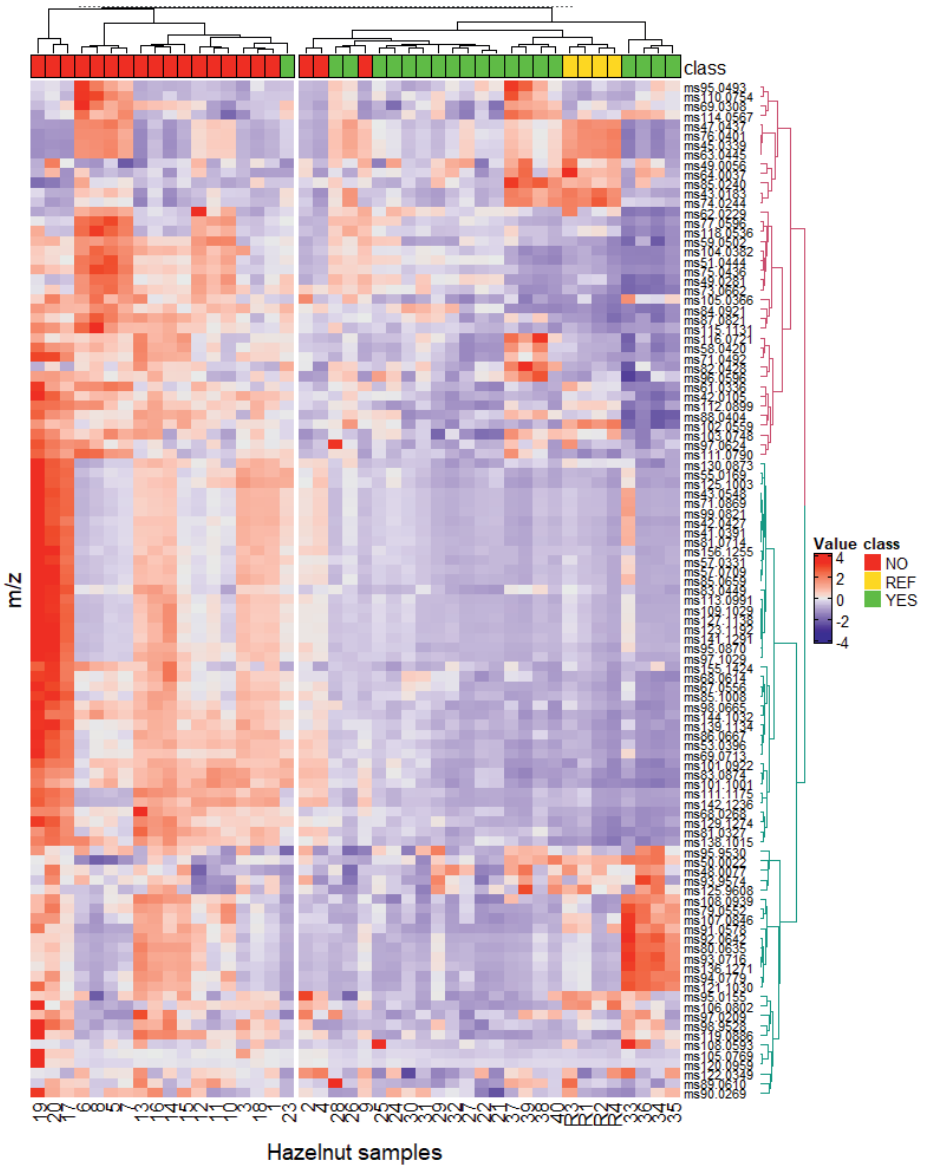



***Figure S5.1:*** *Heat map-illustrating the relative concentration (ppbV) of mass peaks selected from $NO^+$ ionization mode from samples of the blind classification experiment. The color annotation at the top of the heatmap indicates the samples classification from industry. The data for each hazelnut sample were averaged on its replicates and the mass peaks were scaled (mean centering and unit variance). For the samples (columns) firstly, K-mean clustering (k = 2) was applied to split the samples in two groups followed by a hierarchical clustering on the Manhattan distances with a Ward method. For the mass peaks (rows) only hierarchical clustering was applied. Mass peaks were divided in two main clusters highlighted by different colors.*

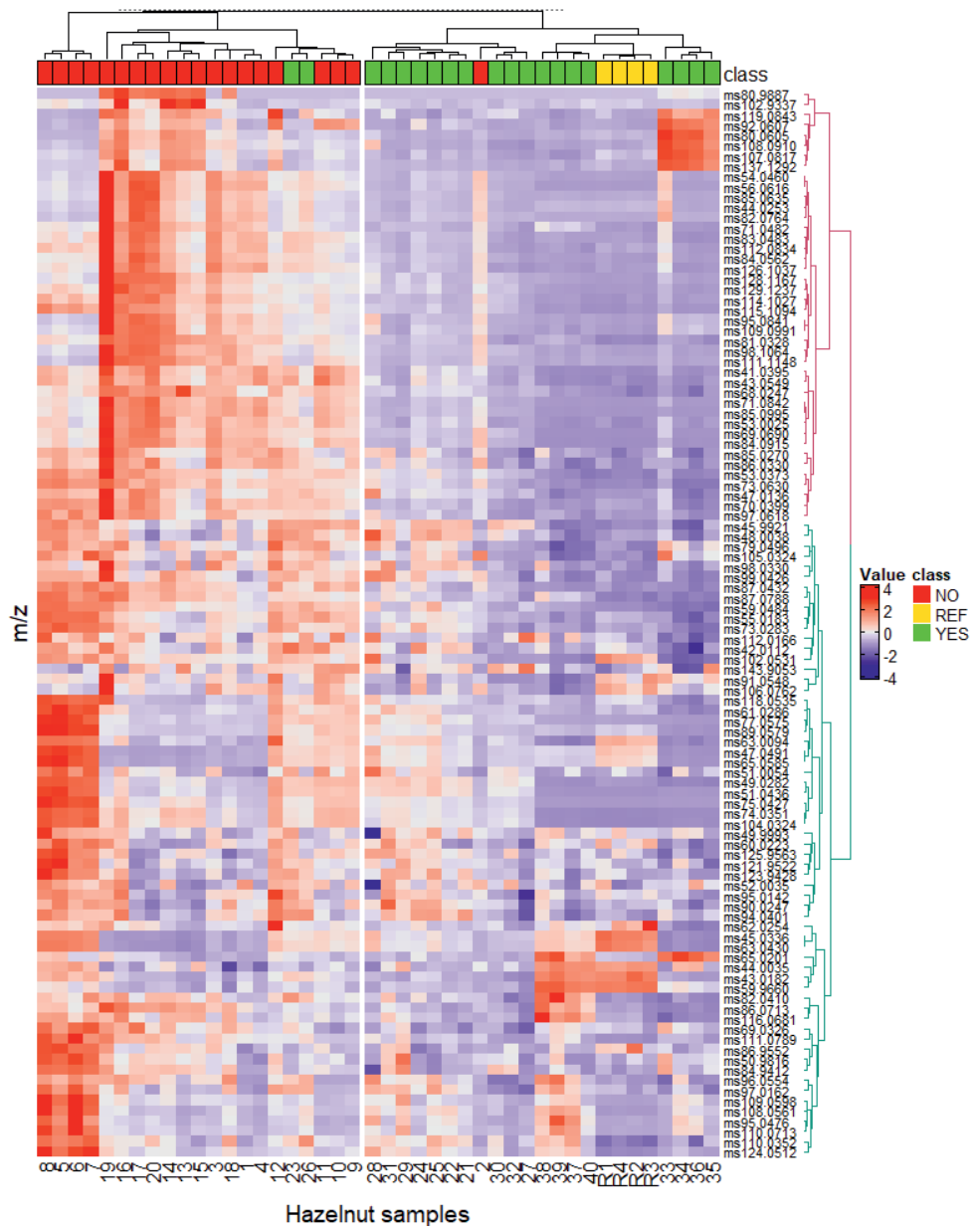



***Figure S5.2**: Heat map-illustrating the relative concentration (ppbV) of mass peaks selected from O2+ ionization mode from samples of the blind classification experiment. The color annotation at the top of the heatmap indicates the samples classification from industry. The data for each hazelnut sample were averaged on its replicates and the mass peaks were scaled (mean centering and unit variance). For the samples (columns) firstly, K-mean clustering (k = 2) was applied to split the samples in two groups followed by a hierarchical clustering on*

*the Manhattan distances with a Ward method. For the mass peaks (rows) only hierarchical clustering was applied. Mass peaks were divided in two main clusters highlighted by different colors.*

**Table S5.1:** Tentatively identified mass peaks in the headspace of raw hazelnuts from first and second experiment. The mass peaks significant different among the classes (P. <0.005 with Bonferroni correction) and with a concentration higher than 0.5 ppbV in at least one of the classes were selected.

| Mass peak | Chemical formula | Tentative ID | 100% YES | 95% YES | 90% YES | 80% YES | 50% YES | 20% YES | 0% YES | YES | LIGHT | DARK | MOLD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ms41.039 | C3H5+ | Alkyl fragment | 34.1±8.2a | 36.8±4.9a | 41.6±6.8a | 48.4±6.4a | 81.9±16.3 b | 91.3±13.7 b | 148.9±29.2 c | 47.7±6.7a | 517±141.2b | 876.2±214.5 c | 197.5±4.3a |
| ms42.02 | | | 31.8±9.4ab | 27.3±8.6a | 26.7±9.4ab | 36±11.8ab | 47.5±14.6 bc | 53.6±16.8 c | 60.9±19.6c | 14.6±1.6a | 33.3±4.2c | 38.4±1.9c | 24±0.7b |
| ms42.034 | C2H3NH+ | Acetonitrile | 7.7±1.3a | 7.2±3.3a | 6.9±3.7a | 7±0.9a | 9.8±1.2a | 9.3±1.2ab | 12.6±1.6b | 15.9±2.8a | 45.6±12.3ab | 331.1±106c | 165.9±42.1 b |
| ms44.023 | C[13]CH3O | Isotope Methyl butanoate/ isotope acetic acid fragment | 12.7±3.8a | 12.3±1.6a | 11.8±2.9a | 14.5±1.6ab | 16.9±2.6 b | 22.4±5.5c | 26.1±4.8d | 14.4±3.1a | 72.9±24.8b | 70.8±16.4b | 30.3±2.1a |
| ms44.059 | C2[13]CH6H+ | Alkyl fragment isotope | 0.4±0.1a | 0.5±0.1a | 0.5±0.1a | 0.6±0.1a | 0.7±0.1b | 0.7±0.1b | 1±0.2c | 0.6±0.1a | 1±0.3ab | 1.2±0.1b | 0.7±0a |
| ms46.038 | C[13]CH4OH + | Acetaldehyde isotope | | | | | | | | 32.2±4.7a | 77.8±14.8b | 93.6±17.5b | 33.7±0.5a |
| ms48.009 | | | 0.7±0.2a | 0.6±0.1a | 0.6±0.1a | 0.7±0.1a | 0.8±0.1a | 0.8±0.1a | 1±0.2b | 0.7±0.1a | 2.3±0.9b | 1.3±0.2ab | 0.7±0.1a |
| ms49.011 | CH4SH+ | Methanethiol | 0.1±0a | 0.2±0ab | 0.3±0b | 0.5±0c | 1.1±0.1d | 1.6±0.2e | 1.7±0.3e | 1±0.1a | 14.9±1.9b | 52.1±10.8c | 23.5±4.2b |
| ms49.028 | CH4O2H+ | | | | | | | | | 0.7±0.1a | 2.4±0.4b | 3.2±0.5c | 2.2±0.4b |
| ms53.005 | C3OH+ | Fragment (ester) | 2.4±0.5a | 2.4±0.5a | 2.7±0.7a | 3.1±0.6a | 4.6±1.6b | 4.8±1.5b | 6.6±2.9c | 1.8±0.3a | 12.7±7.5b | 29.8±4.9c | 10.4±0.4ab |
| ms53.042 | C4H5+ | | 2.7±0.9a | 3.1±0.5a | 3.8±0.7a | 4.4±0.8a | 12±2.2b | 12.2±1.7b | 21.2±4.4c | 2.7±0.7a | 15.6±7.2b | 36.3±6.2c | 15±1.1b |
| ms54.011 | C3HOH+ | | 1.1±0.4a | 1.1±0.4a | 1.2±0.4a | 1.4±0.5a | 2.3±0.8b | 2.5±0.8b | 3.4±1.1c | 1.2±0.1a | 10.4±1.8c | 16.8±1.4d | 5.9±0.2b |
| ms56.029 | C3H3OH+ | | 0.4±0.2a | 0.4±0.1a | 0.4±0.1a | 0.4±0.1ab | 0.7±0.3b | 0.7±0.3b | 1.1±0.4c | 0.6±0.1a | 3.3±0.7c | 5.3±0.4d | 1.7±0.1b |
| ms56.058 | C3[13]CH6H+ | Butanal frag isotope | 0.8±0.2a | 0.9±0.1a | 1.1±0.2a | 1.2±0.2a | 3.4±0.7b | 3.4±0.4b | 5.7±1.2c | 1.1±0.2a | 5.7±1b | 12.2±2.4c | 5.1±0.2b |
| ms57.07 | C3H4OH+ | Common fragment (alcohol, ester, aldehyde) | 8.5±2.2a | 9.1±1.2a | 10.3±1.7a | 12.8±1.6a | 22.5±5.3b | 26.9±4.5b | 41.9±4.4c | 10±1.6a | 297.1±136b | 112.6±112.7 ab | 25.5±0.9a |
| ms58.041 | C3H5OH+ | | 0.4±0.1a | 0.4±0.1a | 0.4±0.1a | 0.5±0.1a | 0.9±0.3b | 0.9±0.2b | 1.3±0.5c | 0.5±0.1a | 3.7±0.7b | 7.6±1.3c | 2.2±0.1b |
| ms60.053 | C2[13]CH6O H+ | 2-propanone isotope | 11.9±2.3ab | 11.2±1.3a | 11.2±1.6ab | 11.9±1.3ab | 13.6±2.2b c | 12.9±1.7a b | 14.9±2.9c | 9.1±1.4a | 24.9±5.7a | 65.6±21.3b | 22.8±3.1a |
| ms62.033 | C[13]CH3OH+ | Acetic acid isotope | 8.9±2.6ab | 8.8±1.3a | 8.3±2.4a | 10±1.2ab | 11.7±1.9b | 15.8±3.8c | 18.2±3.4c | 9.5±2.6a | 48.6±12.5b | 41.3±7.8b | 23.6±1.8a |
| ms63.041 | C2H6O2H+ | Acetaldehyde-water cluster | | | | | | | | 67.6±1.2b | 72.9±4.2b | 77.5±6.6b | 29.4±8.2a |
| ms63.044 | C2H6O2H+ | | | | | | | | | 123.2±14b | 135.6±7.9bc | 144.3±12.3c | 67.8±4.2a |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ms65.021** | $CH_4O_3H^+$ | | | | | | | | | 0.7±0a | 6±2.8b | 0.3±0.3a | 1.1±0.3a |
| **ms65.059** | $C_2H_5OH^*H_3O^+$ | Ethanol cluster | 34.2±13.9a | 26.6±8.1a | 25.4±9.6a | 31.3±7.1a | 35.4±12.4ab | 38±15.3ab | 49±17.3b | 28.6±7.8a | 125.8±47.4ab | 197.6±101.6b | 38.7±2.6a |
| **ms67.056** | $C_5H_7^+$ | $C_5H_7$+ 2-pentenal frag | | | | | | | | 2.8±0.4a | 18.8±3.2b | 43.9±9.5c | 13.2±0.5ab |
| **ms70.073** | $C_4[13]CH_8H^+$ | Isoprene isotope/ Common aldehyde fragment) | 0.5±0.1a | 0.5±0.1a | 0.6±0.1a | 0.7±0.1a | 1.5±0.3b | 1.4±0.2b | 2.3±0.5c | 0.7±0.2a | 14.9±2.9b | 45.9±6.9c | 12.1±0.9b |
| **ms71.049** | $C_4H_6OH^+$ | 2-butenal | 1.7±0.5a | 1.6±0.3a | 1.5±0.4a | 1.6±0.4a | 1.9±0.5a | 2.1±0.5a | 2.4±0.9b | 1.6±0.2a | 7.9±1.2c | 10.2±1.9d | 5.2±0.2b |
| **ms71.086** | $C_5H_{10}H^+$ | 2-pentanol fragment, 3-methyl-1-butanol fragment | 7.8±2a | 9.1±1.3a | 10.5±1.8a | 12.5±2a | 21.9±5.3b | 24±3.8b | 42.7±8.9c | 12±2a | 106.6±30c | 45.8±20b | 12.2±0.8a |
| **ms72.053** | $C_4H_8O^+$ | | | | | | | | | 0.2±0a | 0.9±0.1b | 1.7±0.3c | 0.7±0b |
| **ms74.069** | $C[13]C_3H_9O$ | 2-Butanone isotope / 2-methylpropanal isotope | 1.9±0.5a | 1.8±0.3a | 1.9±0.3a | 2±0.3a | 2.3±0.4ab | 2.2±0.3a | 2.7±0.4b | 2.8±1.6a | 38.7±9.8b | 39±12.5b | 17.6±0.7a |
| **ms75.045** | $C_3H_6O_2H^+$ | Propanoic Acid | 30.2±7.3a | 30.3±3.9a | 32.9±5.8a | 46.3±5.5a | 69.2±15.5b | 102.5±19c | 135.5±38.5d | 13.1±1.9a | 168.4±47.6b | 158.9±42.4b | 57.4±2.1a |
| **ms77.059** | $C_3H_8O_2H^+$ | | 6±2.6ab | 4.8±1.5a | 4.8±1.7ab | 5.3±1.2ab | 7.8±2.7b | 7.8±2.5b | 12.2±2.7c | 5.1±1.4a | 11.2±4.8a | 32.2±10.1b | 4±0.1a |
| **ms78.004** | $C_5HOH^+$ | | | | | | | | | 0±0a | 0.3±0.1a | 1.7±0.4b | 0.3±0.1a |
| **ms79.039** | $C_2H_6O_3H^+$ | | 35.9±20.2a | 32.7±8.2a | 29.8±13.5a | 42.5±9.1a | 50.9±18.1a | 90.7±42.7b | 127.8±44.3c | 61.8±26.8a | 366.8±274.2b | 333.4±131.1ab | 90.3±13.3a |
| **ms81.07** | $C_6H_8H^+$ | Linalool fragment / Monoterpenes fragment | 4.8±1.2a | 6.6±1.6a | 9.2±2.4a | 20.6±3.6b | 58.6±21.8c | 86.9±10.8d | 94.8±6.1d | 33.2±5.8a | 131±34.3b | 122.5±60.7b | 77.4±8.7ab |
| **ms82.075** | $C_5H_7NH^+$ | 2-Methyl-1H-pyrrole | 0.5±0.1a | 0.6±0.1a | 0.7±0.1ab | 1.2±0.2b | 2.6±0.7c | 3.5±0.5d | 4.9±0.9e | 1.8±0.3a | 5.3±0.2b | 8.4±1.3c | 5.6±1.1b |
| **ms83.047** | $C_5H_6OH^+$ | 2-Methylfuran | | | | | | | | 2.2±0.3a | 7.6±1.4b | 20.9±3c | 5.3±1.5ab |
| **ms83.086** | $C_6H_{11}^+$ | Hexanal fragment | 6±2.4a | 9.5±1.6ab | 14.4±4.5ab | 20.4±6b | 91±22.3c | 103.5±13.4c | 133.8±11.3d | 6.8±1.9a | 58.7±17.8b | 18.9±5.3a | 88.2±14.3c |
| **ms84.083** | $C_5[13]CH_{11}^+$ | Isotope hexanal fragment / other? | 0.4±0.1a | 0.6±0.1a | 0.7±0.2a | 1±0.2a | 3.2±0.7b | 3.2±0.4b | 6.5±1.2c | 0.5±0.1a | 20.1±2c | 15.6±1.3b | 19.2±2.3bc |
| **ms85.067** | $C_5H_8OH^+$ | 3-penten-2-one / (E)-2-pentenal | | | | | | | | 16.9±5.1a | 17.6±4.5a | 46±5.1b | 11.4±0.7a |
| **ms85.099** | $C_6H_{13}^+$ | 1-Hexanol fragment (Dehydratation) | 0.5±0.2a | 0.6±0.2a | 0.8±0.3a | 1.5±0.3a | 4.5±1.6b | 4.6±0.9b | 9.9±1.7c | 0.9±0.2a | 2.4±0.1b | 3.6±0.8c | 1.6±0.3ab |
| **ms87.042** | $C_4H_6O_2H^+$ | 2,3-butanedione | 2.3±0.7a | 2.6±0.5a | 2.4±0.3a | 2.8±0.4a | 2.9±1ab | 4.3±1.3bc | 4.4±1.6c | 4.7±3a | 67.3±19.5b | 114.8±42.5c | 120.9±9.3c |
| **ms87.077** | $C_5H_{10}OH^+$ | 2/3-methylbutanal/ 2-pentanone / pentanal | 38.7±10.2a | 38.5±6a | 41.7±8.3a | 42.9±6.5a | 54.3±10.7b | 49.3±8.6ab | 67.3±17.6c | 88±21a | 567.3±152.3b | 1841±156.6c | 264.1±6.7a |
| **ms89.061** | $C_4H_8O_2H^+$ | Butanoic Acid / Ethyl acetate / Acetoin | 5±1.4ab | 4.6±0.6a | 4.5±0.9a | 5.5±0.6ab | 5.8±1.1bc | 6.6±1.4cd | 7.7±1.6d | 6.2±1.1a | 54.4±9.3c | 50.1±2.5c | 31.6±1.4b |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ms91.065** | $C_4H_{10}O_2H^+$ | 2,3-Butanediol | 1±0.4a | 1.1±0.2a | 1.1±0.2a | 1.2±0.2ab | 1.5±0.3bc | 1.6±0.3c | 2.2±0.4d | 1.7±0.7a | 14±5.4a | 41.8±15.4b | 5.1±0.2a |
| **ms92.067** | $C_7H_8+$/ $C_3[13]CH_{10}O_2H+$/* | Fragment/2,3-Butanediol isotope | 0.1±0a | 0.1±0a | 0.1±0a | 0.2±0a | 0.3±0.1b | 0.4±0.1c | 0.5±0.1d | 0.2±0a | 0.9±0.2b | 1.7±0.4c | 0.6±0.1ab |
| **ms93.039** | $C_6H_4OH+$/$C_3H_8OSH+$ | | | | | | | | | 7.4±0.9a | 9.7±1.8b | 10.1±0.9b | 8.3±0.1ab |
| **ms93.069** | $C_7H_8H+$ | Toluene (=Methyl Benzene) / monoterpene fragment | 1.6±1.2a | 2.1±0.8a | 2.1±0.4a | 2.8±0.7a | 5±1b | 5.7±0.6b | 6.4±1.6c | 3.3±0.6a | 6.3±2.4b | 6.4±0.6b | 5.4±0.3ab |
| **ms94.04** | | | | | | | | | | 0.6±0.1a | 0.9±0.1a | 1.9±0.3b | 0.8±0.1a |
| **ms94.075** | $C_7H_9+$ | | 0.1±0a | 0.2±0a | 0.2±0ab | 0.2±0b | 0.3±0.1c | 0.4±0d | 0.5±0.1e | 0.3±0a | 0.7±0.1b | 1±0.1c | 0.8±0.1b |
| **ms95.038** | $C_2H_6O_4H+$ | | 1.7±0.4a | 1.9±0.2ab | 1.9±0.2a | 1.9±0.2ab | 2.2±0.3bc | 2.3±0.3cd | 2.5±0.3d | 1.8±0.1a | 5.3±1c | 6.4±0.5c | 3.7±0.4b |
| **ms95.086** | $C_7H_{11}+$ | Monoterpenes fragment | 0.7±0.1a | 0.9±0.1a | 1±0.2ab | 1.7±0.3b | 3.9±1.2c | 5.1±0.7d | 8.3±1.4e | 2.5±0.3a | 11.2±0.3b | 19.5±4.1c | 13.2±2.4b |
| **ms96.05** | $C_5H_5NOH+$ | 2-formyl pyrrol | | | | | | | | 0.2±0.1a | 0.5±0.3ab | 0.7±0b | 0.3±0ab |
| **ms96.963** | $SO_4H+$ | | 0.1±0a | 0.1±0a | 0.1±0ab | 0.1±0b | 0.3±0.1c | 0.3±0d | 0.5±0.1e | 0.7±0d | 0.4±0.1c | 0.1±0a | 0.2±0b |
| **ms97.029** | $C_5H_4O_2H+$ | Furfural / 4-cyclopentene-1,3-dione | | | | | | | | 1.1±0.1a | 4.6±1b | 9.6±1.7c | 3.2±0.1b |
| **ms97.063** | $C_6H_8OH+$ | 2,5-Dimethylfuran / 3-methyl-2-cyclohexen-1-one | 1.4±0.4a | 1.6±0.2a | 1.6±0.2a | 1.7±0.2ab | 2±0.3bc | 2.1±0.3c | 2.6±0.3d | 2.5±0.3a | 5.2±1.7b | 11.5±1.3c | 4.4±0.4ab |
| **ms97.102** | $C_7H_{13}+$ | Heptanal fragment | 0.6±0.1a | 0.8±0.1a | 1±0.2a | 1.3±0.1a | 2.3±1.3b | 2.7±0.7b | 6±3.3c | 0.9±0.1a | 2.8±0.4c | 1.5±0.1b | 2.5±0.2c |
| **ms98.103** | $C_7H_{14}+$ | 1-Heptanol fragment (dehydratation)/ isotope from heptanal fragment | 0.1±0a | 0.1±0a | 0.1±0a | 0.1±0a | 0.3±0.1b | 0.3±0b | 0.6±0.1c | 0.1±0a | 0.4±0b | 0.6±0c | 0.5±0.1b |
| **ms99.045** | $C_5H_6O_2H+$ | 2-furanmethanol | | | | | | | | 0.4±0.1a | 1.1±0.3b | 1.1±0.1b | 0.9±0b |
| **ms99.083** | $C_6H_{10}OH+$ | 2-Hexenal | 0.5±0.1a | 0.6±0.1a | 0.6±0.1a | 0.7±0.1a | 0.9±0.2b | 1±0.1b | 1.4±0.2c | 0.9±0.1a | 1.8±0.4c | 1.5±0.1bc | 1.2±0.2ab |
| **ms101.058** | $C_5H_8O_2H+$ | 2,3 Pentanedione | 1.2±0.3b | 1.1±0.2b | 1.1±0.2b | 1±0.1b | 1±0.3b | 0.9±0.4b | 0.5±0.2a | 1.7±0.2a | 4.6±1.8b | 2.9±0.3ab | 4.2±0.1b |
| **ms101.097** | $C_6H_{12}OH+$ | Hexanal, 3-methyl-2-pentanone, 2-hexanone | 2.3±0.8a | 3.2±0.5a | 3.9±0.9a | 5±1.1a | 16.1±4.5b | 16±3b | 34±7.9c | 4.1±0.7a | 11±2.6bc | 7.6±1.2b | 14.6±2.4c |
| **ms102.094** | $C_6H_{13}OH+$ | | | | | | | | | 0.4±0.1a | 2.7±0.6b | 19.1±1.7c | 2.1±0.1ab |
| **ms103.077** | $C_5H_{10}O_2H+$ | Pentanoic acid / methyl-butanoic acid | 1.6±0.7ab | 1.6±0.4a | 1.6±0.3a | 1.8±0.4ab | 2.1±0.5ac | 2.2±0.6bc | 2.8±0.6c | 4.3±0.8a | 15.7±4.7b | 20.5±0.8b | 8.4±0.6a |
| **ms105.069** | $C_8H_8H+$ | Styrene | 0.6±0.2ab | 0.7±0.1ab | 0.7±0.1ac | 0.6±0.1a | 0.9±0.2c | 0.8±0.2bc | 1.3±0.3d | 1.7±0.8a | 82.8±4.9c | 87.2±7.2c | 69.1±9b |
| **ms106.076** | $C_7[13]H_8H+$/$C_8H_9+$* | | | | | | | | | 0.2±0.1a | 28.2±11.3b | 17.1±1b | 4±1.3a |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ms107.054** | $C_7H_6OH^+$ | Benzaldehyde | 0.4±0.1a | 0.4±0.1a | 0.4±0.1a | 0.5±0.1ab | 0.5±0.1bc | 0.6±0.1c | 0.7±0.1c | 1.2±0.2a | 6.4±0.8c | 2.5±0.8b | 2.5±0.2ab |
| **ms107.085** | $C_8H_{10}H^+$ | 1,3-Dimethylbenzene (1,2 or 1,4)/ethylbenzene / terpene fragment | 1.2±0.4a | 1.2±0.3a | 1.2±0.2a | 1.5±0.3ab | 1.8±0.5bc | 2.2±0.3c | 2.8±0.7d | 1.4±0.2a | 9.6±2.6b | 3.6±0.9a | 7.4±0.9b |
| **ms108.088** | | | | | | | | | | 0.2±0a | 0.8±0.1b | 1±0.1c | 0.7±0.1b |
| **ms109.076** | $C_6H_8N_2H^+$ | 2-5/6-Dimethyl-pyrazine / ethylpyrazione | | | | | | | | 0.3±0.1a | 2.2±0.4a | 10.1±0.4c | 5.6±2b |
| **ms109.102** | $C_8H_{13}^+$ | Monoterpene fragment | 0.2±0.1a | 0.3±0a | 0.2±0a | 0.4±0.1a | 0.8±0.3b | 0.9±0.1b | 1.4±0.2c | 0.5±0.1a | 1.2±0.2ab | 1.6±0.1b | 1.9±0.7b |
| **ms110.073** | $C_6H_7NOH^+$ | 2-acetylpyrrole | | | | | | | | 0.1±0a | 0.3±0.1b | 0.7±0d | 0.4±0c |
| **ms111.047** | $C_6H_6O_2H^+$ | 2-acetylfuran / 5-methyl furfural | 0.4±0.3ab | 0.5±0.2ab | 0.5±0.1a | 0.5±0.1a | 0.8±0.3ac | 0.8±0.4bc | 0.9±0.4c | 0.6±0.2a | 1.2±0.2a | 3.4±0.5b | 1.3±0.2a |
| **ms111.083** | $C_7H_{10}OH^+$ | 2-Ethyl-5-methylfuran / 2,3,5 – trimethylfuran / octanal fragment | | | | | | | | 11.1±1.6a | 16.8±3.1b | 26.6±2.5c | 7.3±0.6a |
| **ms112.087** | $C_6H_9NOH^+$ | 2-acetyl-1-pyrolline / 1H-pyrrole-2-ethanol | | | | | | | | 0.6±0.1a | 1±0.1b | 1.4±0c | 0.5±0a |
| **ms113.061** | $C_6H_8O_2H^+$ | | | | | | | | | 0.5±0.2a | 0.7±0.5a | 1.4±0b | 0.8±0.1ab |
| **ms113.099** | $C_7H_{12}OH^+$ | 2/4-heptenal /3-hepten-2-one | 0.4±0.1a | 0.5±0.1a | 0.5±0.1a | 0.5±0.1a | 0.8±0.2b | 0.8±0.1b | 1.2±0.2c | 0.6±0.1a | 1.8±0.2b | 3.4±0.5c | 1.3±0.1b |
| **ms115.022** | $C_5H_6OSH^+$ | 2-furfurylthiol | | | | | | | | 0.3±0.0a | 0.4±0.1b | 0.5±0.0b | 0.4±0.0a |
| **ms115.071** | $C_6H_{10}O_2H^+$ | 3,5-Dimethyldihydro-2(3H)-furanone | | | | | | | | 0.3±0a | 1.3±0.6b | 3.2±0.9c | 0.9±0.1ab |
| **ms115.106** | | | 0.1±0.1a | 0.2±0.1a | 0.2±0a | 0.2±0.1a | 0.6±0.2b | 0.7±0.2b | 1.3±0.3c | 0.4±0.2a | 0.8±0.3a | 1.7±0.5b | 0.8±0.1a |
| **ms115.114** | $C_7H_{14}OH^+$ | 2/4-heptanone / heptanal | 1.1±0.3a | 1.4±0.2a | 1.4±0.2a | 1.8±0.3a | 3.4±0.8b | 3.9±0.6b | 6.4±1.2c | 2.1±0.3a | 4.1±0.5b | 3.2±0.7b | 4.3±0.4b |
| **ms117.09** | $C_6H_{12}O_2H^+$ | Hexanoic acid | 0.7±0.2a | 0.7±0.1a | 0.7±0.2a | 0.8±0.1ab | 1±0.2b | 1.2±0.2c | 1.4±0.2c | 1.2±0.5a | 5.5±1.6b | 5.8±1b | 2.4±0.1a |
| **ms119.103** | $C_6H_{15}O_2H^+$ | | 0.2±0.1a | 0.2±0a | 0.3±0.1a | 0.3±0.1a | 1±0.2b | 1±0.2b | 2.2±0.6c | 0.2±0a | 1.2±0.4bc | 1.6±0.6c | 0.8±0.1ab |
| **ms121.072** | $C_8H_8OH^+$ | Phenylacetaldehyde | 0.3±0.1a | 0.3±0.1a | 0.3±0a | 0.4±0.1ab | 0.5±0.1bc | 0.5±0.1c | 0.7±0.1d | 0.6±0.1a | 6.3±1.9c | 15.6±0.8d | 4.4±0.3b |
| **ms123.085** | $C_8H_{10}OH^+$ | 2-phenylethanol | | | | | | | | 0.1±0a | 0.4±0.1b | 0.9±0c | 0.7±0.2c |
| **ms123.119** | $C_9H_{15}^+$ | Fragment nonenal | 0.1±0a | 0.1±0a | 0.1±0a | 0.1±0a | 0.2±0b | 0.2±0b | 0.3±0c | 0.1±0a | 0.3±0a | 0.5±0b | 0.5±0.2b |
| **ms125.055** | $C_7H_8O_2H^+$ | 2-methoxyphenol | | | | | | | | 0.1±0.0a | 0.2±0.0b | 0.2±0.0b | 0.1±0.0a |
| **ms125.097** | $C_8H_{12}OH^+$ | | 0.2±0.1a | 0.2±0a | 0.3±0a | 0.3±0a | 0.5±0.1b | 0.6±0.1b | 0.9±0.2c | 0.4±0.1a | 1±0.1b | 1.7±0.1c | 1±0.1b |
| **ms126.969** | $C_2H_6S_3H^+$ | Dimethyl trisulfide | | | | | | | | 0.3±0.0b | 0.3±0.0ab | 0.3±0.0b | 0.2±0.0a |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ms127.112** | C8H14OH+ | 5-methyl-(E)-2-hepten-4-one / 2-octenal / 2-hexenal,2-ethyl | 0.3±0.1a | 0.3±0a | 0.3±0a | 0.4±0.1a | 0.7±0.2b | 0.8±0.1b | 1.2±0.2c | 0.5±0.1a | 1.2±0.2b | 2±0.1c | 1.1±0.2b |
| **ms129.081** | C7H12O2H+ | 5-propyldihydro-2(3H)-furanone | 0.11±0.03c | 0.12±0.02c | 0.12±0.01c | 0.011±0.02 bc | 0.09±0.03 b | 0.08±0.02 b | 0.04±0.03a | 0.2±0a | 1.1±0.1b | 2.1±0.3c | 0.4±0a |
| **ms129.128** | C8H16OH+ | 3/5-methyl-4-heptanone / Octanal / 2-octanone | 0.6±0.2a | 0.8±0.1a | 0.9±0.1a | 1.2±0.2a | 3±0.9b | 2.9±0.5b | 5±1c | 0.9±0.6a | 3.4±0.5b | 3.9±1b | 3.1±0.2b |
| **ms131.104** | C7H14O2H+ | ethyl 2-methylbutanoate / heptanoic acid | 0.2±0a | 0.2±0a | 0.2±0a | 0.2±0.1a | 0.4±0.1b | 0.5±0.1b | 0.6±0.1c | 0.4±0.1a | 2.6±0.5b | 2.9±0.7b | 0.8±0.1a |
| **ms133.111** | C7H16O2H+ | | | | | | | | | 0.1±0a | 0.6±0.2a | 1.2±0.5b | 0.3±0a |
| **ms135.082** | C9H10OH+ | | | | | | | | | 0.1±0a | 0.5±0.2b | 1.2±0.1c | 0.7±0.2b |
| **ms135.119** | C10H15+ | p-Cymene | 0.1±0a | 0.1±0a | 0.1±0ab | 0.1±0b | 0.2±0.1c | 0.3±0d | 0.4±0.1e | 0.2±0a | 0.9±0.1c | 1.3±0.1d | 0.6±0b |
| **ms137.134** | C10H17+ | Monoterpenes, linalool fragment, 2-decenal fragment | 1.7±0.5a | 2.3±0.7a | 3.4±1a | 8.3±1.7a | 26.3±10.9 b | 44±7.8c | 73.8±17.6d | 14.9±2.7a | 85±9.5b | 168.9±40.7c | 93.3±25.8b |
| **ms139.073** | C8H10O2H+ | 2-methoxy 3,5-dimethylpirazine | | | | | | | | 0.1±0.1a | 0.2±0.5b | 0.3±0.02c | 0.16±0.01a b |
| **ms139.118** | C9H14OH+ | 2,4-nonadienal / 2-pentylfuran | 0.2±0a | 0.2±0ab | 0.2±0ab | 0.3±0b | 0.5±0.1c | 0.6±0.1d | 0.8±0.1e | 0.3±0a | 0.8±0.1b | 1.2±0c | 1.5±0.2d |
| **ms143.107** | C8H14O2H+ | | 0.2±0.1a | 0.2±0a | 0.2±0a | 0.3±0.1a | 0.7±0.2b | 0.6±0.1b | 1±0.2c | 0.2±0.1a | 0.6±0.1c | 0.8±0d | 0.4±0.1b |
| **ms143.146** | C9H18OH+ | 3,5-dimethyl-4-heptanone / 2(3H)-Furanone,5-butyldihydro / nonanal / 2-nonanone | 0.2±0.1a | 0.2±0a | 0.2±0a | 0.3±0.1a | 0.7±0.2b | 0.6±0.1b | 1±0.2c | 0.3±0a | 0.6±0.1b | 0.6±0.1b | 0.9±0.1c |
| **ms145.124** | C8H16O2H+ | Octanoic Acid / butyl butanoate / 2-methylbutyl propanoate | 0.1±0a | 0.1±0a | 0.1±0a | 0.1±0a | 0.1±0b | 0.2±0b | 0.2±0c | 0.1±0a | 0.5±0.1b | 0.8±0.2c | 0.3±0ab |
| **ms147.094** | | | | | | | | | | 0±0a | 0.4±0.1b | 0.8±0.2c | 0.2±0ab |
| **ms153.127** | C10H16OH+ | 2,4-decadienal | | | | | | | | 0.1±0a | 0.5±0b | 0.7±0.1c | 0.4±0b |
| **ms155.148** | C10H18OH+ | 2-decenal / linalool | 0.02±0.01a | 0.02±0.00a | 0.02±0a | 0.03±0a | 0.1±0.01b | 0.1±0b | 0.1±0c | 0.02±0.01a | 0.17±0.03c | 0.16±0.02c | 0.11±0.02b |

6

# What drives *in vivo* aroma release and perception of condiments consumed with carrier foods?

van Eck A.*, Pedrotti M.*, Brouwer R., Supapong A., Fogliano V., Scholten E., Biasioli F., Stieger M.

Authors have contributed equally

*Submitted for publication at Nature Food*

## Abstract

When condiments are combined with carrier foods, aroma release and perception is complex and changes dynamically throughout consumption. This study investigated the effect of condiment and carrier properties on *in vivo* aroma release and perception. Mayonnaises varying in fat content (high/low) and viscosity (high/low) were spiked with two lemon aroma compounds (*limonene, citral*). Carriers differing in moisture absorption capacity (bread, potato) and hardness (hard/soft) were combined with mayonnaises. In-nose aroma release and perception of lemon intensity were assessed simultaneously and dynamically. Mayonnaise properties affected aroma release and perception congruently; higher viscosity decreased aroma release and perception, and higher fat content increased aroma release and perception. When mayonnaises were combined with carriers, aroma release and perception were no longer congruent. Addition of carriers to mayonnaises increased aroma release and decreased perception of aroma intensity. We conclude that cognitive effects are likely to modulate dynamic aroma perception of composite foods.

**Keywords:** nose-space aroma release, sensory perception, mayonnaise, carrier foods

## 6.1 Introduction

Condiments such as spreads, dressings or sauces are key ingredients in many different culinary traditions, and often used to enhance the flavour of bland carrier foods (1). In the last decades, food industry put considerable efforts into development of fat reduced condiments to provide consumers with less energy dense foods. These modifications imply a considerable change in flavour release and perception. Food flavour profiles are partly due to perception of aroma compounds, which are released from the food matrix and reach the olfactory receptors located in the human nasal cavity (retro-nasal pathway) (2). Some aroma compounds remain in the breath air when food is swallowed, and contribute to aroma perception after swallowing (3). Aroma release is a rather complex process, which is influenced by food composition, food structure and dynamic changes thereof during oral processing (4-6).

Fat reduction impacts aroma release and perception in a direct and/or indirect way. Fat or other hydrophobic phases present in the food matrix can bind hydrophobic aroma compounds and reduce their vapor pressure (physicochemical effect). Thus, increasing fat content in the food matrix reduces the release of hydrophobic aroma compounds both in the vapor headspace and in the nose-space during consumption (7-11). Vice versa, in the case of fat reduction, less hydrophobic aroma compounds are retained in the food matrix leading to higher aroma release and intensity perception (12-14). Changing fat content also largely impacts rheological properties of the matrix which can affect aroma release and perception in an indirect way. Higher volume fraction of dispersed fat increases viscosity, which reduces aroma release by slowing down diffusion of aroma compounds into the headspace (15). Hydrocolloids are commonly added to fat-reduced foods to maintain desired rheological properties. Several studies demonstrated that an increase in viscosity through addition of hydrocolloids reduces aroma release and perception (16-23). Hydrocolloids may reduce aroma release and consequently perception by slowing down aroma diffusion or by binding aroma compounds through attractive interactions (physicochemical origin).

Several studies reported congruent effects of food properties (composition, rheological properties) on *in vivo* aroma release and perception (16,18,24), while other studies observed incongruencies between *in vivo* aroma release and perception (25-28). Here, we refer to congruent effects when an increase of *in vivo* aroma release leads to an increase in aroma perception (and a decrease of *in vivo* aroma release to a decrease in perception). We refer to incongruent effects when an increase of *in vivo* aroma release is accompanied by a decrease in aroma perception. In case of flavoured gels, increasing gel hardness did not affect *in vivo* aroma release but decreased perceived aroma intensity (incongruent effect) (28). It has been suggested that cognitive interactions due to cross-modal associations between food texture and aroma perception play a role when incongruent effects were observed (6,29). It is also plausible that changes in oral processing behaviour (e.g. dilution with saliva, structure breakdown by chewing or influence of mouth temperature) induce changes in aroma release and potentially perception (4).

Flavoured condiments such as mayonnaises are usually consumed together with rather plain carrier foods such as potatoes and bread, and dynamic interactions between condiments and carriers can influence aroma release and perception. Addition of bland carrier foods to condiments is generally known to decrease overall perceived flavour intensity of condiments (30-33). Addition of bread or carrots to mayonnaises has been shown to reduce perceived intensities of several mayonnaise-related flavour attributes (33). However, the mechanisms underlying the reduction in flavour perception upon carrier addition are not known. To the best of our knowledge, it has not been investigated whether the decreased flavour perception is due to physiochemical interactions leading to a lower delivery of aroma compounds into the nasal cavity or whether other mechanisms such as different eating behaviours or cognitive interactions due to cross-modal associations come into play. To summarize, it is not well understood how addition of rather plain carriers changes flavour release and perception of condiments.

As shown by our earlier work, not only the presence of a carrier food, but also the type of carrier affects sensory properties of condiments. Crackers decreased overall perceived intensity of a variety of condiments (firm cheese, cheese spread, mayonnaise) to a larger extent than bread (34). Harder carrier foods decreased the perception of mayonnaise to a larger extent than softer carriers(33). Besides sensory attribute intensity, also dynamic perception of sensory properties of condiments were affected by addition of carriers. Mayonnaise-related flavour attributes were perceived as dominant sensations earlier during consumption when combined with softer carriers than when combined with harder carriers. Type and mechanical properties of carrier foods affect static and dynamic sensory properties of food-condiment combinations. We hypothesize that aroma perception of condiment-carrier combinations is driven by physicochemical effects on aroma release (condiment aroma release is decreased as condiment aroma compounds bind to the carrier).

This study aimed to understand the relation between in-nose aroma release and dynamic aroma perception of condiments (mayonnaise) when consumed with and without carriers (bread, potatoes). Mayonnaise properties were varied by varying fat content (high, low) and viscosity (high, low) to understand the effect of different physicochemical properties on in-nose aroma release and perception. Carrier foods with different moisture absorption capacity (bread, potato) varying in texture (soft, hard) were tested to investigate the role of carrier type on aroma release and perception of condiments (Figure 6.1).

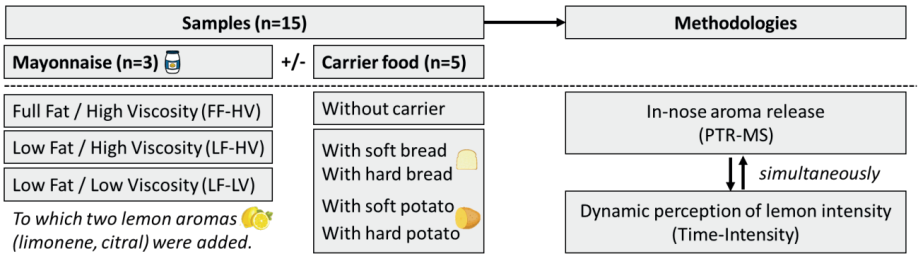



***Figure 6.1:*** *Experimental design outlining the approach. Mayonnaises varying in fat content (high/low) and viscosity (high/low) were tested without carrier food (n=3) and together with carrier foods differing in moisture absorption capacity (bread, potato) and hardness (hard/soft). Mayonnaises were spiked with two lemon aroma compounds (limonene, citral), which allowed to characterize in-nose aroma release and dynamic lemon intensity perception.*

## 6.2 Materials and Methods

### 6.2.1 | Samples

Three different mayonnaises varying in fat content and viscosity were prepared, namely full fat/high viscosity (FF-HV; 69% w/w oil; Calvé De echte, Unilever, The Netherlands), low fat/high viscosity (LF-HV), and low fat/low viscosity (LF-LV) mayonnaises. For the low fat mayonnaises, a 2.5 or 1.0% xanthan in water solution (E415, Pit&Pit bvba, Belgium) was gradually spooned into the FF-HV mayonnaise following a 1.6:1.0 weight ratio to create the LF-HV or LF-LV mayonnaises (26.5% w/w oil) with similar oil droplet sizes, respectively. Two lemon aroma compounds varying in hydrophobicity, citral ($M_w$ = 152 g/mol , *log P* = 2.76, 1 mg/g mayonnaise) and limonene ($M_w$ = 136 g/mol, *log P* = 4.2, 1 mg/g mayonnaise), were gently mixed into the mayonnaises using a spatula. The addition of these compounds made the mayonnaise easier to track during the proton transfer reaction mass spectrometry (PTR-MS) analysis and easier to be perceived by the panellists. The two compounds were chosen based on their aroma, their different physical/chemical properties and their masses after some preliminary measurements on volatile organic compounds (VOCs) emissions on both mayonnaises and carriers to verify interferences. Mayonnaises were served at a weight of 2 g.

Mayonnaises were assessed alone and in combination with different carrier foods. Two commercial carrier foods were used, namely bread (Plaisir de mie toastbrood, Jacquet®, France) and potatoes (Waxy potatoes, Albert Heijn, The Netherlands). Mayonnaise on bread represents a simplified model food for sandwiches and mayonnaise on potato represents a simplified model food for salads. Bread cubes without crust (35x35x8 mm) were served fresh and oven-dried for 40 min at 100°C (Venti-line, VWR®) to obtain two bread samples with varying properties. Peeled potato cubes (30x12x12 mm) were cooked sous-vide at 90°C for 15 and 45 min to obtain two potato samples with varying properties. Carrier-mayonnaise combinations were

prepared just before serving to minimize moisture transfer of the mayonnaises into the carriers before consumption.

Table 6.1 presents an overview of the composition and product properties of the mayonnaises (fat content, viscosity, oil droplet size) and the carrier foods (firmness, water activity). Mayonnaises properties were measured each morning before data collection (n=10 days of data collection) and carrier properties were measured for each new preparation batch (n=4 batches) to ensure that samples were stable over the data collection period. To determine the viscosity of the mayonnaises, mayonnaises were sheared at shear rates ranging from 1 $s^{-1}$ to 1000 $s^{-1}$ after a resting period of 5 minutes using a rheometer (MCR 301 Rheometer, Anton Paar Benelux BVBA, Belgium) equipped with an Inset I-PP50/SS plate and a CP50-1 cone. The oil droplet size of the mayonnaises ($D_{3,2}$) was measured by light scattering (Mastersizer 2000, Malvern Instruments) in triplicate using the refractive index of sunflower oil (1.469). To determine the firmness of the carrier foods, uniaxial compression tests were performed with a Texture Analyser (TA.XT Plus, Stable Micro Systems, United Kingdom) fitted with a 50 kg load cell, a cylindrical plate with a diameter of 100 mm and a constant speed of 1 mm/s. Bread samples were compressed until 20% strain, and the mean force needed to compress the bread samples was calculated. Potato samples were compressed until 50% stain and the mean fracture stress of the potatoes was calculated. The water activity of the carrier foods was measured using a LabMaster aw (Novasina®).

**Table 6.1:** Product properties of mayonnaises varying in fat content and viscosity (A) and the carrier foods bread (B) and potato (C) varying in preparation methods (mean±SD).

| (A) Mayonnaise properties | Full fat / High viscosity (FF-HV) | Low fat / High viscosity (LF-HV) | Low fat / Low viscosity (LF-LV) |
|---|---|---|---|
| Fat content (w/w %) | 70 | 27 | 27 |
| Viscosity at 1 $s^{-1}$ (Pa·s) | 84±19 | 73±12 | 11±3 |
| Viscosity at 10 $s^{-1}$ (Pa·s) | 13±3 | 10±1 | 2±1 |
| Viscosity at 100 $s^{-1}$ (Pa·s) | 2.1±0.4 | 1.3±0.2 | 0.3±0.1 |
| $D_{3,2}$ (µm) | 10.9±2 | 7.9±1 | 7.1±1 |

| (B) Bread properties | Fresh bread | Dried bread |
|---|---|---|
| Compression force (N) | 5±2 | 100±38 |
| $A_w$ | 0.92±0.02 | 0.20±0.16 |

| (C) Potato properties | Soft potato | Semi-hard potato |
|---|---|---|
| Fracture stress | 40±17 | 198±132 |
| $A_w$ | 0.98±0.01 | 0.99±0.00 |

### 6.2.2 | Subjects

A group of 14 Caucasian, European females (23±3 years,) participated in the study. An homogeneous group of subjects was selected based on their mechanically stimulated saliva flow rate (1.4±0.6 g/min, mean±SD) (60), size of the oral cavity (73.5±10.4 g water, mean±SD) (61) and natural eating time of the samples (16±5 s, mean±SD) (34), which were assessed during one selection session of one hour based on procedures described elsewhere. In addition, they had non-smoking habits (self-reported), good dental health (self-reported), and were consumers of mayonnaise, bread and potato on a regular basis. All subjects gave written informed consent, completed the study and received financial compensation for participation.

### 6.2.3 | Chewing protocol

Subjects were instructed to follow a chewing protocol to minimize the influence of individual differences in mastication behaviour on aroma release and perception throughout consumption. Subjects were instructed to consume each sample within one bite, and to swallow after 20 seconds of consumption (timer was shown on the screen). In the case of mayonnaises alone, they were instructed to swirl samples in their mouth. In the case of mayonnaise-carrier combinations, they were instructed to chew the sample with a frequency of 1 chew/s (i.e. approximately 20 chews) using a metronome and the timer on the screen. Furthermore, subjects were asked to raise their hand each time they swallowed, which was recorded by the researcher. In addition, they were asked to keep their mouth closed during all the evaluations.

### 6.2.4 | Nose-space analysis, data extraction and peak selection

*In vivo* aroma release was measured using a commercial PTR-MS instrument (Ionicon Analytik GmbH, Innsbruck, Austria) equipped with a time of flight and a quadrupole ion guide (PTR-QiToF). $H_3O^+$ was used as precursor ion, and the ionization conditions were the following: 1000 V drift voltage, 60.0 °C drift temperature, 3.8 mbar drift pressure, resulting in an E/N ratio of 133 Td. Acquisition was set to 1 spectrum per second. Sampling was carried out via a heated (95 °C) inlet tube with an inlet flow of 45.02 sccm. The mass resolution (m/Δm) was at least 5000.

The nose-space experimental set up was adapted from previous PTR-MS in-nose studies (50,62,63) For each measurement, laboratory air was sampled for 20 s. After that, subjects were asked to insert two Teflon tubes (diameter: 6.8 mm, length: 6.4 cm, connected to the heated inlet tubes) in the nose. They were asked to breath normally through their nose, and subjects' breath was sampled for 60 s. Then they consumed the samples for 20 s. After swallowing the sample, subjects kept on breathing for 90 s. This led to a total sampling time of 190 s (Figure 6.2). Samples were assessed in triplicate by each subject.

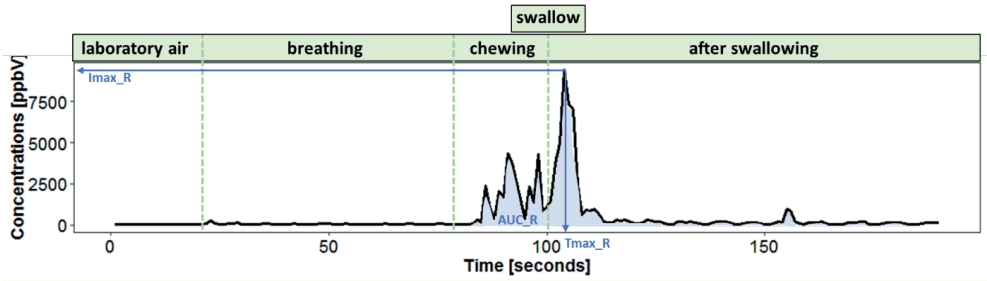
PTR-MS data were treated with TOFO office software (Department of Food Quality and Nutrition, Edmund Mach Foundation) as described in Cappellin *et al.* (2011). A total of 247 mass peaks were extracted from 20 *m/z* to 250 *m/z* and in-nose concentration was calculated. From that, 73 peaks were selected for the further analysis based on pilot experiment report, literatures and the high concentration of the release curve

for the relevant aroma compounds of citral, limonene, mayonnaise, food carriers and the exhaled gases from participants. In the work, only mass peaks corresponding to the two lemon aroma compound are considered: *m/z* 138.139 and 153.131 tentatively identified as the limonene isotope ($^{13}CC_9H_{16}H^+$) and citral ($C_{10}H_{16}OH^+$) were chosen as representative examples, respectively. The *m/z* 81.070 ($C_6H_9^+$) and 135.119 ($C_{10}H_{15}^+$) were chosen as main fragments of limonene and citral. For each mass peaks a release curve was obtained by plotting between peak concentration (ppbV) and time (seconds). Each release curve, was divided in 4-time separate windows: lab air session (1-20 s), breathing (21-80 s), mastication session (81 s to first swallowing point) and post-swallowing session (first swallowing point until 195 s). Each part of the curve was averaged for all the panel and superposed to create an average release curve for each sample. For comparing the different mayonnaises aroma release and the food carrier interactions, the baseline (signal before the sample was ingested) was then subtracted and three main parameters were extracted from each individual release curve: the area under the curve (AUC_R), the maximum concentration ($I_{max}$_R) and the time to reach the maximum concentration ($T_{max}$_R).

### 6.2.5 | Time-intensity (TI) sensory methodology

Dynamic lemon aroma intensity of mayonnaises was determined using the time-intensity (TI) methodology (64). Subjects were instructed to place the sample in the mouth and simultaneously click the *start* button. Then, they continuously scored the lemon intensity over time by moving the cursor horizontally on a 100 mm unstructured line scale anchored from *not at all* to *very* (Eye Question software, version 4.11.19). The total duration of the evaluation was set at 110 s, meaning that subjects evaluated lemon intensity during chewing (approximately 20 s) and after the sample had been swallowed (approximately 90 s). Intensity scores were recorded with an interval time of 500 ms. From the time-intensity profiling, the total area under the curve (AUC_S), the maximum perceived intensity ($I_{max}$_S) and the time to maximum intensity ($T_{max}$_S) were obtained (Figure 6.2). In the present study, Liu & MacFie standardization was applied to correct for individual signature curves (58,65).

**(A)**




**(B)**

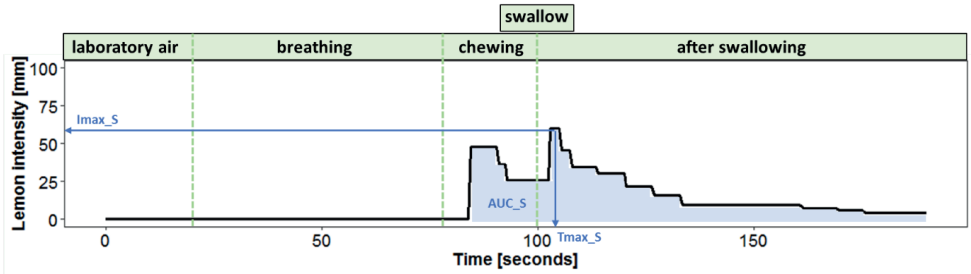



***Figure 6.2:*** *Example of an aroma release curve (A) and a time intensity curve (B), in which the different sampling procedures and panel instructions are indicated in green. Parameters obtained from the curves are shown in blue.*

### 6.2.6 | Experimental approach

Subjects participated in 10 sessions over a period of one month. Subjects were firstly trained over four sessions of 1 hour, after which dynamic aroma perception and *in vivo* aroma release were determined simultaneously by using TI and PTR-MS during the subsequent six sessions of maximum 1.5 hours.

During the four training sessions, subjects were acquainted with the chewing protocol (training 1 and 2) and the TI methodology (training 3 and 4). The first training started with an introduction to the chewing protocol (section 6.2.3), after which the subjects practiced the protocol. During the second training session, subjects were familiarized with the nose tubes used to connect the subjects' nasal cavity with the PTR-MS. During this session, subjects continued practicing the chewing protocol while having the nose tubes in their nose. The third session was used to introduce TI methodology to the subjects, after which they practiced with the tasting protocol, nose tubes and TI methodology using mayonnaises spiked with lemon aroma. The fourth session was a pilot experiment, during which they practiced with the tasting protocol, nose tube and TI methodology for all samples included in the present study.

During the six data collection sessions, subjects were requested to not eat, drink, or brush their teeth two hours before the experiment and to not wear perfume or lotion. All samples were assessed following a 3x5 design: three mayonnaises with five carrier conditions (without carrier, with fresh bread, with oven-dried

bread, with shortly cooked potato, with long cooked potato). Samples were assessed in triplicate leading to a total of 45 nose-space measurements and sensory analyses for each subject. Each replicate was assessed over two sessions. Within each replicate, samples were presented in a random order following a completely randomized design. Samples were presented with three digits codes and served on a spoon to facilitate easy intake. Between each sample, subjects cleansed their palate for at least 6 minutes using cold water, hot water and tongue scrapers to aid the removal of oil from their tongue. No other palate cleansers were used, since they might affect the volatile release of follow-up samples.

### 5.3.7 | Statistical data analyses

Results were reported as mean values with standard error (n=14 subjects, in triplicate). Outliers (Z-score>3.29 or Z-score<-3.29) were removed from the data. To investigate the effect of mayonnaise properties, linear mixed models were performed on a subset of the data including the data of the single mayonnaises (i.e. without carriers) only. For this analysis, *mayonnaise* was set as fixed effect and *subject, replicate, serving order* and *session were set* as random effects using Lmer package (66). To investigate the interplay between mayonnaise properties and carrier properties, linear mixed models were performed with *mayonnaise, carrier* and *mayonnaise:carrier interaction* as fixed effects and *subject, replicate, serving order* and *session* as random effects. This analysis was performed for bread and potato carrier separately. In addition, Multiple Factor Analysis (MFA) was performed on the selected mass peaks from PTR-MS analysis and on the Time-Intensity data by using FactoMineR package (67). Only the AUC was used in this case and data were scaled to unit variance before performing the analysis (67). R language (RStudio, version 1.0.143) was used to perform all statistical tests. Significance level of $p<0.05$ was chosen.

## 6.3 Results

### 6.3.1 | In-nose aroma release and dynamic lemon perception of mayonnaises without carriers: effect of viscosity and fat content

Dynamic aroma release and dynamic lemon intensity profiles of mayonnaises without carrier foods are shown in Figure 6.3. Table 6.2 provides a summary of all aroma release (AUC_R, $I_{max}$_R and $T_{max}$_R) and perception (AUC_S, $I_{max}$_S and $T_{max}$_S) parameters. As can be seen from Figure 6.3A – 6.3D, limonene and citral display different release profiles. While limonene was released fast, resulting in a sharp peak (Figure 6.3A, 6.3B), citral was released slowly throughout consumption (later $T_{max}$_R, see Table 6.2) resulting in a later and broader peak (Figure 6.3C, 6.3D). Citral has a higher boiling temperature and lower vapor pressure due to its higher molecular weight and its molecular structure, resulting in a lower volatility than limonene. Mayonnaise viscosity (LF-HV vs. LF-LV) clearly affected in-nose aroma concentrations (Figure 6.3A, 6.3C) and dynamic lemon intensity perception (Figure 6.3E). *In vivo* limonene release, *in vivo* citral release and lemon intensity perception decreased with increasing mayonnaise viscosity. For example, in case of

limonene (*m/z* 138.139), AUC_R decreased by 69% and $I_{max}$_R decreased by 74% with increasing viscosity (Table 6.2). Congruently, with respect to sensory perception, AUC_S decreased by 31% and $I_{max}$_S decreased by 23%. The times to reach the maximum concentration and intensity ($T_{max}$_R, $T_{max}$_S) were not significantly affected by mayonnaise viscosity.

Mayonnaise fat content (FF-HV vs. LF-HV) also affected in-nose aroma concentration (Figure 6.3B, 6.3D) and dynamic lemon intensity perception (Figure 6.3F). *In vivo* limonene release, *in vivo* citral release and lemon intensity perception decreased upon fat reduction of mayonnaises from 70 to 27 wt%. For example, AUC_R of limonene (*m/z* 138.139) decreased by 72% and AUC_S decreased by 45% with decreasing fat content. Similar trends were found for the $I_{max}$ values (Table 6.2). A reduction of fat content slowed down the release of limonene ($p<0.05$, $T_{max}$_R), but no significant effect was observed for citral release.

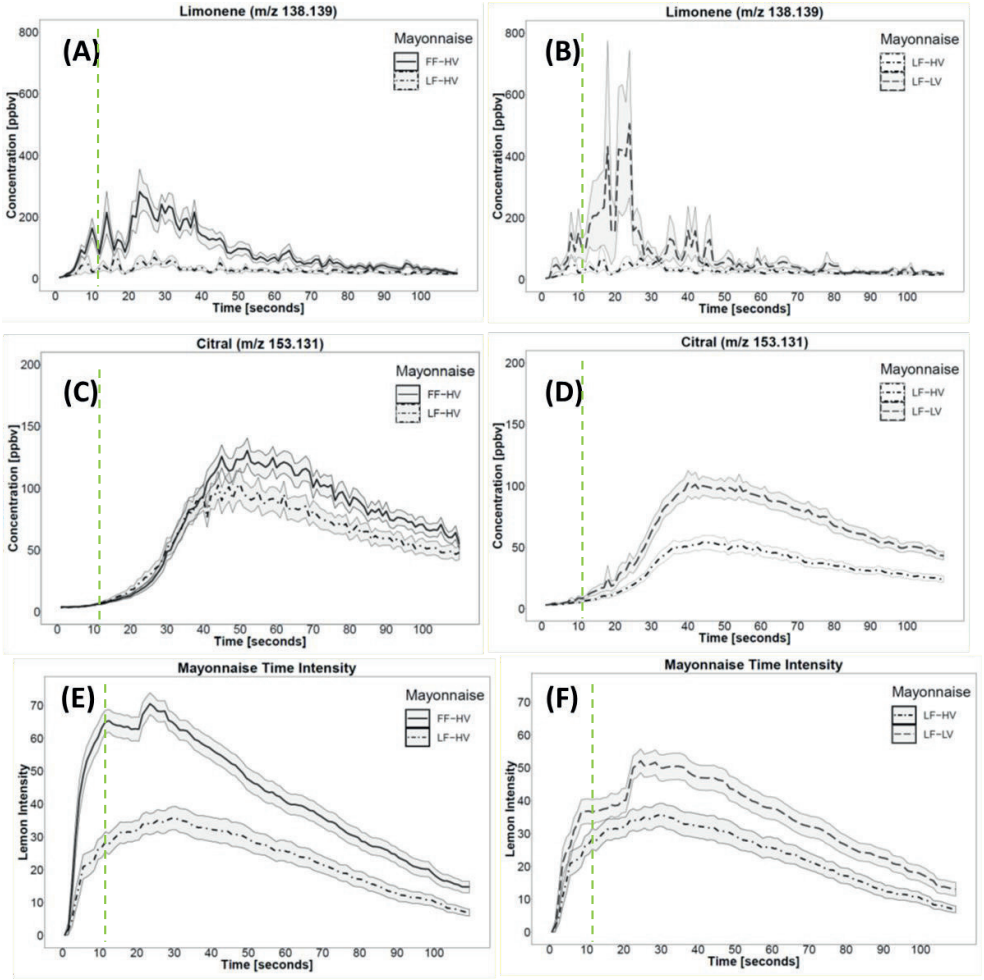



***Figure 6.3:*** *Averaged in-nose limonene release (m/z = 138.139) (A, B), in-nose citral release (m/z = 153.131) (C, D) and lemon intensity perception (E, F) during mastication and after swallowing for mayonnaise varying in viscosity and fat content (n=14 subjects, in triplicate). Mayonnaise differing in viscosity (LF-HV and LF-LV) are presented on the left (A, C, E), and mayonnaise varying in fat content (FF-HV and LF-HV) are presented on the right (B, D, F). The shaded bars represent the standard error of the mean. The moment of swallowing is indicated as dashed line at 20 s.*

**Table 6.2:** Summary of parameters (mean±SE) describing in vivo limonene release, in vivo citral release and dynamic lemon intensity perception for mayonnaises varying in fat content (FF = full fat, LF = low fat) and viscosity (HV = high viscosity, LV = low viscosity). The release parameters AUC_R, $I_{max}$_R, $T_{max}$_R correspond to the area under the curve, the maximum concentration and time to reach the maximum concentration. The sensory parameters AUC_S, $I_{max}$_S and $T_{max}$_S correspond to the total area under the curve, the maximum perceived intensity and the time to reach the maximum perceived intensity.

| | | Mayonnaise | | FF-HV | | | | LF-HV | | | | LF-LV | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AUC** | | *F* | *p* | *mean* | ± | *SE* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | |
| AUC_R (ppbV·s) | *m/z 81.070* | 17.0 | <0.001 | 94331 | ± | 6988 | *a* | 2584 | ± | 3907 | *b* | 72744 | ± | 14208 | *a* |
| *limonene* | *m/z 138.139* | 10.3 | <0.001 | 9617 | ± | 701 | *a* | 2695 | ± | 407 | *b* | 8571 | ± | 2003 | *a* |
| AUC_R (ppbV·s) | *m/z 135.119* | 25.8 | <0.001 | 4210 | ± | 277 | *b* | 3228 | ± | 321 | *b* | 6226 | ± | 424 | *a* |
| *Citral* | *m/z 153.131* | 23.9 | <0.001 | 7550 | ± | 538 | *b* | 6007 | ± | 608 | *b* | 11767 | ± | 850 | *a* |
| AUC_S (mm·s) | | 29.0 | <0.001 | 9198 | ± | 506 | *a* | 5096 | ± | 489 | *c* | 7362 | ± | 566 | *B* |
| **$I_{max}$** | | *F* | *p* | *mean* | ± | *SE* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | |
| $I_{max}$_R (ppbV) | *m/z 81.070* | 9.4 | <0.001 | 8521 | ± | 785 | *a* | 2586 | ± | 468 | *b* | 8085 | ± | 1616 | *a* |
| *limonene* | *m/z 138.139* | 5.9 | <0.01 | 857 | ± | 80 | *ab* | 269 | ± | 50 | *b* | 1030 | ± | 271 | *a* |
| $I_{max}$_R (ppbV) | *m/z 135.119* | 26.3 | <0.001 | 88 | ± | 5 | *b* | 72 | ± | 7 | *b* | 134 | ± | 8 | *a* |
| *citral* | *m/z 153.131* | 24.8 | <0.001 | 173 | ± | 12 | *b* | 145 | ± | 14 | *b* | 267 | ± | 16 | *a* |
| $I_{max}$_S (mm) | | 30.6 | <0.001 | 77 | ± | 3 | *a* | 48 | ± | 4 | *c* | 62 | ± | 4 | *b* |
| **$T_{max}$** | | *F* | *p* | *mean* | ± | *SE* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | |
| $T_{max}$_R (s) | *m/z 81.070* | 4.7 | <0.05 | 29 | ± | 2 | *b* | 39 | ± | 3 | *a* | 34 | ± | 3 | *ab* |
| *limonene* | *m/z 138.139* | 3.8 | <0.05 | 28 | ± | 2 | *b* | 38 | ± | 3 | *a* | 33 | ± | 3 | *ab* |
| $T_{max}$_R (s) | *m/z 135.119* | 0.0 | NS | 51 | ± | 2 | | 49 | ± | 2 | | 50 | ± | 3 | |
| *citral* | *m/z 153.131* | 0.3 | NS | 56 | ± | 2 | | 52 | ± | 2 | | 52 | ± | 2 | |
| $T_{max}$_S (s) | | 4.8 | <0.05 | 33 | ± | 3 | *b* | 39 | ± | 3 | *ab* | 43 | ± | 3 | *a* |

*Lower case letters: significant differences between mayonnaise varying in fat content and viscosity (p<0.05).*

### 6.3.2 | In-nose aroma release and dynamic lemon perception of mayonnaise with carrier foods

Figure 6.4 shows averaged in-nose limonene release, in-nose citral release and perceived lemon intensity curves for FF-HV mayonnaise without and with carrier foods. Addition of carriers affected release and perception of the other two mayonnaises (LF-HV and LF-LV) in a relatively similar way (data not shown). In-nose limonene and citral release parameters (AUC_R, $I_{max}$_R and $T_{max}$_R) and perceived lemon parameters (AUC_S, $I_{max}$_S and $T_{max}$_S) of mayonnaises without and with carriers are presented in Table 6.3. Overall, in-nose limonene and citral release increased with the addition of food carriers, whereas simultaneous lemon intensity perception of mayonnaises decreased. Bread and potato affected aroma release and perception of mayonnaises differently. The results of bread and potato addition are therefore reported separately in the following sub sections.

### 6.3.3 | Effect of bread addition on in-nose aroma release and aroma perception of mayonnaises

Addition of bread increased in-nose limonene and citral release of mayonnaises, regardless of bread texture (Figure 6.4A, 6.4C and Table 6.3A). For example, in case of limonene (*m/z* 138.139), AUC_R increased by 136% and 144% after addition of soft and hard bread, respectively ($p<0.001$; $p<0.001$). For citral (*m/z* 135.199 and 153.131), AUC_R increased with addition of bread, but this effect was only significant for LF-HV mayonnaise. Similar trends were observed for $I_{max}$_R values. Bread texture (soft vs. hard) did not affect limonene and citral release concentrations significantly.

The time to reach maximum aroma concentration ($T_{max}$_R) was affected by addition of bread, regardless of bread texture (Table 6.3A). Overall, $T_{max}$_R was reached earlier for mayonnaise-bread combinations than for mayonnaises consumed without bread. These differences in $T_{max}$_R were significant for LF-HV but not for FF-HV nor LF-LV.

Addition of bread decreased lemon intensity perception of mayonnaises (Figure 6.4E, Table 6.3A). However, the effect of bread on lemon intensity perception was not the same for each mayonnaise (significant mayonnaise:bread interaction). For FF-HV mayonnaise, AUC_S and $I_{max}$_S were lowered by 11 and 8% with soft bread ($p>0.05$; $p>0.05$) and by 21 and 22% with hard bread ($p=0.003$; $p=0.001$). For LF-LV mayonnaise, AUC_S and $I_{max}$_S decreased by 10 and 10% with soft bread ($p>0.05$; $p>0.05$) and by 22 and 15% with hard bread ($p=0.030$; $p>0.05$). No significant effect was observed for LF-HV. Hence, bread hardness partly affected lemon intensity perception of mayonnaises. $T_{max}$_S was not significantly affected by the addition of bread.

### 6.3.4 | Effect of potato addition on in-nose aroma release and aroma perception of mayonnaises

Addition of potato to mayonnaises increased both limonene and citral release (Figure 6.4B, 6.4D, Table 6.3B). For example, in case of limonene release (*m/z* 138.139), AUC_R increased by 45 and 43% after addition of soft and hard potato ($p<0.001$ and $p<0.001$). In case of citral release (*m/z* 135.119), AUC_R

increased by 8% with soft potato ($p>0.05$) and by 21% with hard potato ($p<0.001$). Hence, potato texture (soft vs. hard) affected citral release concentrations. Similar trends were observed for $I_{max}$_R.

The time to reach maximum aroma concentration of mayonnaises ($T_{max}$_R) was affected by the addition of potatoes, regardless of potato texture (Table 6.3B). On average, $T_{max}$_R was reached in less time after the addition of potato. Regarding sensory perception, presence of potato carriers decreased perceived lemon intensity. AUC_S decreased by 19% with addition of soft potato ($p<0.001$) and by 17% with addition of hard potato ($p=0.002$). Similar effects were observed for $I_{max}$_S, but the effect was significant for soft potato (reduction by 15% $p<0.001$) but not for hard potato (reduction by 9%; $p>0.05$). $T_{max}$_S was not significantly affected by addition of potato carriers.

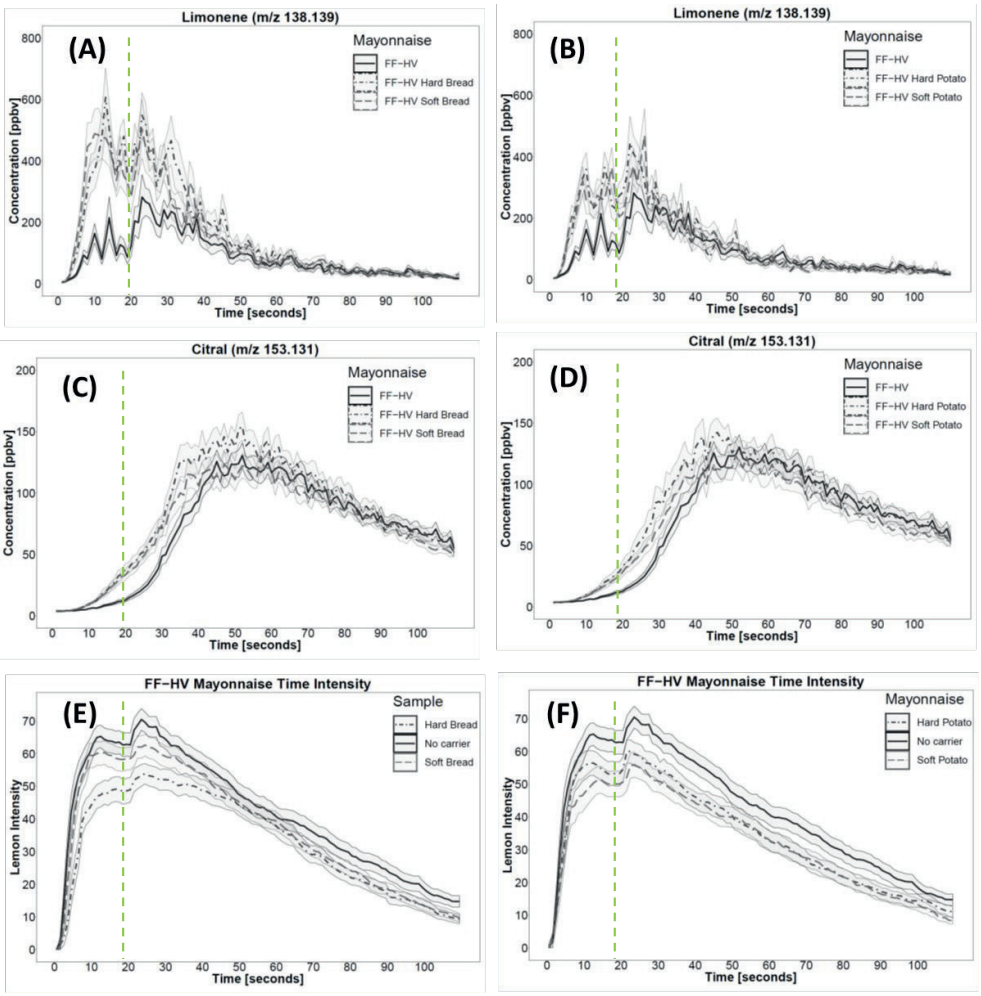



***Figure 6.4:*** *Averaged in-nose limonene release (m/z = 138.139) (A, B), in-nose citral release (m/z = 153.131) (C, D) and lemon intensity perception (E, F) during mastication and after swallowing for mayonnaise without and with different food carriers (n=14 subjects, in triplicate). Mayonnaise (i.e. FF-HV mayonnaise) with bread carriers (soft, hard) is presented in yellow on the left (A, C, E), and the mayonnaise with potato carriers (soft, hard) is presented in pink on the right (B, D, F). The shaded bars represent the standard error of the mean. The moment of swallowing is shown as adashed line at 20 s.*

**Table 6.3**: Summary of parameters (mean±SE) describing in vivo limonene release, in vivo citral release and dynamic lemon intensity perception for mayonnaises without and with carrier foods. The effects of bread (A) and potato (B) are presented separately. The release parameters AUC_R, Imax_R, Tmax_R correspond to the area under the curve, the maximum concentration and time to reach the maximum concentration. The sensory parameters AUC_S, Imax_S and Tmax_S correspond to the total area under the curve, the maximum perceived intensity and the time to reach the maximum perceived intensity.

**(A) Mayonnaises without and with bread**

| | | Bread | | Bread:Mayo | | | No carrier | | | | With soft bread | | | | With hard bread | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AUC** | | *F* | *p* | *F* | *p* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | |
| AUC_R (ppbV·s) *limonene* | *m/z 81.070* | 63.1 | p<0.001 | 1.1 | NS | | 64613 | ± | 6001 | *B* | 149536 | ± | 9825 | *A* | 158206 | ± | 9186 | *A* |
| | *m/z 138.139* | 42.4 | p<0.001 | 1.3 | NS | | 6995 | ± | 770 | *B* | 16553 | ± | 1278 | *A* | 17085 | ± | 1173 | *A* |
| AUC_R (ppbV·s) *citral* | *m/z 135.119* | 13.1 | p<0.001 | 4.5 | p<0.01 | *FF-HV* | 4210 | ± | 277 | *A* | 4532 | ± | 248 | *A* | 5119 | ± | 307 | *A* |
| | | | | | | *LF-HV* | 3228 | ± | 321 | *B* | 5970 | ± | 400 | *A* | 5562 | ± | 450 | *A* |
| | | | | | | *LF-LV* | 6226 | ± | 424 | *A* | 6766 | ± | 423 | *A* | 6590 | ± | 460 | *A* |
| | *m/z 153.131* | 6.2 | p<0.01 | 6.0 | p<0.001 | *FF-HV* | 7550 | ± | 538 | *A* | 7833 | ± | 477 | *A* | 8909 | ± | 595 | *A* |
| | | | | | | *LF-HV* | 6007 | ± | 608 | *B* | 10828 | ± | 775 | *A* | 9953 | ± | 859 | *A* |
| | | | | | | *LF-LV* | 11767 | ± | 850 | *A* | 11571 | ± | 736 | *A* | 10902 | ± | 821 | *A* |
| AUC_S (mm·s) | | 7.0 | p<0.01 | 4.0 | p<0.01 | *FF-HV* | 9198 | ± | 506 | *A* | 8214 | ± | 437 | *AB* | 7307 | ± | 488 | *B* |
| | | | | | | *LF-HV* | 5096 | ± | 489 | *A* | 5844 | ± | 490 | *A* | 5515 | ± | 481 | *A* |
| | | | | | | *LF-LV* | 7362 | ± | 566 | *A* | 6608 | ± | 522 | *AB* | 5767 | ± | 484 | *B* |
| **Imax** | | *F* | *p* | *F* | *p* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | |
| $I_{max}$_R (ppbV) *limonene* | *m/z 81.070* | 34.4 | p<0.001 | 0.5 | NS | | 6414 | ± | 658 | *B* | 12880 | ± | 926 | *A* | 13414 | ± | 937 | *A* |
| | *m/z 138.139* | 17.9 | p<0.001 | 1.4 | NS | | 720 | ± | 99 | *B* | 1613 | ± | 175 | *A* | 1533 | ± | 147 | *A* |
| $I_{max}$_R (ppbV) *citral* | *m/z 135.119* | 11.2 | p<0.001 | 2.3 | NS | | 97 | ± | 5 | *B* | 121 | ± | 5 | *A* | 128 | ± | 7 | *A* |
| | *m/z 153.131* | 4.5 | p<0.05 | 4.8 | p<0.001 | *FF-HV* | 173 | ± | 12 | *A* | 176 | ± | 12 | *A* | 203 | ± | 14 | *A* |
| | | | | | | *LF-HV* | 145 | ± | 14 | *B* | 248 | ± | 19 | *A* | 223 | ± | 20 | *A* |
| | | | | | | *LF-LV* | 267 | ± | 16 | *A* | 268 | ± | 19 | *A* | 250 | ± | 22 | *A* |
| $I_{max}$_S (mm) | | 9.1 | p<0.001 | 3.5 | p<0.01 | *FF-HV* | 77 | ± | 3 | *A* | 71 | | 3 | *AB* | 61 | | 3 | *B* |
| | | | | | | *LF-HV* | 48 | ± | 4 | *A* | 48 | | 3 | *A* | 48 | | 3 | *A* |
| | | | | | | *LF-LV* | 62 | ± | 4 | *A* | 56 | | 3 | *A* | 52 | | 4 | *A* |
| **Tmax** | | *F* | *p* | *F* | *p* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | | *mean* | ± | *SE* | |
| $T_{max}$_R (s) *limonene* | *m/z 81.070* | 32.8 | p<0.001 | 4.2 | p<0.01 | *FF-HV* | 29 | ± | 2 | *A* | 24 | ± | 2 | *A* | 26 | ± | 2 | *A* |
| | | | | | | *LF-HV* | 39 | ± | 3 | *A* | 24 | ± | 2 | *B* | 22 | ± | 2 | *B* |
| | | | | | | *LF-LV* | 34 | ± | 3 | *A* | 21 | ± | 1 | *B* | 23 | ± | 2 | *B* |
| | *m/z 138.139* | 30.4 | p<0.001 | 4.0 | p<0.01 | *FF-HV* | 28 | ± | 2 | *A* | 24 | ± | 2 | *A* | 25 | ± | 1 | *A* |
| | | | | | | *LF-HV* | 38 | ± | 3 | *A* | 23 | ± | 2 | *B* | 22 | ± | 2 | *B* |
| | | | | | | *LF-LV* | 33 | ± | 3 | *A* | 19 | ± | 1 | *B* | 24 | ± | 2 | *B* |
| $T_{max}$_R (s) *citral* | *m/z 135.119* | 6.3 | p<0.01 | 1.3 | NS | | 50 | ± | 1 | *A* | 43 | ± | 1 | *B* | 46 | ± | 1 | *AB* |
| | *m/z 153.131* | 1.4 | NS | 0.7 | NS | | 54 | ± | 1 | | 52 | ± | 1 | | 55 | ± | 1 | |
| $T_{max}$_S (s) | | 0.3 | NS | 1.4 | NS | | 38 | ± | 2 | | 38 | ± | 2 | | 40 | ± | 2 | |

*Capital letters: significant differences between mayonnaise without/with bread carriers (p<0.05)*

**(B) Mayonnaises without and with potato**

| | | Potato | | Potato:Mayo | | | No carrier | | | With soft potato | | | With hard potato | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AUC** | | *F* | *p* | *F* | *p* | | *mean* ± *SE* | | | *mean* ± *SE* | | | *mean* ± *SE* | | |
| AUC_R (ppbV·s) *limonene* | *m/z 81.070* | 32.5 | p<0.001 | 2.2 | NS | | 64613 ± 6001 | | *B* | 118421 ± 8157 | | *A* | 118637 ± 7441 | | *A* |
| | *m/z 138.139* | 21.8 | p<0.001 | 1.9 | NS | | 6995 ± 770 | | *B* | 12697 ± 1015 | | *A* | 12350 ± 803 | | *A* |
| AUC_R (ppbV·s) *citral* | *m/z 135.119* | 9.0 | p<0.001 | 2.2 | NS | | 4552 ± 227 | | *B* | 4957 ± 195 | | *B* | 5722 ± 246 | | *A* |
| | *m/z 153.131* | 4.5 | p<0.05 | 3.1 | p<0.05 | *FF-HV* | 7550 ± 538 | | *A* | 7476 ± 527 | | *A* | 8091 ± 605 | | *A* |
| | | | | | | *LF-HV* | 6007 ± 608 | | *B* | 8365 ± 577 | | *AB* | 9536 ± 772 | | *A* |
| | | | | | | *LF-LV* | 11767 ± 850 | | *A* | 10706 ± 634 | | *A* | 12201 ± 658 | | *A* |
| AUC_S (mm·s) | | 10.5 | p<0.001 | 2.1 | NS | | 7236 ± 335 | | *A* | 6085 ± 307 | | *B* | 6200 ± 300 | | *B* |
| **Imax** | | *F* | *p* | *F* | *p* | | *mean* ± *SE* | | | *mean* ± *SE* | | | *mean* ± *SE* | | |
| $I_{max}$_R (ppbV) *limonene* | *m/z 81.070* | 20.0 | p<0.001 | 2.4 | NS | | 6414 ± 658 | | *B* | 11860 ± 927 | | *A* | 11370 ± 898 | | *A* |
| | *m/z 138.139* | 10.4 | p<0.001 | 1.7 | NS | | 720 ± 99 | | *B* | 1373 ± 151 | | *A* | 1361 ± 155 | | *A* |
| $I_{max}$_R (ppbV) *citral* | *m/z 135.119* | 9.1 | p<0.001 | 2.3 | NS | | 97 ± 5 | | *B* | 110 ± 5 | | *B* | 127 ± 6 | | *A* |
| | *m/z 153.131* | 5.4 | p<0.01 | 2.8 | p<0.05 | *FF-HV* | 173 ± 12 | | *A* | 172 ± 14 | | *A* | 182 ± 14 | | *A* |
| *Capital* | | | | | | *LF-HV* | 145 ± 14 | | *B* | 204 ± 15 | | *AB* | 233 ± 20 | | *A* |
| *letters:* | | | | | | *LF-LV* | 267 ± 16 | | *A* | 260 ± 17 | | *A* | 295 ± 19 | | *A* |
| $I_{max}$_S (mm) | | 9.8 | p<0.001 | 1.4 | NS | | 62 ± 2 | | *A* | 54 ± 2 | | *B* | 57 ± 2 | | *AB* |
| **Tmax** | | *F* | *p* | *F* | *p* | | *mean* ± *SE* | | | *mean* ± *SE* | | | *mean* ± *SE* | | |
| $T_{max}$_R (s) *limonene* | *m/z 81.070* | 20.1 | p<0.001 | 1.5 | NS | | 34 ± 1 | | *A* | 25 ± 1 | | *B* | 27 ± 1 | | *B* |
| | *m/z 138.139* | 16.1 | p<0.001 | 1.5 | NS | | 33 ± 1 | | *A* | 24 ± 1 | | *B* | 27 ± 1 | | *B* |
| $T_{max}$_R (s) *citral* | *m/z 135.119* | 5.8 | p<0.01 | 0.7 | NS | | 50 ± 1 | | *A* | 45 ± 1 | | *B* | 46 ± 1 | | *B* |
| | *m/z 153.131* | 1.9 | NS | 0.2 | NS | | 54 ± 1 | | | 51 ± 1 | | | 51 ± 1 | | |
| $T_{max}$_S (s) | | 0.2 | NS | 1.0 | NS | | 38 ± 2 | | | 38 ± 2 | | | 37 ± 2 | | |

*significant differences between mayonnaise without/with bread carriers (p<0.05)*

### 6.3.5 | Results overview: in-nose aroma release and sensory perception of mayonnaise-carrier combinations

A Multiple Factor Analysis (MFA) analysis was conducted to summarize the effect of mayonnaise viscosity, mayonnaise fat content, carrier addition and carrier texture on aroma release and perception of mayonnaises (Figure 6.5). PC1 explained 44.9% of total variance and accounted mainly for differences in mayonnaise fat content (Figure 6.5B). In this case, the 95% confidence ellipses highlight two clusters: one with full fat mayonnaise (FF-HV) and the other with the two low fat mayonnaises (LF-HV and LF-LV). PC2 explained 34.3% of total variance and accounted for sample differences in viscosity (Figure 6.5A) and carrier addition (Figure 6.5C). In this case, the 95% confidence ellipses highlight two clusters: one with low viscosity mayonnaises (LF-LV) and one with high viscosity mayonnaises (LF-HV and FF-HV). Figure 6.5C highlights a difference between food carriers: bread samples are positioned further away from the single mayonnaises than potato samples, indicating that bread had a larger overall impact on lemon aroma release and intensity perception than potato.

To summarize, increasing mayonnaise viscosity or decreasing mayonnaise fat content reduced lemon aroma release and simultaneously lemon intensity perception. The two lemon aroma compounds (limonene, citral) had slightly different release patterns, with limonene being released faster and with higher

concentration due to its higher volatility than citral. When mayonnaises were combined with carriers, aroma release and perception were no longer congruent. Addition of bread and potato to mayonnaises enhanced lemon aroma release and decreased simultaneous lemon intensity perception. When comparing the different carrier foods, addition of bread increased lemon aroma release concentrations more than potato. Bread hardness did not influence lemon aroma release, but harder bread tended to decrease lemon intensity perception to a larger extent than soft bread. Potato hardness did not influence aroma release, but softer potato tended to decrease lemon intensity perception slightly more than harder potato.

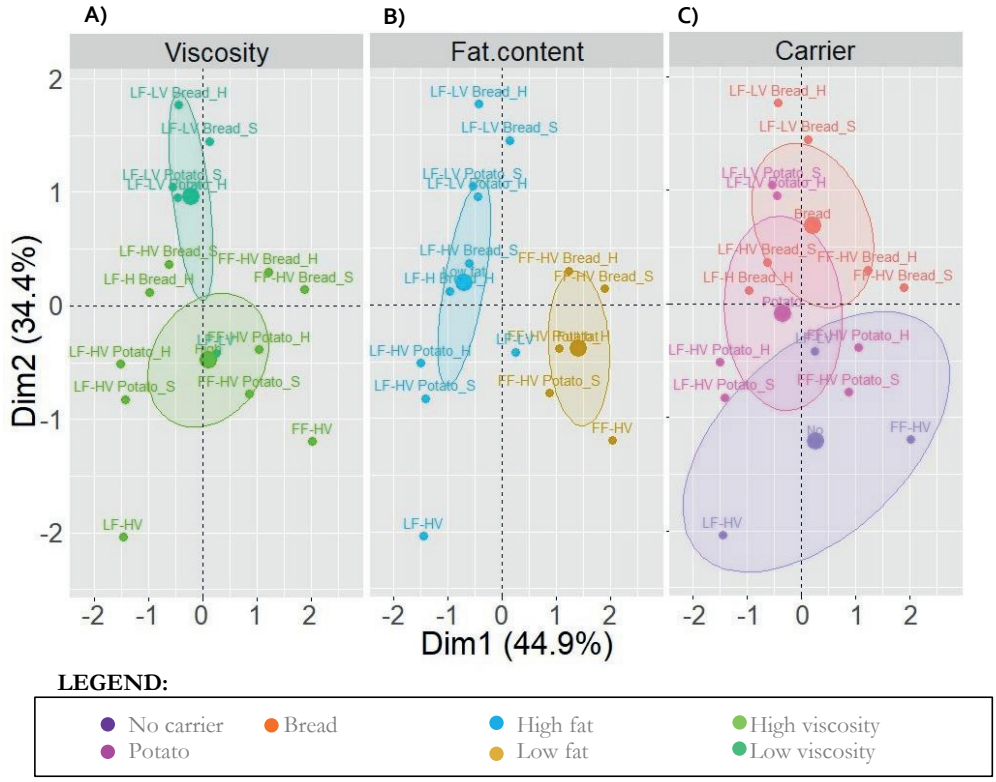



***Figure 6.5:*** *Scatter plot of multiple factor analysis (MFA) of in-nose aroma release and dynamic lemon perception data, in which the mayonnaise viscosity effect (A), the mayonnaise fat content effect (B) and the carrier effect (C) are highlighted using different colours.*

## 6.4 Discussion

Regarding the in-nose aroma release of condiment-carrier combinations, we see that aroma release from condiments (mayonnaises) is enhanced when consumed together with carriers (bread or potatoes) compared to consumption without carriers. We hypothesized that aroma perception of condiment-carrier combinations is driven by physicochemical effects on aroma release i.e. that condiment aroma release is

decreased as condiment aroma compounds bind to carriers. Although such interaction might have occurred through physical non-covalent bonds between carriers and condiments, a higher concentration of aroma compounds was released in the nose when carrier foods were added to mayonnaises. This indicates that for composite foods other mechanisms contribute more to in-nose aroma release than binding of aroma compounds. We suggest that differences in food oral processing between mayonnaise and mayonnaise in combination with carriers explain the increase in in-nose aroma release. Mayonnaise-carrier combinations required chewing to safely break down the food before swallowing, whereas the mayonnaises without carriers were swirled around in the mouth without chewing following standardized consumption protocols (mayonnaise with carrier: chew with 1 chew/s for 20 s; mayonnaise without carrier: swirl in mouth for 20 s; see section 6.2.3). The chewing required to masticate the mayonnaise-carrier combinations apparently induced more aroma release. Moreover, as a result of chewing and mixing, the surface area of mayonnaise-carrier combinations might have increased since the carrier might have been broken down into multiple smaller bolus pieces. Consequently, the mayonnaise would be distributed over a larger area which could have led to a higher transfer of aroma compounds from the mayonnaise to the vapor phase. This could explain why total aroma released increased upon addition of carriers to mayonnaises. This was also reflected in the time required to reach the maximum aroma concentration ($T_{max}$_R), which was faster in case of the carrier-mayonnaise combinations than for single mayonnaises (Table 6.3). In addition, the velum-tongue border has been observed to open more frequently during consumption of solid foods than liquid foods (35), which could increase the ability of aroma compounds to pass to the nasal cavity ahead of swallowing. Such an effect of oral processing behavior on *in vivo* aroma release is consistent with previous studies (36-41). Addition of solid carrier foods to condiments thus increases oral movements, in-mouth food manipulations and food's surface area, and therefore favours an increase in in-nose aroma release of condiments throughout consumption.

Higher in-nose aroma release with the addition of carrier foods was still maintained after participants swallowed the sample. Such effect might be explained by differences in bolus properties and consequent oral retention. When mayonnaise is consumed on its own, it is mixed with saliva leading to a liquid-like bolus that is easily swallowed. We assume that little product remains in the mouth after swallowing (42-44). When mayonnaise is consumed with a carrier, it is mixed with both the carrier and saliva leading to a relatively cohesive solid bolus that easily sticks to different oral surfaces (teeth, tongue, palate) upon swallowing. We speculate that in this case more product remains in the mouth which likely leads to longer aroma release into the nasal cavity after swallowing.

The type of carrier food (bread versus potato) affected *in vivo* aroma release of mayonnaises, since bread increased nasal aroma concentrations to a larger extent than potatoes (Figure 6.4, Table 6.3). We suggest that this result could be partly explained by a difference in moisture absorption capacity between bread and potato. Bread is a dry, low water content product, so it absorbs moisture from the mayonnaises (34). This

effectively increases the oil content in the mayonnaises, and decreases the volume of the continuous aqueous phase, through which the aroma compounds need to diffuse before reaching the air phase. Consequently, the aroma compounds are assumed to transfer faster into the nasal cavity for breads. Another explanation for the lower release of aroma compounds from potato can be found in the properties of the starch in cooked potatoes. Potatoes contain starch granules, which are gelatinized upon cooking. The gelatinization leads to release of amylose from the granules into the continuous phase, whereas amylopectin resides mostly within the granules. Consequently, starch (mainly amylose) becomes available for interactions with hydrophobic aroma compounds after cooking through hydrophobic interactions. It is known that gelatinized starch retains hydrophobic aroma compounds including limonene to a larger extent than starch granules (45). Such interactions can limit aroma release and could explain the lower release for potato. Together these results show that mayonnaise aroma release depended on the properties of the carriers it is combined with.

The texture of carrier foods did not significantly influence mayonnaise aroma release. It is important to note that a standardized consumption protocol was used (section 6.2.3), meaning that both soft and hard carrier foods were chewed 20 times at the same chewing frequency. This did not allow participants to adapt oral behavior based on texture, and presumably resulted in similar nasal air flows and release patterns, and this could be the reason why we see no effect of texture on release. In case of free eating, differences in aroma release of mayonnaises depending on the texture of the carrier food might occur, since softer foods generally require fewer chews than harder foods, likely to result in different nasal air flows which in turn can affect in-nose aroma release. For example, in case of cheese, firmer cheeses were chewed for a longer time and broken down into more bolus pieces by which both the release rate and the total amount of released aroma were increased (46,47).

Inter-individual variation between subjects is known to affect oral behavior, aroma release and perception (48-50). To alleviate such subject variation, we selected a homogenous panel (young, female, Caucasian) and standardized their way of chewing by training with a chewing protocol (section 6.2.3). A next step would involve studies investigating aroma release of condiment-carrier combinations among participants with different eating behaviours (slow vs. fast eaters), as eating rate is known to affect bolus formation, sensory perception and food intake (51,52).

Additionaly, mayonnaise properties (viscosity, fat content) were observed to influence aroma release considerably. As both viscosity and fat content have been shown to influence aroma release of single foods in previous studies, these results are discussed only shortly here. Increasing mayonnaise viscosity by adding more xanthan resulted in lower aroma release and perception (LF-LV vs. LF-HV). Viscosity is known to play a relevant role in aroma release, as diffusion rate of aroma compounds is hindered by an increase in viscosity (21,53). In addition, xanthan has been suggested to physically interact with hydrophobic aroma compounds by trapping them into a so-called "hydrophobic cavity" (19,54). Decreasing mayonnaise fat content while

keeping the same viscosity resulted in decreased aroma release and perception (FF-HV and LF-HV). A similar observation was reported by Wendin *et al.*, who found that decreased fat content tended to decrease the perceived lemon intensity in mayonnaise (55). However, these results do not support the general theory that the partitioning of hydrophobic aromas into aqueous phases and air is greatly reduced with increasing fat/oil content (8,9,12). This discrepancy may be due to different factors. Firstly, aroma compounds may interact with xanthan in low fat emulsions (LF-HV), which was added to compensate for the difference in viscosity due to the reduction of fat. So even though lowering oil content could provide the expected increase in aroma release, interactions with xanthan might have been more pronounced, eventually leading to a decrease in aroma release. Secondly, the FF-HV mayonnaise contains a higher number of fat droplets when compared to the LF-HV. This results in more interfacial area between oil and the continuous aqueous phase, and therefore interaction with the saliva may be increased and eventual transfer to the air phase. This may ultimately lead to a higher aroma release and an accompanying higher aroma concentration in the nose-space (56).

To summarize, our study highlights that aroma release from mayonnaises is enhanced when they are consumed together with carrier foods such as bread or potatoes. Intuitively, one would expect that this increase in aroma release would be reflected in an increase in aroma perception. However, when looking at the sensory perception of the mayonnaise-carrier combinations, carrier addition actually decreased perceived aroma intensity of mayonnaises (Figure 6.4E-6.4F, Table 6.3). This decrease in perceived intensity is in line with previous studies, indicating that flavour intensity of soy sauce and mayonnaise decreased with addition of solid carrier foods (30,33). The present study shows that the lower perceived intensity is not due to a lower delivery of aroma compounds into the nasal cavity, as aroma release was increased with addition of carriers (Figure 6.4A-6.4D, Table 6.3). This misalignment between perception and aroma release in case of mayonnaise-carrier combinations indicates that carriers modify condiment perception via other ways independent of actual in-nose aroma concentrations. We therefore suggest that cognitive effects play a pivotal role in the modulation of condiment-carrier perception, i.e. consumers pay more attention to texture and/or chewing with the presence of carriers, whereupon the aroma of condiments appears to be less intense. Recently, White *et al.* (2020) stressed that the influence of cognitive processes on sensory evaluation should be considered more by food scientists (57). They revealed that consumer perception is shaped by the way attention is distributed among sensory sensations. This phenomenon has been mainly discussed in the light of aroma-taste mixtures, showing that attention was directed to one or a few elements of multisensory mixtures. In the present study, condiments were evaluated in combination with solid carrier foods, which added another dimension (i.e. texture / the process of chewing) to the aroma perception of the mayonnaises. We argue that cognitive attention was thereby drawn to the process of chewing. In case of condiment-carrier combinations, focusing on a specific task (e.g. chewing) might thus limit conscious

perception of other senses (e.g. aroma) present, and this cognitive influence is important to keep in mind in sensory evaluation of complex foods.

Although such a cognitive effect seems plausible, it is important to acknowledge a possible sensory dumping effect (a well-known limitation of the Time-Intensity methodology) (58). Carriers with different texture properties were added to mayonnaises with lemon aroma, and participants were asked to evaluate lemon intensity only. Subjects probably perceived differences in texture, and where asked to evaluate lemon aroma intensity only which might have led to the projection of perceived differences and changes in texture into lemon intensity. To minimize potential dumping effect, the perceived textural differences were carefully discussed during the multiple training sessions. Subsequently, the panel practiced with the evaluation of aroma intensity, while being aware of the possible differences in texture. In this context, it is known that transfer of aroma compounds into the nasal cavity follows swallow breath (59). Thus, aroma perception is known to increase just after swallowing. When looking at our Time-Intensity data (Figure 6.3E-6.3F, 6.4E-6.4F), we observe a consistent increase in perceived lemon intensity just after 20 s. This demonstrates that our panel functioned very well since they clearly perceived this increase in aroma after swallowing, which strongly suggests that our panel was capable to perceive and evaluate aroma intensity. Furthermore, previous studies using RATA on similar composite foods demonstrated similar to our study that flavour intensity of mayonnaise decreased with addition of carrier foods (30,33). In these studies, texture and flavour attributes were evaluated so dumping effects can be excluded. We therefore assume the sensory dumping effect in our study to be small. Hence, the influence of cognitive effects on sensory perception of complex foods must be considered.

The novelty of the present study is the fact that simultaneous aroma release and perception was assessed for condiment-carrier combinations and not only in model foods or single foods. Combining condiments and carrier foods increases the complexity of the food consumed, which is more representative of the common consumption context. In summary, in-nose aroma release matched perceived aroma intensity when mayonnaise was consumed alone (i.e. when higher aroma concentrations were released in the nose, also higher perceived aroma intensity values were reported). This was not the case for more complex foods such as condiment-carrier combinations. Addition of carriers increased in-nose aroma release but decreased the perceived aroma intensity of mayonnaises. Since this decreased aroma perception was not due to a lower delivery of aroma compounds into the nasal cavity, we conclude that aroma release alone does not explain sensory perception of composite foods. In case of composite foods, cognitive effects are likely to modulate perception of more complex food combinations, which supports the idea that not only physicochemical characteristics, but also consumers' cognitive mode should be considered in food design with excellent consumer appreciation.

## 6.5 References


1 Sikora, M., Badrie, N., Deisingh, A. K. & Kowalski, S. Sauces and Dressings: A Review of Properties and Applications. Critical reviews in food science and nutrition 48, 50-77, doi:10.1080/10408390601079934 (2008).

2 Robert-Hazotte, A. et al. Ex vivo real-time monitoring of volatile metabolites resulting from nasal odorant metabolism. Scientific Reports 9, 2492, doi:10.1038/s41598-019-39404-x (2019).

3 Mayr, D., Märk, T., Lindinger, W., Brevard, H. & Yeretzian, C. Breath-by-breath analysis of banana aroma by proton transfer reaction mass spectrometry. International Journal of Mass Spectrometry 223-224, 743-756, doi:https://doi.org/10.1016/S1387-3806(02)00967-3 (2003).

4 Buettner, A. & Beauchamp, J. Chemical input–Sensory output: Diverse modes of physiology–flavour interaction. Food Quality and Preference 21, 915-924 (2010).

5 Poinot, P., Arvisenet, G., Ledauphin, J., Gaillard, J.-L. & Prost, C. How can aroma–related cross–modal interactions be analysed? A review of current methodologies. Food Quality and Preference 28, 304-316 (2013).

6 Tournier, C., Sulmont-Rossé, C. & Guichard, E. Flavour perception: aroma, taste and texture interactions. Food 1, 246-257 (2007).

7 Carrapiso, A. I. Effect of fat content on flavour release from sausages. Food Chemistry 103, 396-403, doi:https://doi.org/10.1016/j.foodchem.2006.07.037 (2007).

8 Frank, D., Appelqvist, I., Piyasiri, U., Wooster, T. J. & Delahunty, C. Proton transfer reaction mass spectrometry and time intensity perceptual measurement of flavour release from lipid emulsions using trained human subjects. Journal of agricultural and food chemistry 59, 4891-4903, doi:10.1021/jf104376b (2011).

9 González-Tomás, L., Bayarri, S., Taylor, A. J. & Costell, E. Flavour release and perception from model dairy custards. Food Research International 40, 520-528, doi:https://doi.org/10.1016/j.foodres.2006.10.002 (2007).

10 Linforth, R., Cabannes, M., Hewson, L., Yang, N. & Taylor, A. Effect of Fat Content on Flavour Delivery during Consumption: an in Vivo Model. Journal of agricultural and food chemistry 58, 6905-6911, doi:10.1021/jf100643d (2010).

11 Wendin, K., Ellekjær, M. R. & Solheim, R. Fat Content and Homogenization Effects on Flavour and Texture of Mayonnaise with Added Aroma. LWT - Food Science and Technology 32, 377-383, doi:https://doi.org/10.1006/fstl.1999.0562 (1999).

12 Arancibia, C., Jublot, L., Costell, E. & Bayarri, S. Flavour release and sensory characteristics of o/w emulsions. Influence of composition, microstructure and rheological behavior. Food Research International 44, 1632-1641, doi:https://doi.org/10.1016/j.foodres.2011.04.049 (2011).

13 Miettinen, S. M., Hyvonen, L. & Tuorila, H. Timing of intensity perception of a polar vs nonpolar aroma compound in the presence of added vegetable fat in milk. Journal of agricultural and food chemistry 51, 5437-5443, doi:10.1021/jf0342066 (2003).

14 Miettinen, S.-M., Tuorila, H., Piironen, V., Vehkalahti, K. & Hyvönen, L. Effect of Emulsion Characteristics on the Release of Aroma As Detected by Sensory Evaluation, Static Headspace Gas Chromatography, and Electronic Nose. Journal of agricultural and food chemistry 50, 4232-4239, doi:10.1021/jf0107205 (2002).

15 Cook, D. J., Hollowood, T. A., Linforth, R. S. & Taylor, A. J. Oral shear stress predicts flavour perception in viscous solutions. Chemical Senses 28, 11-23 (2003).

16 Baek, I., Linforth, R. S., Blake, A. & Taylor, A. J. Sensory perception is related to the rate of change of volatile concentration in-nose during eating of model gels. Chemical Senses 24, 155-160 (1999).

17 Baines, Z. V. & Morris, E. R. Flavour/taste perception in thickened systems: the effect of guar gum above and below c*. Food Hydrocolloids 1, 197-205, doi:https://doi.org/10.1016/S0268-005X(87)80003-6 (1987).

18 Boland, A. B., Delahunty, C. M. & van Ruth, S. M. Influence of the texture of gelatin gels and pectin gels on strawberry flavour release and perception. Food Chemistry 96, 452-460, doi:https://doi.org/10.1016/j.foodchem.2005.02.027 (2006).

19 Bylaite, E., Adler-Nissen, J. & Meyer, A. S. Effect of xanthan on flavour release from thickened viscous food model systems. Journal of agricultural and food chemistry 53, 3577-3583, doi:10.1021/jf048111v (2005).

20 Cook, D. J., Hollowood, T. A., Linforth, R. S. T. & Taylor, A. J. Oral Shear Stress Predicts Flavour Perception in Viscous Solutions. Chemical Senses 28, 11-23, doi:10.1093/chemse/28.1.11 (2003).

21 Malone, M. E., Appelqvist, I. A. M. & Norton, I. T. Oral behaviour of food hydrocolloids and emulsions. Part 2. Taste and aroma release. Food Hydrocolloids 17, 775-784, doi:https://doi.org/10.1016/S0268-005X(03)00098-5 (2003).

22 Roberts, D. D., Elmore, J. S., Langley, K. R. & Bakker, J. Effects of Sucrose, Guar Gum, and Carboxymethylcellulose on the Release of Volatile Flavour Compounds under Dynamic Conditions. Journal of agricultural and food chemistry 44, 1321-1326, doi:10.1021/jf950567c (1996).

23 Xu, J. et al. Effect of xanthan gum on the release of strawberry flavour in formulated soy beverage. Food Chem 228, 595-601, doi:10.1016/j.foodchem.2017.02.040 (2017).

24 Saint-Eve, A. et al. Flavoured yogurt complex viscosity influences real-time aroma release in the mouth and sensory properties. Journal of agricultural and food chemistry 54, 7794-7803 (2006).

25 Cook, D. J., Hollowood, T. A., Linforth, R. S. & Taylor, A. J. Correlating instrumental measurements of texture and flavour release with human perception. International journal of food science & technology 40, 631-641 (2005).

26 Hollowood, T., Linforth, R. & Taylor, A. The effect of viscosity on the perception of flavour. Chemical Senses 27, 583-591 (2002).

27 Lethuaut, L., Weel, K. G. C., Boelrijk, A. E. M. & Brossard, C. D. Flavour Perception and Aroma Release from Model Dairy Desserts. Journal of agricultural and food chemistry 52, 3478-3485, doi:10.1021/jf035488c (2004).

28 Weel, K. G. et al. Flavour release and perception of flavoured whey protein gels: perception is determined by texture rather than by release. Journal of agricultural and food chemistry 50, 5149-5155 (2002).

29 Tournier, C. et al. A study on texture-taste-aroma interactions: Physico-chemical and cognitive mechanisms. International Dairy Journal 19, 450-458, doi:10.1016/j.idairyj.2009.01.003 (2009).

30 Cherdchu, P. & Chambers, E. Effect of carriers on descriptive sensory characteristics: A case study with soy sauce. Journal of Sensory Studies 29, 272-284, doi:10.1111/joss.12100 (2014).

31 Meinert, L., Frøst, M. B., Bejerholm, C. & Aaslyng, M. D. Enhancing the sensory quality of vegetables by decreasing some less-desired sensory properties with low-fat pork gravy. Journal of Culinary Science and Technology 9, 113-131, doi:10.1080/15428052.2011.584496 (2011).

32 Paulsen, M. T., Ueland, T., Nilsen, A. N., Öström, Å. & Hersleth, M. Sensory perception of salmon and culinary sauces - An interdisciplinary approach. Food Quality and Preference 23, 99-109, doi:10.1016/j.foodqual.2011.09.004 (2012).

33 van Eck, A., Fogliano, V., Galindo-Cuspinera, V., Scholten, E. & Stieger, M. Adding condiments to foods: How does static and dynamic sensory perception change when bread and carrots are consumed with mayonnaise? Food Quality and Preference 73, 154-170, doi:10.1016/j.foodqual.2018.11.013 (2019).

34 van Eck, A. et al. Oral processing behavior and dynamic sensory perception of composite foods: Toppings assist saliva in bolus formation. Food Quality and Preference 71, 497-509, doi:10.1016/j.foodqual.2018.05.009 (2019).

35 Buettner, A., Beer, A., Hannig, C., Settles, M. & Schieberle, P. Physiological and analytical studies on flavour perception dynamics as induced by the eating and swallowing process. Food Quality and Preference 13, 497-504, doi:https://doi.org/10.1016/S0950-3293(02)00052-6 (2002).

36 Aprea, E., Biasioli, F., Gasperi, F., Märk, T. D. & van Ruth, S. In vivo monitoring of strawberry flavour release from model custards: effect of texture and oral processing. Flavour and Fragrance Journal 21, 53-58, doi:10.1002/ffj.1702 (2006).

37 Doyennette, M., Aguayo-Mendoza, M. G., Williamson, A.-M., Martins, S. I. F. S. & Stieger, M. Capturing the impact of oral processing behaviour on consumption time and dynamic sensory perception of ice creams differing in hardness. Food Quality and Preference 78, 103721, doi:https://doi.org/10.1016/j.foodqual.2019.103721 (2019).

38 Hansson, A., Giannouli, P. & van Ruth, S. The Influence of Gel Strength on Aroma Release from Pectin Gels in a Model Mouth and in Vivo, Monitored with Proton-Transfer-Reaction Mass Spectrometry. Journal of agricultural and food chemistry 51, 4732-4740, doi:10.1021/jf030050y (2003).

39 Pionnier, E., Chabanet, C., Mioche, L., Le Quéré, J.-L. & Salles, C. 1. In Vivo Aroma Release during Eating of a Model Cheese: Relationships with Oral Parameters. Journal of agricultural and food chemistry 52, 557-564, doi:10.1021/jf030544v (2004).

40 Raithore, S. & Peterson, D. G. Effects of polyol type and particle size on flavour release in chewing gum. Food chemistry 253, 293-299, doi:10.1016/j.foodchem.2018.01.123 (2018).

41 Tarrega, A., Yven, C., Sémon, E. & Salles, C. Aroma release and chewing activity during eating different model cheeses. International Dairy Journal 18, 849-857, doi:https://doi.org/10.1016/j.idairyj.2007.09.008 (2008).

42 Camacho, S., den Hollander, E., van de Velde, F. & Stieger, M. Properties of oil/water emulsions affecting the deposition, clearance, and after-feel sensory perception of oral coatings. Journal of agricultural and food chemistry 63, 2145-2153 (2015).

43 Camacho, S., Liu, K., Van Der Linden, A., Stieger, M. & Van De Velde, F. Formation, Clearance and Mouthfeel Perception of Oral Coatings Formed by Emulsion-Filled Gels. Journal of texture studies 46, 399-410 (2015).

44 Camacho, S., Van Riel, V., De Graaf, C., Van De Velde, F. & Stieger, M. Physical and sensory characterizations of oral coatings of oil/water emulsions. Journal of agricultural and food chemistry 62, 5789-5795 (2014).

45 Boutboul, A., Giampaoli, P., Feigenbaum, A. & Ducruet, V. Influence of the nature and treatment of starch on aroma retention. Carbohydrate polymers 47, 73-82 (2002).

46 Repoux, M. et al. Combined effect of cheese characteristics and food oral processing on in vivo aroma release. Flavour and fragrance journal 27, 414-423 (2012).

47 Tarrega, A., Yven, C., Sémon, E. & Salles, C. In-mouth aroma compound release during cheese consumption: Relationship with food bolus formation. International Dairy Journal 21, 358-364, doi:10.1016/j.idairyj.2010.12.010 (2011).

48 Charles, M., Rosselin, V., Beck, L., Sauvageot, F. & Guichard, E. Flavour Release from Salad Dressings: Sensory and Physicochemical Approaches in Relation with the Structure. Journal of agricultural and food chemistry 48, 1810-1816, doi:10.1021/jf9906533 (2000).

49 Labouré, H., Repoux, M., Courcoux, P., Feron, G. & Guichard, E. Inter-individual retronasal aroma release variability during cheese consumption: Role of food oral processing. Food Research International 64, 692-700, doi:https://doi.org/10.1016/j.foodres.2014.07.024 (2014).

50 Pedrotti, M., Spaccasassi, A., Biasioli, F. & Fogliano, V. Ethnicity, gender and physiological parameters: Their effect on in vivo flavour release and perception during chewing gum consumption. Food Research International 116, 57-70, doi:10.1016/j.foodres.2018.12.019 (2019).

51 Devezeaux de Lavergne, M., van de Velde, F. & Stieger, M. Bolus matters: the influence of food oral breakdown on dynamic texture perception. Food & Function 8, 464-480, doi:10.1039/c6fo01005a (2017).

52 Robinson, E. et al. A systematic review and meta-analysis examining the effect of eating rate on energy intake and hunger. Am J Clin Nutr 100, 123-151, doi:10.3945/ajcn.113.081745 (2014).

53 Weterings, M., Bodnár, I., Boom, R. M. & Beyrer, M. A classification scheme for interfacial mass transfer and the kinetics of aroma release. Trends in Food Science & Technology (2019).

54 Milas, M., Rinaudo, M., Knipper, M. & Schuppiser, J. L. Flow and viscoelastic properties of xanthan gum solutions. Macromolecules 23, 2506-2511 (1990).

55 Wendin, K. et al. Low-fat mayonnaise: influences of fat content, aroma compounds and thickeners. Food Hydrocolloids 11, 87-99, doi:https://doi.org/10.1016/S0268-005X(97)80015-X (1997).

56 Tarrega, A., Yven, C., Semon, E., Mielle, P. & Salles, C. Effect of Oral Physiology Parameters on In-Mouth Aroma Compound Release Using Lipoprotein Matrices: An In Vitro Approach. Foods 8, 106 (2019).

57 White, T. L., Thomas-Danguin, T., Olofsson, J. K., Zucco, G. M. & Prescott, J. Thought for Food: Cognitive Influences on Chemosensory Perceptions and Preferences. Food Quality and Preference, 103776 (2019).

58 Lawless, H. T. & Heymann, H. Sensory evaluation of food: principles and practices. (Springer Science & Business Media, 2010).

59 Buettner, A., Beer, A., Hannig, C. & Settles, M. Observation of the Swallowing Process by Application of Videofluoroscopy and Real-time Magnetic Resonance Imaging—Consequences for Retronasal Aroma Stimulation. Chemical Senses 26, 1211-1219, doi:10.1093/chemse/26.9.1211 (2001).

60 Engelen, L., Fontijn-Tekamp, A. & Van Der Bilt, A. The influence of product and oral characteristics on swallowing. Archives of oral biology 50, 739-746, doi:10.1016/j.archoralbio.2005.01.004 (2005).

61 Alsanei, W. A. & Chen, J. Studies of the Oral Capabilities in Relation to Bolus Manipulations and the Ease of Initiating Bolus Flow. Journal of Texture Studies 45, 1-12, doi:10.1111/jtxs.12041 (2014).

62 Charles, M. et al. Understanding flavour perception of espresso coffee by the combination of a dynamic sensory method and in-vivo nosespace analysis. Food Research International 69, 9-20, doi:https://doi.org/10.1016/j.foodres.2014.11.036 (2015).

63 Heenan, S. et al. PTR-TOF-MS monitoring of in vitro and in vivo flavour release in cereal bars with varying sugar composition. Food Chemistry 131, 477-484, doi:https://doi.org/10.1016/j.foodchem.2011.09.010 (2012).

64 Cliff, M. & Heymann, H. Development and use of time-intensity methodology for sensory evaluation: A review. Food Research International 26, 375-385, doi:https://doi.org/10.1016/0963-9969(93)90081-S (1993).

65 Liu, Y.-H. & MacFie, H. Methods for averaging time–intensity curves. Chemical Senses 15, 471-484 (1990).

66 lmerTest: Tests in Linear Mixed Effects Models (2016).

67 FactoMineR: An R Package for Multivariate Analysis (2008).

7

# Ethnicity, gender and physiological parameters: their effect on *in vivo* flavour release and perception during chewing gum consumption

Pedrotti M., Spaccasassi A., Biasioli F., Fogliano V.

*Food Research International,* 2019, 116

DOI: 10.1016/j.foodres.2018

## Abstract

In this study, the impact of physiological parameters, ethnicity and gender on flavour perception and flavour release of chewing gum was investigated. Proton transfer reaction mass spectrometry in-nose monitoring of volatile organic compounds was coupled to discontinuous time intensity sensory evaluation for mint flavour and sweetness perception. Each of the 29 subjects, 14 European and 15 Chinese panellists (13 male and 16 females, age 24 ± 1.4 years old) consumed the samples in triplicates. Physiological parameters (oral cavity volume, salivary flow, acetone and isoprene concentration and fungiform papillae density) were measured. Significant differences for *in vivo* flavour release between Chinese and European panellists after 90 s of consumption and after the gum was removed from the mouth were found. Significant differences were observed also in flavour and sweetness perception while no gender effect was detected.
In this work, for the first time an effect of ethnicity on in-nose flavour release monitored through PTR-MS was noticed during chewing gum consumption, in agreement with the findings from sensory evaluation. Single physiological parameters do not explain the relation between flavour in nose release and perception during consumption.


**Keywords:** flavour release, *in-vivo* analysis, PTR-MS, chewing gum, cross-cultural

## 7.1 Introduction

Chewing gum is not only a valuable commodity – 1.74 trillion sticks of chewing gum are produced annually (1) – but also an ideal model food that can be used for drug delivery and tailored to investigate oral processing. It is a combination of water-insoluble phase, the gum base, and other ingredients like powdered sugars, various softeners and flavourings (1). Although flavourings make up only a small amount (from 0.4 to 1.0% p/w) of the final gum formula, they are fundamental for its perceived quality. During consumption, volatile organic compounds (VOCs) are transferred to saliva and to the air phase in the oral cavity being eventually transported to the olfactory receptors in the nasal cavity via the respiratory flow (retro nasal olfaction) (2).

Aroma release and perception are dynamic processes which depend not only on flavour compounds, but also on matrix, oral processing and subject characteristics (3). For these reasons, chewing gum oral processing can be properly investigated by combining simultaneously dynamic sensory and instrumental techniques (4).

As far as sensory investigation is concerned, different dynamic sensory methods have been developed and applied to investigate the temporal dimensions of sensory sensations during food consumption such as Time Intensity (TI), Temporal Dominance of Sensations (TDS), Progressive Profile (PP) and more recently the Temporal Check-all-That-Apply (TCATA) method (5–13). Among these, time Intensity (TI) methods have been amply used for chewing gum sensory evaluation in many different researches (14–19). According to Duizer et *al.*, with a slowly changing product like chewing gum, it is also possible to have a dual-attribute TI (20). Another possibility for evaluating multiple sensory attributes during time is the discrete time-intensity (DTI) method where panellists are asked for repeated rating of single or few attributes at discrete time points (21–23).

*In vivo* VOCs analysis provides information on the molecules that interact with our receptors by analyzing the air coming out of the nostrils (24) but faces different analytical challenges: the analysis should be fast (sampling time below 1 s), several compounds should be measured simultaneously and possibly at low concentration (25). In the last years, different mass spectrometry approaches were employed to investigate chewing gum aroma release with the so called *in-nose* or nose-space analysis (17,18,26–31). Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) was the first analytical approach that was applied for in-nose analysis and has been used to monitor menthol and menthone release from peppermint oil in chewing gum during consumption (29,32,33) and other compounds like cinnamaldehyde, carvone, piperitone and jasmine (17,18). Proton transfer reaction mass spectrometry (PTR-MS) and, in particular the version equipped with a time of flight mass Analyser (PTR-ToF-MS), thanks to its high time resolution, can also be applied for breath analysis and nose-space measurement during consumption of food (30,34). Compared to APCI-MS, PTR-MS separates the formation of the precursor ions from the ionization region allowing for a more controlled ionization (35). The combination of PTR-MS analysis with TI is one of the best option to investigate flavour perception (36) and to verify how flavour

perception is controlled by flavour release and/or by cognitive processes. PTR-MS was used to characterize menthol release from different aroma encapsulation systems in mint flavoured chewing gum (37) and used on a mechanical device simulating mastication to perform a mass balance of both volatiles and non-volatiles added to chewing gum (38).

Flavour release is influenced by different physiological parameters (39) which play an important role in retro nasal flavour release: salivary flow, breathing, mastication, and swallowing. The interaction of VOCs with saliva and oral mucosa may be the reason of inter-subject variability of the concentration of aroma in the nose-space (40). Data on salivary flow, breathing cycle, and mouth volume can be correlated with flavour release and perception (41). Flavour release is controlled not only by flavour concentration, but also by many other intrinsic and extrinsic factors as dissolution rate, sugar release, air bolus contact area, mass transfer coefficient in the bolus, sex and age (41–44). Cultural origin and, consequently, life experiences might also impact flavour perception (45–49). Instrumental in-nose-space monitoring may contribute to dissect possible effects of physiological parameters and cross-cultural differences and help to explain the complex phenomena of retro nasal perception.

The present work, aims at investigating the impact of ethnicity, gender and physiological parameters, on flavour perception and release of mint chewing gum through in nose PTR-MS approach coupled to DTI sensory analysis and collection of physiological parameters like stimulated salivary flow, volume of oral cavity and fungiform papillae density.

## 7.2 Material and methods

### 7.2.1 | Product

Samples were commercial mint flavour chewing gum (weight 1.0 ± 0.2 g) without any sugar coating or flavour encapsulation. The sweetener used in the product was sorbitol.

### 7.2.2 | Subjects

14 Caucasian-European and 15 Asian-Chinese panellists (13 male and 16 female) were selected among students from Wageningen University who lived most of their life in their own country. Chinese panellists moved out from their own country around 3 months before the experiments. European panellists were originally from the Netherlands or they moved there from their own European country since maximum 1 year. Personal data and privacy signatures were collected through a questionnaire. All participants were non-pregnant, non-smoking and with no history of oral perception disorders or olfactory impairments. Recruited panellists had similar age (24 ± 1.4 years). Information on familiarity, consumption frequency and liking of the product were collected during the first training. The ethical committee of Wageningen University provided an official opinion that the EC approval is not needed for this study.

### 7.2.3 | Design of the study

Before realizing the measurements, subjects underwent two training session of one our each During the training sessions, subjects were introduced to the experimental design, the experimental set-up and to the usage of the Labeled Magnitude Scale (LMS) according to Green *et al.* (1996) (50). Three kind of mint flavour commercial gums with different flavour intensity (low, medium and high) were used for training on attributes evaluation (sweetness and flavour intensity) and scale usage. Each trial lasted 5 min: subjects were introduced to instrumental set-up and they were instructed to breathe deeply and regularly. Oral cavity volume was measured in triplicate. The study was composed by two experimental sessions of 1 h each were three gums were evaluated. During the first experimental session, the salivary flow was initially measured. Then two of the three measurements per subject were done with a break in between the two replicates. During the second experimental session, salivary flow was measured again and the third replicate was consumed. At the end of this experimental session, pictures of the tongue for the papillary count were also taken. All participants were instructed to avoid drinking, eating and perfumes usage for 2 h before sessions. The experimental design is summarized in Figure 7.1.

### 7.2.4 | Physiological parameters

Three main parameters were considered: oral cavity volume, stimulated salivary flow and fungiform papillae density. The maximum oral capacity of each subject was measured in triplicate and then average volume, maximum volume and standard deviation were determined as described by Alsanei & Chen (51). Stimulated salivary flow (g/l) was measured with the parafilm method (52) by using 0.29 g Parafilm (American National Can, Chicago, IL, USA). The salivary flow evaluation was done for 6 min. The first minute only unstimulated saliva was measured. From the second minute a piece of parafilm was introduced in the mouth and participants were asked to chew regularly. Saliva expectoration was done at the end of each minute. Fungiform papillae count was performed by taking pictures of participants' tongues according to Monteleone *et al.* (53).

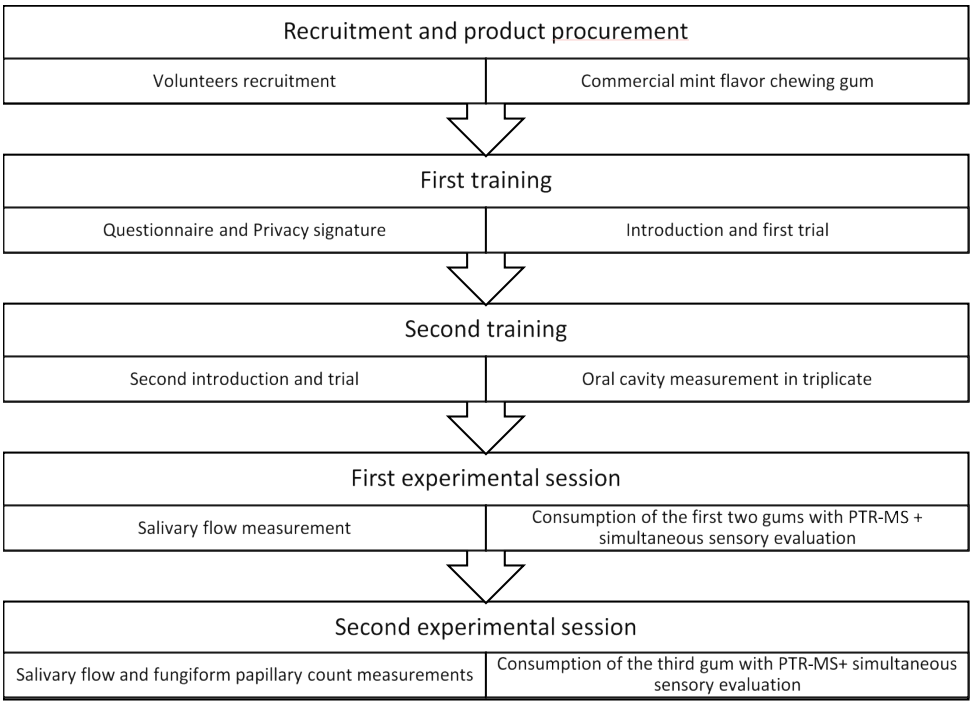



**Figure 7.1:** Schematic representation of the experimental design.

### 7.2.5 | Nose-space analysis

The experimental set up was adapted from previous PTR-MS in nose studies (28,30). Laboratory air was sampled for 20 s without any interaction with subject. After 20 s panellists were asked to insert two Teflon tubes (6.8 mm of diameter and 6.4 cm long at room temperature, then heated at 95 °C) in the nose and start breathing normally through the nose (mouth closed). Their breath was sampled for 60 s. At the end of this minute, participants received the gum and started chewing it. Nose-space was analysed for the next 7 min. At the seventh minute panellists were asked to remove the gum from the mouth and keep on breathing for two more minutes without product. This was done in triplicate for each subject. During all the evaluations, the judges had to keep their mouth closed. No mastication protocol was prescribed.

### 7.2.6 | PTR-MS conditions

A commercial PTR-MS instrument (Ionicon Analytik GmbH, Innsbruck, Austria) equipped with a time of flight and a quadrupole ion guide (PTR-QiToF) was used for volatile analysis. The ionization conditions, with $H_3O^+$ as precursor ion, were the following: 670 V drift voltage, 91.5 °C drift temperature, 1.87 mbar drift pressure, resulting in an E/N ratio of 139 Td. Acquisition was set to 1 spectrum per second. Sampling was carried out via a heated (95 °C) inlet tube with an inlet flow of 45 sccm.

### 7.2.7 | Discontinuous time evaluation of sensory perception

Simultaneously to the PTR-MS in-nose analysis, panellists rated the perceived flavour and sweetness intensities at different time intervals by using a LMS. The scale was constructed by taking the frame developed by Green *et al.* (1996) (50). To avoid the compression effect that can occur with a general labeled magnitude scale and that could led to less differentiation power among subject (23), for each scale the anchor words explained during the training session were adopted. Subjects were asked to simultaneously rate perception of chewing gum sweetness and minty flavour at different time intervals (10s, 30 s, 1 min, 2 min, 3 min, 5 min, 7 min and 8 min). Eye-Question software (version 4.8) was used to collect the data through a laptop that was put in front of the subjects. Water was provided to the assessors to cleanse their palate between samples.

## 7.3 Data analysis

### 7.3.1 | Physiological and sensory data analysis

Pictures of the tongue were processed by using ImageJ software (open source license) and the Matlab (Mathsworks, ver. R2015a) scripts according to the procedure described by *Piochi* et al. (54). Sensory data were log-transformed accordingly to the semi-log nature of the scale (50). Assumption of homogeneity of variance and normality distribution were checked by using Levene and Shapiro tests for sensory data. Welch's *t*-test was then applied on both sensory and physiological data to find differences between the mean values of different classification groups. To explore each set of data, descriptive statistic was also computed for each situation. Linear regression models were used to investigate the effect of physiological parameters on both sweetness and flavour perception.

### 7.3.2 | PTR-QiToF-MS data treatment analysis and peak selection

PTR-MS data were treated with TOFO office software (Department of Food Quality and nutrition, Edmund Mach Foundation) according to Cappellin *et al.* (55). A total of 45 mass peaks were obtained, ranging from *m/z* 20 to *m/z* 250. A further selection of the mass peaks was then realized. Firstly, peaks attributed to $^{13}C$ isotopologues and water clusters were discarded. Then, to identify the product volatile compounds, signals from the panellists baseline breath (60 s) were compared with the ones that appeared as soon as panellists started masticating the gum (adaptation from (31)). Mass peak identification was based on literature available for aroma compounds contained in mint essential oil usually employed as flavour in minty chewing gum (56–58). In this paper, the experimental *m/z* values are reported up to the third decimal place.

### 7.3.3 | PTR-MS data processing and statistical analysis

For each relevant mass peak selected, means in parts per billion by volume [ppbV] concentrations for 20 s time bins were computed and used to both visualize trends and to statistically compare groups. This allowed to stabilize the data because of the high variability due to the breathing cycle, which characterize *in vivo* data.

As for sensory data, descriptive statistic was computed for each situation and assumption of homogeneity of variance and normality distribution were checked by using Levene and Shapiro tests. Welch's *t*-test was used to find differences between the mean values of different classification groups at the different time bins for the relevant mass peaks. Analysis of variance (ANOVA) was used to investigate difference in flavour perception and flavour release during time. Linear regression models were used to investigate the effect of physiological parameters on flavour release and perception. Principal component analysis (PCA) was conducted on the scaled data set corresponding to 29 rows (the 29 subjects) and 14 columns (oral cavity volume, salivary flow and 12 selected mass peaks) to find data latent structure according to indications reported elsewhere (59).

### 7.3.4 | Software

Data were analysed with R (version 3.4.3) (R foundation for statistical computing, Vienna, Austria), ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2009) and Microsoft Excel (version 2016).

## 7.4 Results

### 7.4.1 | Physiological parameters

Cumulative demographic and physiological data (Chinese vs European and female vs male) are presented in Table 7.1 while information for single panellists is provided as supplementary materials (Table S7.1). All the participants were familiar with the product but two Chinese panellists.

Differences between the different groups in physiological parameters were checked by running Welch's two sample *t*-tests. The only significant difference found was between male and female in the volume of the oral cavity ($p < .05$). Male panellists presented a higher volume of oral cavity which is in agreement with previous studies (60,61). No significant correlation between oral cavity volume, salivary flow and flavour release was found. The same was found for the number of fungiform papillae and sweetness perception.

**Table 7.1:** Physiological parameters. Average Oral Cavity Volume (O.C·V.) is the mean value of the three measurements of the oral cavity volume (ml) with the associated standard deviation. For O.C·V. and salivary flow, average values for male and females are presented in brackets.

| Group | Participants | Age | Average O.C.V [ml] | Salivary flow [g/min] | N. Papillae |
|---|---|---|---|---|---|
| **Caucasian-European** | 15 | 23.8 ± 1.2 | 93.6 ± 19.2 | 4.3 ± 2.0 | 52.5 ± 16.9 |
| **Asian-Chinese** | 15 | 23.3 ± 1.5 | 81.9 ± 16.8 | 3.4 ± 1.3 | 60.0 ± 21.7 |
| **Males** | 14 | 23.9 ± 1.6 | 101.3 ± 17.4 | 4.4 ± 2.7 | 59.5 ± 23.9 |
| **Females** | 16 | 23.2 ± 0.9 | 77.2 ± 13.7 | 3.9 ± 1.4 | 52.9 ± 13.8 |

### 7.4.2 PTR-MS in nose-space analysis

In Table S7.2 the mass peaks obtained by PTR-MS analysis are presented. The peak selection processes identified 6 volatile organic compounds related to the peppermint oil: these compounds and some of their fragments were considered for further statistical analysis and are presented in Table 7.2. Additionally, three mass peaks signals, associated to breath compounds ($m/z$ = 44.996, $m/z$ = 60.053 and $m/z$ = 69.069 identified as carbon dioxide, acetone isotope and isoprene respectively) produced normally by humans were selected to monitor panellists' breathing.

**Table 7.2:** Mass peaks list selected from the PTR-MS analysis. The following compounds and some of their fragments were used for further statistical analysis.

| Monitored Molecule | Sum Formula (protonated) | Peak (m/z) | Fragments | Reference |
|---|---|---|---|---|
| *Chewing gum flavour compounds* | | | | |
| **Monoterpens** | C10H16H+ | 137.133 | 67.054, 81.068, 93.069, 95.086, 109.101 | (Heenan et al., 2012; Maleknia, Bell, & Adams, 2007; Masi et al., 2017; Tani, Hayward, & Hewitt, 2003; Sine Yener et al., 2015) |
| **(–) Menthol** | C10H14OH+ (dehydration) | 139.148 | 83.085 | (Davidson et al., 1999; Heenan et al., 2012; Španěl & Smith, 1998) |
| **Menthofuran** | C10H14OH+ | 151.112 | | (Dimandja, Stanfill, Grainger, & Patterson, 2000; Kumar, Shukla, & Samad, 2014; Masi et al., 2017) |
| **1,8 –cineole / (+ \| –) menthone** | C10H18OH+ | 155.144 | | (Heenan et al., 2012; Masi et al., 2017; Steeghs, 2004) |
| **Menthyl acetate** | C12H2202H+ | 198.162 | 81.068, 83.085, 139.148 | (Heenan et al., 2012; Masi et al., 2017) |
| *Breath compounds* | | | | |
| **Carbon dioxide** | C02H+ | 44.996 | | (Moser et al., 2005) |
| **Acetone (isotope)** | C2[13]CH6OH+ | 60.053 | | (Heenan et al., 2012; Moser et al., 2005) |
| **Isoprene** | C5H8O2H+ | 69.069 | | (Moser et al., 2005) |

Figure 7.2 shows the dynamic emission of the flavour compounds in time: in this case it is not decreasing over the course of product consumption (7 min) and, it appears, in most of the measurements, quite constant regardless the panellist's origin and gender.

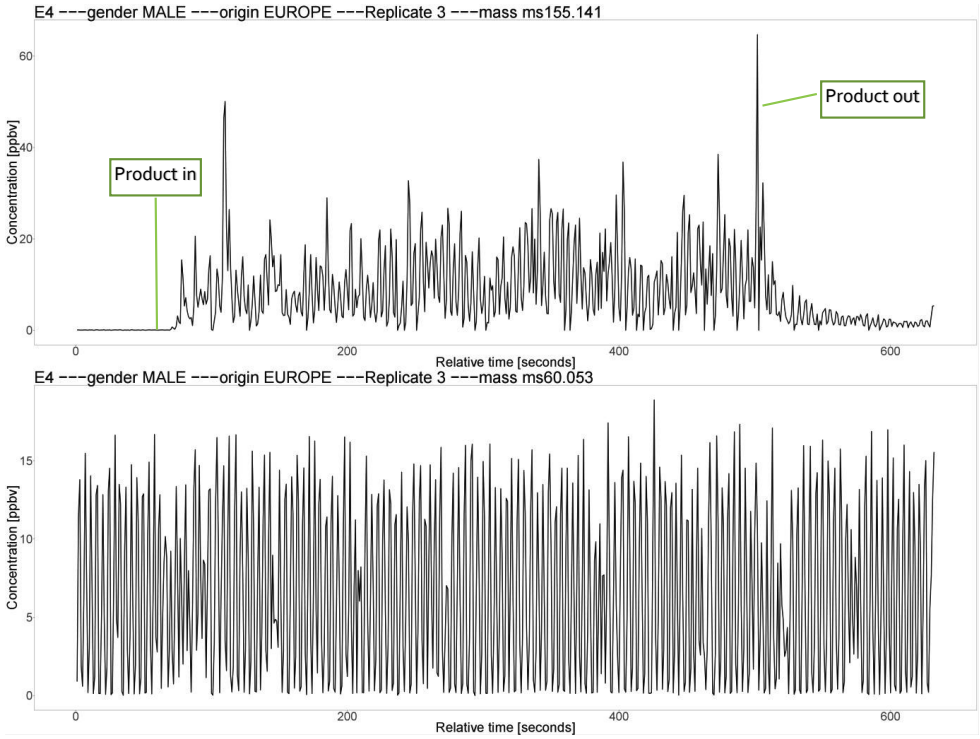



***Figure 7.2:*** *Concentration [ppbV] during time of the mass peak 155.141 (top panel) and 60.053 (bottom panel) identified respectively as + | – Menthone / 1,8-cineole and as the acetone isotope. Time at which the product starts to be consumed and its removal from the mouth are indicated on the upper part of the figure.*

When subjects were asked to remove the gum from the mouth, a spike in flavour release was often observed for most mass peaks. For this reason, no maximum intensity ($I_{max}$) or time at which maxim intensity occurred ($t_{max}$) was considered in the analysis. Moreover spikes are also associated to swallowing events (Fig. 7.2) (62,63).

In Figure 7.3 the flavour release curves of different mass peaks for the different groups and subgroups are summarized.

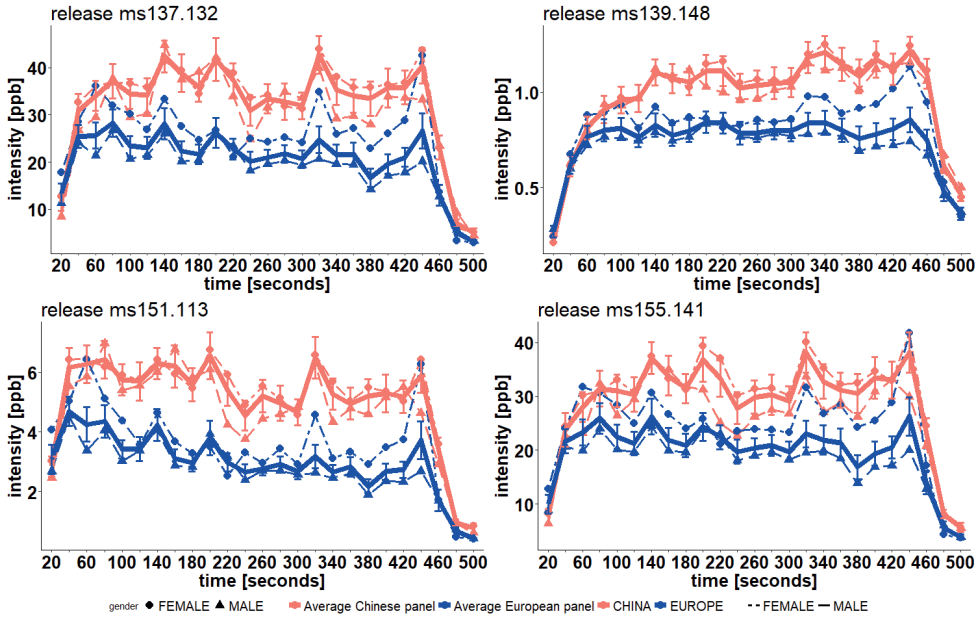



***Figure 7.3**: Average release during time for some of the relevant mass peak: (A) m/z = 137.134 ($C_{10}H_{16}H^+$) Monoterpens (B) m/z = 139.148 ($C_{10}H_{19}^+$) (–)Menthol / Fragment of Menthyl Acetate. (C) m/z = 151.113 ($C_{10}H_{14}OH^+$) Menthofuran. (D) 155.141($C_{10}H_{18}OH^+$) 1–8 Cineole /Menthone. Distinction between Chinese and European panellists are shown based on colour while distinction based on sex is indicated by different line types and symbols.*

The aroma compounds *m/z* 137.133 and 151.113, rapidly increased at the beginning of mastication reaching a maximum around 40 s, then they slowly decrease. On the other hand, *m/z* 139.148 and 155.141 increase rapidly at the beginning of the consumption and then continue to increase until the moment the product was discarded. The most striking result highlighted in Fig. 7.3 is that Chinese panellists presented a higher concentration of the aroma compound in the retro nasal cavity than European panellists. Computed bins of 20 s were compared between the groups and the p. values obtained from the Welch's two samples *t*-test are reported in Table 7.3 for mass peak m/z 137.133, which represents monoterpenes. This phenomenon is not connected to a specific compound: all the other mass peaks representative of the other aroma compounds showed the same trend (Table S7.3 – S7.6). Differences between Caucasian and Chinese panellists are statistically significant (*p* value < .05) generally after 80 s of consumption. The differences in flavour concentration disappear only after the chewing gum was discarded (approx. 450 s).

**Table 7.3:** Welch's t-test p values. For each time interval Chinese and Europeans and the total of males and females are compared for the aroma release of *m/z* 137.133. * and ^ shows time bins that reported a p.value < .01 for the origin effect and gender respectively.

| Time intervals | Mean Chinese [ppb] | Mean European [ppb] | Mean Female [ppb] | Mean Male [ppb] |
|---|---|---|---|---|
| **0–20 s** | 11.4 ± 8 | 13.2 ± 10 | 14.2 ± 10 | 10.5 ± 6 |
| **20–40s** | 30.9 ± 20 | 25.4 ± 14 | 31.5 ± 21 | 25 ± 16 |
| **40–60s^** | 34.1 ± 20 | 25.8 ± 16 | 36.1 ± 21 | 23.9 ± 13 |
| **60–80s** | 37.2 ± 23 | 28.2 ± 17 | 35.5 ± 22 | 30.1 ± 19 |
| **80–100 s*^** | 34.5 ± 20 | 23.4 ± 13 | 34.8 ± 19 | 23.4 ± 13 |
| **100–120 s*** | 34.2 ± 22 | 23 ± 14 | 33.3 ± 20 | 24.1 ± 18 |
| **120–140 s*** | 42.4 ± 21 | 28.1 ± 20 | 39 ± 22 | 31.8 ± 21 |
| **140–160 s*** | 38.9 ± 25 | 22.3 ± 13 | 36.1 ± 20 | 25.5 ± 21 |
| **160–180 s*** | 35.9 ± 19 | 21.7 ± 12 | 31.6 ± 17 | 26.2 ± 17 |
| **180–200 s*** | 41.7 ± 28 | 26.2 ± 19 | 37.3 ± 23 | 31 ± 26 |
| **200–220 s*** | 37.4 ± 22 | 22.7 ± 14 | 33.8 ± 20 | 26.6 ± 18 |
| **220–240 s*^** | 31 ± 20 | 20.2 ± 13 | 31.2 ± 20 | 20.2 ± 13 |
| **240–260 s*** | 33.4 ± 19 | 20.9 ± 10 | 31.3 ± 19 | 23.3 ± 13 |
| **260–280 s*** | 32.8 ± 24 | 21.8 ± 11 | 30 ± 18 | 24.8 ± 17 |
| **280–300 s*** | 32 ± 17 | 20.9 ± 11 | 29.2 ± 15 | 23.7 ± 16 |
| **300–320 s*^** | 42.8 ± 25 | 24.8 ± 17 | 41.4 ± 26 | 26.6 ± 17 |
| **320–340 s*^** | 35.3 ± 19 | 21.5 ± 14 | 34.5 ± 20 | 22.6 ± 14 |
| **340–360 s*** | 34 ± 22 | 21.7 ± 15 | 33.4 ± 23 | 22.6 ± 14 |
| **360–380 s*^** | 33.4 ± 23 | 16.7 ± 12 | 32.1 ± 24 | 18.4 ± 12 |
| **380–400 s*** | 35.7 ± 25 | 19.7 ± 12 | 33.4 ± 22 | 22.2 ± 19 |
| **400–420 s*^** | 35.8 ± 22 | 21 ± 12 | 34.5 ± 21 | 22.7 ± 16 |
| **420–440 s*^** | 40.4 ± 23 | 26.6 ± 22.9 | 43.4 ± 26 | 24.2 ± 17 |
| | | Gum out | | |
| **440–460 s*** | 23.1 ± 16 | 13 ± 13 | 5.3 ± 3 | 6.7 ± 4.2 |
| **460–480 s** | 7.1 ± 4 | 5 ± 3 | 4.5 ± 5 | 3.7 ± 2 |
| **480–500 s** | 5 ± 3 | 3.2 ± 2 | 14.2 ± 11 | 10.5 ± 8 |

From Fig. 7.3 it is also possible to see that females presented a higher release then males for both origin groups. However, the differences in aroma release found between gender were smaller than the one found for origin: significant differences (p.value < .01) were found just for few time bins. The significance of

the origin effect was confirmed also by two-way ANOVA (p.value < .01) for the origin but not for the gender at a certain time interval.

In Fig. 7.4, a PCA analysis performed using all the relevant mass peaks and physiological parameters is shown.

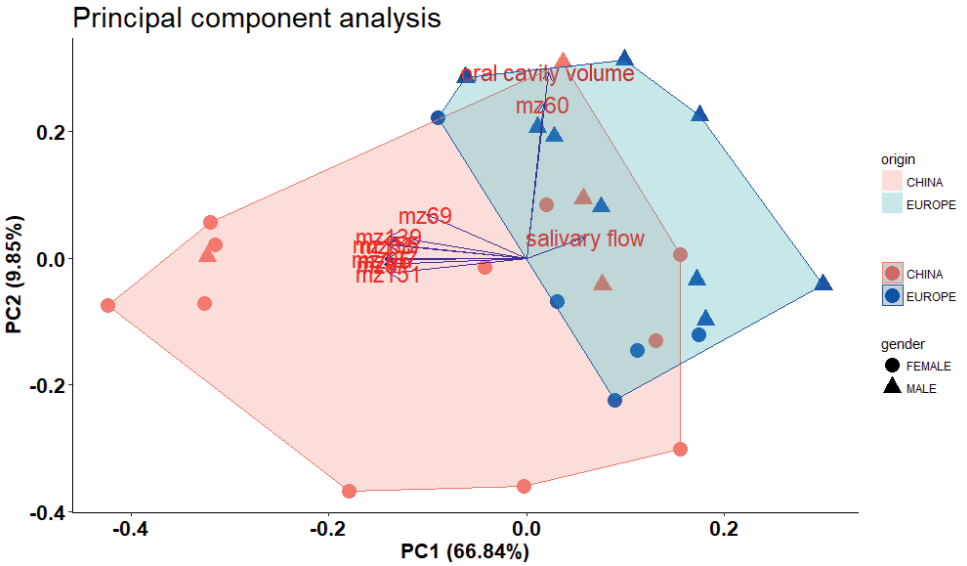



***Figure 7.4**: Principal component analysis biplot. PC1 is represented by flavour compounds release, PC2 by physiological parameters and acetone level in the breath. The two clusters (Chinese and European panellists) are highlighted with frames of different colours while gender distinction is highlighted based on shape.*

The first two components explained 77% of variance. Half of the Chinese panel clearly cluster on the left side of the plot, which represents a higher flavour release for all mass peaks of the chewing gum. Oral cavity volume and acetone concentration did not correlate with flavour release, which is represented by PC2 while mass peaks responsible for the mint aroma separated European from Chinese panellists. Even though differences in acetone level were found between the two groups, this mass peak does not seem to influence the chewing gum VOCs release (Figure 7.4). It can also be noticed that salivary flow is negatively correlated with aroma release mass peaks signals.

### 7.4.4 | Sensory evaluation during time

The dynamic perception of the overall mint flavour and sweetness divided by origin and gender is shown in Fig. 7.5. After the first two minutes of consumption, the perception of both sweetness and flavour decreases during time in a similar fashion. To investigate more in detail, perception evolution during time, a 1-way ANOVA was performed on the evaluated time intervals (10, 30, 60, 120, 180, 300, 420, 480 s). A significant effect of time is present (p.value < .05) on both flavour and sweetness. A pairwise *t*-test with a

Bonferroni correction found out that a significant decrease in perception occurred at 180 s while no significant differences were found between the last three sensory evaluations (300, 420 and 480 s).

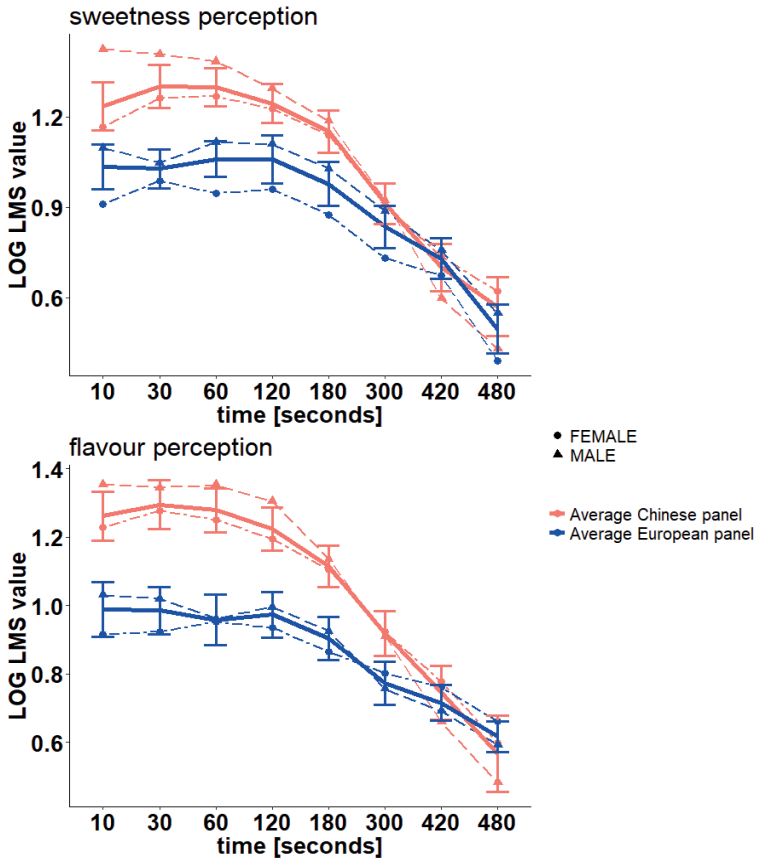



***Figure 7.5:*** *Perceived intensity of overall mint flavour and sweetness during time. Distinction based on gender and origin are highlighted by using different colours, different line shapes and symbols.*

From Figure 7.5 it is also possible to observe that, especially at the beginning of the consumption, the Chinese panel has higher scores than the European panel for both the sensory attributes while gender differences are less evident. 2-way ANOVA found a significant effect (p.value < .01) only for origin and not for gender. Perception comparison at each evaluation time between Chinese and European panellists was then made by using Welch's two sample t-test. The output of the analysis is summarized in Table 7.4, which also shows that Chinese panellists perceived both attributes higher than European panellists at the beginning of the consumption: the means' differences are significant at 10, 30, and 60 s of evaluation for attribute flavour and significant at 30 and 60 s for attribute sweetness ($p$ value < .05). The same procedure was applied to check differences in perception of flavour and sweetness for gender: no significant differences were found.

**Table 7.4** Welch's t-test output for comparison of flavour and sweetness perception means between the origin of the panellists. * indicate that there is a significant difference (p.value < .05).

| EVALUATION TIME | ATTRIBUTE | Log MEAN CHINESE | Log MEAN EUROPEAN |
|---|---|---|---|
| **10 s** | FLAVOUR | 2.97 ± 0.6* | 2.41 ± 0.6* |
| | SWEETNESS | 2.91 ± 0.6 | 2.49 ± 0.6 |
| **30 s** | FLAVOUR | 3.04 ± 0.6* | 2.41 ± 0.5* |
| | SWEETNESS | 3.05 ± 0.6* | 2.47 ± 0.5* |
| **60 s** | FLAVOUR | 3.00 ± 0.5* | 2.37 ± 0.6* |
| | SWEETNESS | 3.05 ± 0.6 * | 2.53 ± 0.5* |
| **120 s** | FLAVOUR | 2.47 ± 0.5 | 2.43 ± 0.5 |
| | SWEETNESS | 2.93 ± 0.5 | 2.54 ± 0.5 |
| **180 s** | FLAVOUR | 2.88 ± 0.5 | 2.40 ± 0.5 |
| | SWEETNESS | 2.79 ± 0.6 | 2.37 ± 0.6 |
| **300 s** | FLAVOUR | 2.24 ± 0.5 | 2.00 ± 0.5 |
| | SWEETNESS | 2.23 ± 0.6 | 2.08 ± 0.5 |
| **420 s** | FLAVOUR | 1.90 ± 0.6 | 1.88 ± 0.4 |
| | SWEETNESS | 1.82 ± 0.6 | 1.87 ± 0.5 |
| **480 s** | FLAVOUR | 1.61 ± 0.8 | 1.68 ± 0.3 |
| | SWEETNESS | 1.60 ± 0.7 | 1.46 ± 0.5 |

In Figure 7.6 are presented on the same plot the means of the in nose concentration of different mass peaks and the flavour perception scores during time by centring and scaling the values. In this way it is possible to visually compare the trends of the two aspects during time. The mass peak *m/z* = 151.113 identified as menthofuran, follows the same decreasing trend of the overall mint flavour perception. The other mass peaks (*m/z* = 137.133, 139.148) present a different trend. In particular, the mass peak of monoterpenes, after reaching a maximum remains constant until the removal of the product. The same trend was observed for other mass peaks (*m/z* = 81.069 and 155.141) On the other hand, the mass peak identified as menthol (and fragment of menthyl acetate) shows an increasing trend for all the mastication duration till the removal of the product.

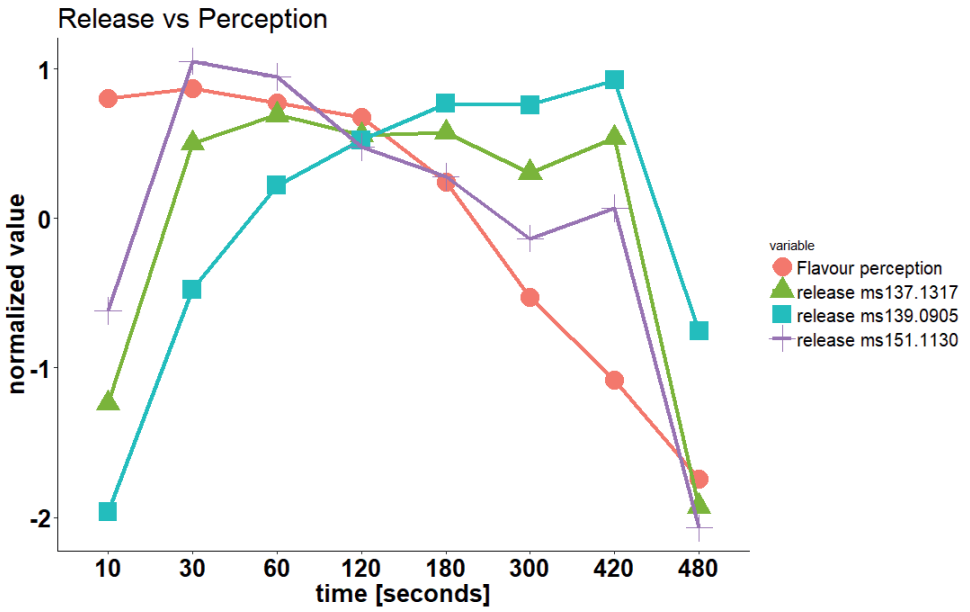



***Figure 7.6:*** *Mean volatile releases of different mass peaks in nose concentration and mint flavour perception intensity (dTI curve) for all the panel during product consumption. All data were centred and scaled for allowing to visually compare the trends.*

## 7.5 Discussion

The present results highlight a significant ethnicity effect in terms of both sensory perception and flavour release. During chewing gum consumption, Chinese panellists had a higher perception of both sweetness and overall mint flavour which parallel an higher in nose concentration of the flavour compounds. Gender had a limited effect as only a marginal significant difference related to the oral cavity volume was found. As expected high inter-individual variability, reflected in the nose-space signal, was observed (28). Despite of these inter-individual differences, differences in both sensory perception and *in vivo* flavour release between Chinese and European panellists were measured.

Physiological parameters have a limited role in explaining these differences. In line with previous studies, a weak negative correlation between salivary flow and flavour release (33) and a weak positive correlation between density of taste papillae and sweetness perception were found (64,65). The fact that no strong and significant correlation between physiological parameters and flavour release was found may indicate that single physiological parameters are not good predictors of such a complex phenomenon, where probably a larger combination of different factors is required (43,66). Oral cavity volume did not clearly explain the variability in both flavour perception and release. Male panellists had a significant larger oral capacity than female, although in this case it was found to have a limit impact on the amount of aroma release. Finally,

Chinese panellists show a lower average of saliva flow rate (even though not statistically significant) when compared to Europeans.

During the 7 min of consumption the different compounds have slightly different trends for the measured in nose concentration. These differences are probably due to each aroma compound concentration in the chewing gum and to the compound physical and chemical properties included the affinity for saliva (i.e., the dissolution) and salivary components (i.e., molecular interaction, enzymatic degradation) that are key parameters in flavour perception (67). For *m/z* = 155.141 and *m/z* = 139.148 corresponding respectively to menthone (and 1,8-Cineole) and menthol, the trends are in line with previous studies where similar chewing gum (without sugar coating and with not encapsulated peppermint oil as aroma) were used (29,37,57). The mass peak *m/z* = 151.113 identified as menthofuran, is the only compound showing a decreasing trend. It may be due to its lower concentration in the food matrix that leads to its gradual consumption during the mastication.

For the further discussion, the gum consumption is broken up in three different portions of time: first 90 s, from 90 to 420 s and from 420 to 500 s. Generally, at the beginning of consumption (first time interval) differences in flavour concentration are not observed while in the second and third ones differences are observed for in-nose flavour concentration (see Table 7.3). The pulse of exhalation just before chewing gum removal (Figure 7.2) it is most likely due to the "swallow-breath" phenomenon which occurs when the epiglottis and trachea re-opens during the pharyngeal stage of the swallowing. This situation leads to the access of odorants in the nasal cavity, and ultimately, to an aroma pulse (39).

In the third time interval, the gum was removed from the mouth and differences between the Chinese and the European panels were still present, especially, immediately after the product was discarded. After the removal, aroma compounds remains in saliva for a variable amount of time (33): at this stage, differences in flavour release might be due to different volume and chemical composition of saliva or/and mucosa rather than different chewing behaviour because no mastication was performed in those last time intervals. In fact, flavour compounds release, can be influenced by many different processes such as adsorption to salivary proteins and mucous membranes, enzymatic reaction, non-covalent or covalent binding and saliva buffering capacity (68–70). In our case, all the aroma compounds under investigations have an hydrophobic nature (log *P* = 2.5 to 3.6) and it is therefore possible that individual differences in saliva protein concentrations resulted in a different reduction of hydrophobic compound release due to the aromatic compounds binding by saliva proteins (71).

Moreover, differences in flavour release in the second and third time intervals may be due to the lower average of saliva flow rate of the Chinese panellists. Higher salivary flow was related to a lower flavour release of mint aromas during both *in vivo* consumption (33) and in amodel mouth system where larger saliva volumes decreased release of aroma compounds (70,72–75). However, it should be noticed that in literature contrasting results were found on the role of salivary flow on flavour release: Guinard *et al.* found that

salivary flow influences only the rate of release but not the lasting and the intensity of aroma released during chewing gum consumption (76) while Pionnier *et al.* (77,78) observed no significant influence of salivary flow rate on VOCs release.

We then hypothesize, that the observed ethnicity differences in flavour release are partially given by different salivary flow. As well differences in saliva composition might play a role in explaining ethnicity differences in flavour release. Saliva proteins can interact with aroma compounds through both covalent and non-covalent bonds and consequently influence their partitioning between the liquid and air phases (71). Moreover, salivary protein like proline-rich proteins, cystatins, mucins salivary amylases and esterase are able to bind or degrade food volatile, to retain molecules in the liquid phase or favour their release by enzymatic hydrolysis of ester bounds (69,70,79,80). A great inter-individual variation in both the flow and the salivary composition (e.g. mucins, proline-rich proteins, sodium, amylolytic, proteolytic and lipolytic activities) is present in humans, which has been related to differences in both flavour release and perception (67,81). For instance, inter-individual differences in the quantity of menthone released during the consumption of a sweet mint table could partly be explained by the total protein concentrations in subjects saliva (43). Diet and environment have been shown to affect saliva composition. For example, the expression and the levels of salivary amylase protein are affected by the type of diet (i.e. low or high-starch) (82) while vegetarian diet was found to have an effect on saliva secretion rate, buffer capacity and sodium concentration (83). Saliva composition and activity (like total antioxidant capacity ad amylolysis) are also correlated to carbohydrate intake (84). As well, the consumption of relatively large amounts of coffee and broccoli increases the activity of certain salivary enzymes like the cytosolic class 3 aldehyde dehydrogenase, some gluthanione S-transferases and DT-diphorase (85).

Composition of the oral microbiota may also have an effect on aroma perception and release (69,81). Analysis of the salivary microbiome has revealed a high diversity between individuals even if variation between geographical locations and ethnicity are still unclear (86–88).

Therefore, differences in diets, environment and genetic expression have all a relevant role in determining saliva composition in terms of both oral microbiota and proteins. These differences then may help in partially explaining the observed differences in flavour release between the two groups (Chinese and Europeans).

Another factor that may explain the differences in flavour release are the different eating behaviours. In this study it was decided to have a free protocol for chewing, breathing and swallowing to not overstress participants, to let intra-subject and inter-subject variability emerge if present and also based on the indications found by Leclercq and Blancher (89). However, it cannot be excluded that differences in eating behaviours may also play a role in different flavour release from the chewing gum. The mastication rate, the number of chews and the chew amplitude are all positively correlated with high concentration of volatiles in

the nose (33,73,75,77,90). Moreover, menthone release during the consumption of sweet mint tables is influenced by oral processing which induced, or did not induce, the opening of the velum (43).

For the sweetness and flavour perception the participants evaluations are in line with previous researches: a maximum after few minutes followed by a decrease in both sweetness and flavour intensity (17,18,20,37,57). Flavour perception during time statistically decreases already at 180 s while this does not happen for in-nose VOC concentration that reaches a peak and then remains constant until the product is removed from the mouth. This indicates that sensory perception, in many cases, is rather influenced by other factors such as sucrose concentration in the mouth, cross-modal perception, textural properties of the food matrix and, ultimately, sensory adaptation during time (29,91,92).

Sweetness release may have played a role in determine this difference in trends as it is reported by Davidson et al. who found that the sensory flavour perception is more controlled by sweetness concentration in the mouth rather than menthone concentration in the nose-space (29).

When coming to group comparison for the sensory attributes, our findings are in accordance with what was found by Murray *et al.* who compared food choices and preferences between Chinese and European-origin Australian consumer and the first rated sensory attributes significantly higher than Australian subjects (93). As a matter of fact, sweetness perception differs across different cultures for genetic variability, liking and food-specific discrimination ability reasons (94,95) and significant differences in retro nasal identification were found among culture (47).

As well, the difference in salivary flow previously highlighted may explain not only the lower aroma release but also the lower sensory perception for the flavour intensity in the European group. It has been hypothesized that saliva can dilute taste stimuli and decrease taste (67) even if the precise impact of saliva on aroma perception has never been directly investigated (69).

In our study, it is interesting to note that the difference in both sweetness and flavour perception is significant at the beginning of the consumption, while the in-nose concentration is not significantly different. This suggests that the higher release may not be the only reason behind the higher perception but also cultural difference in the scale's usage and different previous experienced of the product played a role (96,97). Lack of difference between male and female in sensory evaluation of flavour intensity is consistent with the results from VOCs in-nose concentration.

*In vivo* studies are needed to investigate flavour release since they account for continuous changes in volume, composition, and viscosity of food bolus during mastication (39). However, this work presented some limitations. First, for practical reasons, the adopted experimental design can be considered ethnocentric in terms of environment, language and context. The sensory output may then be biased by this factor (96,98). Secondly, the carryover effect of the product was not investigated in detail. Previous analysis on similar products indicated that carryover effects were detected till 1 h after consumption (33). Extra attention should be paid when working with long lasting compounds like menthone and menthol. However,

in our case rinsing procedures reduced the aroma residuals risks. Finally, it should be considered that the study was conducted on a relativly small sample and further studies in this direction should aim at a higher number of participants to have a better representation of population variability. As well, future studies should be toward the investigation of saliva and mucosa composition, their role on *in vivo* flavour release and perception, and how do they differ among ethnic groups. Moreover, the possibility to include data collection about eating behavior during consumption (e.g. mastication rate, number of chews), may add information about the origin of the flavour release differences that were observed. Finally, it is important, for future research to consider also a different kind of perception investigation, with a less ethnocentric experimental design. It should rather be used an emic approach, based on categories and meaning understood internally by both ethnic groups.

## 7.6 Conclusion

A significant difference between Chinese and Europeans has been observed for in nose flavour release for aroma compounds related to mint flavoured chewing gum. The same differences were found for sweetness and flavour perception. These differences were not explained by the physiological parameter considered, as they did not explain the variability in flavour release and perception. The mechanism underlying the different aroma persistence in the oral and nasal cavity of Chinese and European panellists is not yet clear. Further research is needed in both the underlying mechanisms and the ethnical variability (69). However, this paper provides both methodological and fundamental insights for further investigation in geographical variability in flavour release and perception. In terms of ethnical variability in *in vivo* flavour release, this article presents a totally new field of research which has not been investigated before. Clarifying inter-individual differences, not only represents a step forward for the tuning of food products for different markets, but it can be also a step forward for a more mechanistic understanding of what is happening in consumer's mouth during product consumption.

## 7.7 Acknowledgments

Thanks to Eva Ketel, Arianne Van Eck and Dr. Markus Stieger who provided ideas and suggestions during the experimental design.

## 7.8 References


1. Konar N, Palabiyik I, Toker OS, Sagdic O. Chewing gum: Production, quality parameters and opportunities for delivering bioactive compounds. Trends Food Sci Technol. 2016;55:29–38.
2. Guichard E, Salles C. Retention and release of taste and aroma compounds from the food matrix during mastication and ingestion. Flavor: From Food to Behaviors, Wellbeing and Health. Elsevier Ltd; 2016. 3–22 p.
3. Fisk ID. Aroma release. In: Flavour Development, Analysis and Perception in Food and Beverages [Internet]. 2015. p. 105–23. Available from: http://linkinghub.elsevier.com/retrieve/pii/B9781782421030000060
4. Gierczynski I, Laboure H, Guichard E. In vivo aroma release of milk gels of different hardnesses: Inter-individual differences and their consequences on aroma perception. J Agric Food Chem. 2008;56(5):1697–703.
5. Castura JC, Antúnez L, Giménez A, Ares G. Temporal Check-All-That-Apply (TCATA): A novel dynamic method for characterizing products. Food Qual Prefer. 2016;
6. Déléris I, Saint-Eve A, Dakowski F, Sémon E, Le Quéré JL, Guillemin H, et al. The dynamics of aroma release during consumption of candies of different structures, and relationship with temporal perception. Food Chem. 2011;127(4):1615–24.
7. Di Monaco R, Miele NA, Volpe S, Picone D, Cavella S. Temporal Sweetness Profile of MNEI and Comparison with Commercial Sweeteners. J Sens Stud. 2014;29(6):385–94.
8. Dinnella C, Masi C, Zoboli G, Monteleone E. Sensory functionality of extra-virgin olive oil in vegetable foods assessed by Temporal Dominance of Sensations and Descriptive Analysis. Food Qual Prefer. 2012;26(2):141–50.
9. Esmerino EA, Castura JC, Ferraz JP, Tavares Filho ER, Silva R, Cruz AG, et al. Dynamic profiling of different ready-to-drink fermented dairy products: A comparative study using Temporal Check-All-That-Apply (TCATA), Temporal Dominance of Sensations (TDS) and Progressive Profile (PP). Food Res Int. 2017;101:249–58.
10. Hutchings SC, Foster KD, Grigor JMV, Bronlund JE, Morgenstern MP. Temporal dominance of sensations: A comparison between younger and older subjects for the perception of food texture. Food Qual Prefer. 2014;31(1):106–15.
11. Lenfant F, Loret C, Pineau N, Hartmann C, Martin N. Perception of oral food breakdown. The concept of sensory trajectory. Appetite. 2009;52(3):659–67.
12. Morais EC, Pinheiro ACM, Nunes CA, Bolini HMA. Multiple Time-Intensity Analysis and Temporal Dominance of Sensations of Chocolate Dairy Dessert Using Prebiotic and Different High-Intensity Sweeteners. J Sens Stud. 2014;29(5):339–50.
13. Rodrigues JF, Gonçalves CS, Pereira RC, Carneiro JDS, Pinheiro ACM. Utilization of temporal dominance of sensations and time intensity methodology for development of low-sodium Mozzarella cheese using a mixture of salts. J Dairy Sci [Internet]. 2014;97(8):4733–44. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030214003841
14. Galmarini M V., Symoneaux R, Visalli M, Zamora MC, Schlich P. Could Time-Intensity by a trained panel be replaced with a progressive profile done by consumers? A case on chewing-gum. Food Qual Prefer. 2016;48:274–82.
15. McGowan BA, Lee SY. Comparison of methods to analyze time-intensity curves in a corn zein chewing gum study. Food Qual Prefer. 2006;17(3–4):296–306.
16. Ovejero-López I, Bro R, Bredie WLP. Univariate and multivariate modelling of flavour release in chewing gum using time-intensity: A comparison of data analytical methods. Food Qual Prefer. 2005;16(4):327–43.
17. Potineni R V., Peterson DG. Influence of flavor solvent on flavor release and perception in sugar-free chewing gum. J Agric Food Chem. 2008;56(9):3254–9.

18. Potineni R V., Peterson DG. Mechanisms of flavor release in chewing gum: Cinnamaldehyde. J Agric Food Chem. 2008;56(9):3260–7.
19. Santos MG, Carpinteiro DA, Thomazini M, Rocha-Selmi GA, da Cruz AG, Rodrigues CEC, et al. Coencapsulation of xylitol and menthol by double emulsion followed by complex coacervation and microcapsule application in chewing gum. Food Res Int [Internet]. 2014;66:454–62. Available from: http://dx.doi.org/10.1016/j.foodres.2014.10.010
20. Duizer LM, Bloom K, Findlay CJ. Dual-attribute time-intensity sensory evaluation: A new method for temporal measurement of sensory perceptions. Food Qual Prefer. 1997;8(4):261–9.
21. Delarue J, Loescher E. Dynamics of food preferences: A case study with chewing gums. Food Qual Prefer. 2004;15(7-8 SPEC.ISS.):771–9.
22. Kuesten C. Time – Intensity Using Discrete Time Points. 2017;182–236.
23. Lawless HT, Heymann H. Sensory Evaluation of Food. Elementary Food Science. 2010. 600 p.
24. Biasioli F, Yeretzian C, Gasperi F, Mark TD. PTR-MS monitoring of VOCs and BVOCs in food science and technology. Trac-Trends Anal Chem. 2011;30(7):968–77.
25. Taylor AJ, Linforth RST. On-Line Monitoring of Flavour Processes. In: Food Flavour Technology: Second Edition. 2010. p. 266–95.
26. Avison SJ. Real-time flavor analysis: Optimization of a proton-transfer-mass spectrometer and comparison with an atmospheric pressure chemical ionization mass spectrometer with an MS-nose interface. J Agric Food Chem. 2013;61(9):2070–6.
27. Boland AB, Delahunty CM, Van Ruth SM. Influence of the texture of gelatin gels and pectin gels on strawberry flavour release and perception. In: Food Chemistry. 2006.
28. Charles M, Romano A, Yener S, Barnabà M, Navarini L, Märk TD, et al. Understanding flavour perception of espresso coffee by the combination of a dynamic sensory method and in-vivo nosespace analysis. Food Res Int. 2015;69.
29. Davidson JM, Linforth RST, Hollowood TA, Taylor AJ. Effect of sucrose on the perceived flavor intensity of chewing gum. J Agric Food Chem. 1999;47(10):4336–40.
30. Heenan S, Soukoulis C, Silcock P, Fabris A, Aprea E, Cappellin L, et al. PTR-TOF-MS monitoring of in vitro and in vivo flavour release in cereal bars with varying sugar composition. Food Chem. 2012;131(2):477–84.
31. Romano A, Cappellin L, Ting V, Aprea E, Navarini L, Gasperi F, et al. Nosespace analysis by PTR-ToF-MS for the characterization of food and tasters: The case study of coffee. Int J Mass Spectrom [Internet]. 2014;365–366:20–7. Available from: http://dx.doi.org/10.1016/j.ijms.2013.12.001
32. Blee N, Linforth R, Yang N, Brown K, Taylor A. Variation in aroma release between panellists consuming different types of confectionary. Flavour Fragr J. 2011;26(3):186–91.
33. Haahr AM, Bardow A, Thomsen CE, Jensen SB, Nauntofte B, Bakke M, et al. Release of peppermint flavour compounds from chewing gum: Effect of oral functions. Physiol Behav. 2004;82(2–3):531–40.
34. Deleris I, Saint-Eve A, Semon E, Guillemin H, Guichard E, Souchon I, et al. Comparison of direct mass spectrometry methods for the on-line analysis of volatile compounds in foods. J Mass Spectrom. 2013;48(5):594–607.
35. Biasioli F, Yeretzian C, Mark TD, Dewulf J, Van Langenhove H. Direct-injection mass spectrometry adds the time dimension to (B)VOC analysis. Trac-Trends Anal Chem. 2011;30(7):1003–17.
36. Biasioli F, Gasperi F, Aprea E, Endrizzi I, Framondino V, Marini F, et al. Correlation of PTR-MS spectral fingerprints with sensory characterisation of flavour and odour profile of "Trentingrana" cheese. Food Qual Prefer. 2006;17(1–2):63–75.
37. Sostmann K, Potineni R, Blancher G, Zhang X, Espinosa-Diaz M, Antenucci RN. Aroma release from encapsulation systems in chewing gum. Expr Multidiscip Flavour Sci [Internet]. 2008;154–6. Available from:

https://home.zhaw.ch/~yere/weurman2008.htm

38. Krause AJ, Henson LS, Reineccius GA. Use of a chewing device to perform a mass balance on chewing gum components. Flavour Fragr J. 2011;26(1):47–54.
39. Salles C, Chagnon MC, Feron G, Guichard E, Laboure H, Morzel M, et al. In-mouth mechanisms leading to flavor release and perception. Crit Rev Food Sci Nutr. 2011;51(1):67–90.
40. Buettner A, Otto S, Beer A, Mestres M, Schieberle P, Hummel T. Dynamics of retronasal aroma perception during consumption: Cross-linking on-line breath analysis with medico-analytical tools to elucidate a complex process. Food Chem. 2008;108(4):1234–46.
41. Doyennette M, Déléris I, Féron G, Guichard E, Souchon I, Trelea IC. Main individual and product characteristics influencing in-mouth flavour release during eating masticated food products with different textures: Mechanistic modelling and experimental validation. J Theor Biol. 2014;340:209–21.
42. Muñoz-gonzález C, Feron G, Guichard E, Rodríguez- JJ. Understanding the role of saliva on aroma release from wine by using static and dynamic headspace conditions. :1–48.
43. Repoux M, Sémon E, Feron G, Guichard E, Labouré H. Inter-individual variability in aroma release during sweet mint consumption. Flavour Fragr J. 2012;27(1):40–6.
44. Déléris I, Saint-Eve A, Guo Y, Lieben P, Cypriani ML, Jacquet N, et al. Impact of swallowing on the dynamics of aroma release and perception during the consumption of alcoholic beverages. Chem Senses. 2011;36(8):701–13.
45. Ayabe-Kanamura S, Schicker I, Laska M, Hudson R, Distel H, Kobayakawa T, et al. Differences in perception of everyday odors: a Japanese-German cross- cultural study. Chem Senses. 1998;23(1):31–8.
46. Chrea C, Valentin D, Sulmont-Rossé C, Ly Mai H, Hoang Nguyen D, Abdi H. Culture and odor categorization: Agreement between cultures depends upon the odors. Food Qual Prefer. 2004;15(7-8 SPEC.ISS.):669–79.
47. Croy I, Hoffmann H, Philpott C, Rombaux P, Welge-Luessen A, Vodicka J, et al. Retronasal testing of olfactory function: An investigation and comparison in seven countries. Eur Arch Oto-Rhino-Laryngology. 2014;271(5):1087–95.
48. Prescott J. Comparisons of taste perceptions and preferences of Japanese and Australian consumers: overview and implications for cross-cultural sensory research. Food Qual Prefer. 1998;9(6):393–402.
49. Yeretzian C, Pollien P, Lindinger C, Ali S. Individualization of Flavor Preferences: Toward a Consumer-centric and Individualized Aroma Science. Compr Rev Food Sci Food Saf. 2004;
50. Green BG, Dalton P, Cowart B, Shaffer G, Rankin K, Higgins J. Evaluating the "labeled magnitude scale" for measuring sensations of taste and smell. Chem Senses. 1996;21(3):323–34.
51. Alsanei WA, Chen J. Studies of the Oral Capabilities in Relation to Bolus Manipulations and the Ease of Initiating Bolus Flow. J Texture Stud. 2014;45(1):1–12.
52. Gaviao MB, Engelen L, Van der Bilt a. Chewing behaviour and salivary secretion. Eur J Oral Sci. 2004;112:19–24.
53. Monteleone E, Spinelli S, Dinnella C, Endrizzi I, Laureati M, Pagliarini E, et al. Exploring influences on food choice in a large population sample: The Italian Taste project. Food Qual Prefer. 2017;59:123–40.
54. Piochi M, Monteleone E, Torri L, Masi C, Almli VL, Wold JP, et al. Comparing manual counting to automated image analysis for the assessment of fungiform papillae density on human tongue. Chem Senses. 2017;42(7):553–61.
55. Cappellin L, Biasioli F, Granitto PM, Schuhfried E, Soukoulis C, Costa F, et al. On data analysis in PTR-TOF-MS: From raw spectra to data mining. Sensors and Actuators B-Chemical. 2011;155(1):183–90.
56. Hawrył MA, Skalicka-Woniak K, Wieboda R, Niemiec M, Stępak K, Waksmundzka-Hajnos M, et al. GC-MS fingerprints of mint essential oils. Open Chem. 2015;13(1):1326–32.
57. Ovejero-López I, Haahr AM, Van Den Berg F, Bredie WLP. Flavor release measurement from gum model

system. J Agric Food Chem. 2004;52(26):8119–26.
58. Rohloff J. Monoterpene composition of essential oil from peppermint (Mentha x piperita L.) with Regard to leaf position using solid-phase microextraction and gas chromatography/mass spectrometry analysis. J Agric Food Chem. 1999;47(9):3782–6.
59. Granato D, Putnik P, Kovačević DB, Santos JS, Calado V, Rocha RS, et al. Trends in Chemometrics: Food Authentication, Microbiology, and Effects of Processing. Compr Rev Food Sci Food Saf. 2018;17(3):663–77.
60. Lawless HT, Bender S, Oman C, Pelletier C. Gender, age, vessel size, cup vs. straw sipping, and sequence effects on sip volume. Dysphagia. 2003;18(3):196–202.
61. Nascimento WV, Cassiani RA, Dantas RO. Gender effect on oral volume capacity. Dysphagia. 2012;27(3):384–9.
62. Buettner A, Beer A, Hannig C, Settles M, Schieberle P. Physiological and analytical studies on flavor perception dynamics as induced by the eating and swallowing process. Food Qual Prefer. 2002;13(7–8):497–504.
63. Ingham KE, Linforth RST, Taylor AJ. The effect of eating on aroma release from mint-flavoured sweets. Flavour Fragr J. 1995;10(1):15–24.
64. Miller IJ, Reedy FE. Variations in human taste bud density and taste intensity perception. Physiol Behav. 1990;47(6):1213–9.
65. Segovia C, Hutchinson I, Laing DG, Jinks AL. A quantitative study of fungiform papillae and taste pore density in adults and children. Dev Brain Res. 2002;138(2):135–46.
66. Mishellany-Dutour A, Woda A, Labouré H, Bourdiol P, Lachaze P, Guichard E, et al. Retro-Nasal Aroma release is correlated with variations in the in-mouth air cavity volume after empty deglutition. PLoS One. 2012;7(7).
67. Canon F, Neiers F, Guichard E. Saliva and flavour perception : perspectives. 2018;
68. Buettner A, Schieberle P. In ¯ uence of mastication on the concentrations of aroma volatiles Ð some aspects of ¯ avour release and ¯ avour perception. 2000;71:347–54.
69. Ployon S, Morzel M, Canon F. The role of saliva in aroma release and perception. Food Chem. 2017;226:212–20.
70. Roberts DD, Aeree TE. Simulation of Retronasal Aroma Using a Modified Headspace Technique: Investigating the Effects of Saliva, Temperature, Shearing, and Oil on Flavor Release. J Agric Food Chem. 1995;43(8):2179–86.
71. van Ruth S, Grossmann I, Geary M, Delahunty CM. Interactions between Artificial Saliva and 20 Aroma Compounds in Water and Oil Model Systems. J Agric Food Chem [Internet]. 2001;49(5):2409–13. Available from: %5C%5CRobsrv-05%5Creference manager%5CArticles%5C3358.pdf
72. Feron G, Ayed C, Qannari EM, Courcoux P, Laboure H, Guichard E. Understanding aroma release from model cheeses by a statistical multiblock approach on oral processing. PLoS One. 2014;9(4).
73. Hansson A, Giannouli P, Van Ruth S. The influence of gel strength on aroma release from pectin gels in a model mouth and in vivo, monitored with proton-transfer-reaction mass spectrometry. J Agric Food Chem. 2003;51(16):4732–40.
74. Mehinagic E, Prost C, Demaimay M. Optimization of extraction of apple aroma by dynamic headspace and influence of saliva on extraction of volatiles. J Agric Food Chem. 2004;52(16):5175–82.
75. Van Ruth SM, Roozen JP. Influence of mastication and saliva on aroma release in a model mouth system. In: Food Chemistry. 2000. p. 339–45.
76. Guinard JX, Zoumas-Morse C, Walchak C, Simpson H. Relation between saliva flow and flavor release from chewing gum. Physiol Behav. 1997;61(4):591–6.
77. Pionnier E, Chabanet C, Mioche L, Le Quéré JL, Salles C. 1. In Vivo Aroma Release during Eating of a Model Cheese: Relationships with Oral Parameters. J Agric Food Chem. 2004;52(3):557–64.
78. Pionnier E, Nicklaus S, Chabanet C, Mioche L, Taylor AJ, Le Quéré JL, et al. Flavor perception of a model cheese:

Relationships with oral and physico-chemical parameters. Food Qual Prefer. 2004;15(7-8 SPEC.ISS.):843–52.
79. Friel EN, Taylor AJ. Effect of salivary components on volatile partitioning from solutions. J Agric Food Chem. 2001;49(8):3898–905.
80. Taylor AJ. Volatile flavor release from foods during eating. Crit Rev Food Sci Nutr. 1996;36(8):765–84.
81. Piombino P, Genovese A, Esposito S, Moio L, Cutolo PP, Chambery A, et al. Saliva from obese individuals suppresses the release of aroma compounds from wine. PLoS One. 2014;
82. Perry G, Dominy N, Claw K, Lee A. Diet and the evolution of human amylase gene copy number variation. Nat Genet. 2007;39(10):1256–60.
83. Johansson G, Birkhed D. Effect of a long-term change from a mixed to a lactovegetarian diet on human saliva. Arch Oral Biol. 1994;39(4):283–8.
84. Méjean C, Morzel M, Neyraud E, Issanchou S, Martin C, Bozonnet S, et al. Salivary composition is associated with liking and usual nutrient intake. PLoS One. 2015;10(9).
85. Sreerama, Hedge S. Identification of a Class 3 Aldehyde Dehydrogenase in Human Saliva and Increased Levels of This Enzyme, Glutathione S-Transferases, and DT-Diaphorase in the Saliva of Subjects Who Continually Ingest Large Quantities of Coffee or Broccoli. Clin Cancer Res. 1995;1:1153–63.
86. Mason MR, Nagaraja HN, Camerlengo T, Joshi V, Kumar PS. Deep Sequencing Identifies Ethnicity-Specific Bacterial Signatures in the Oral Microbiome. 2013;8(10):1–7.
87. Nasidze I, Li J, Quinque D, Tang K, Stoneking M. Global diversity in the human salivary microbiome. 2009;636–43.
88. Li J, Quinque D, Horz H, Li M, Rzhetskaya M, Raff JA, et al. Comparative analysis of the human saliva microbiome from different climate zones : Alaska , Germany , and Africa. 2014;1–13.
89. Leclercq S, Blancher G. Multimodal sensory integration during sequential eating-linking chewing activity, aroma release, and aroma perception over time. Chem Senses. 2012;37(8):689–700.
90. Mestres M, Kieffer R, Buettner A. Release and perception of ethyl butanoate during and after consumption of whey protein gels: Relation between textural and physiological parameters. J Agric Food Chem. 2006;54(5):1814–21.
91. Overbosch P, Afterof WGM, Haring PGM. Flavor release in the mouth. Food Rev Int. 1991;7(2):137–84.
92. Weel KGC, Boelrijk AEM, Alting AC, Van Mil PJJM, Burger JJ, Gruppen H, et al. Flavor release and perception of flavored whey protein gels: Perception is determined by texture rather than by release. J Agric Food Chem. 2002;50(18):5149–55.
93. Murray JM, Easton K, Best DJ. A study of Chinese-Origin and European-Origin Australian consumers' texture preferences using a novel extruded product. J Sens Stud. 2001;16(5):485–504.
94. Fushan AA, Simons CT, Slack JP, Drayna D. Association between Common Variation in Genes Encoding Sweet Taste Signaling Components and Human Sucrose Perception. 2017;(October):579–92.
95. Holt SHA, Cobiac L, Beaumont-smith NE, Easton K, Best DJ. Dietary habits and the perception and liking of sweetness among Australian and Malaysian students : A cross-cultural study. 2000;11.
96. Douglas SP, Samuel Craig C. The changing dynamic of consumer behavior: implications for cross-cultural research. Int J Res Mark. 1997;14(4):379–95.
97. Yeh L., Kim K., Chompreeda P, Rimkeeree H, Yau NJ., Lundahl D. Comparison in Use of the 9-Point Hedonic Scale between Americans, Chinese, Koreans, and Thai. Food Qual Prefer. 1998;9(6):413–9.
98. Ares G. Methodological issues in cross-cultural sensory and consumer research. Food Qual Prefer. 2018;64:253–63.

## 7.9 Supplementary materials

**Table S7.1:** Physiological parameters. Average Oral Cavity Volume (O.C.V.) is the mean value of the three measurements of the oral cavity volume (ml) and the standard deviation. Max O.C.V represents the maximum oral cavity volume among the measurements. In number of papillae (N° papillae), NA value represents pictures where the image resolution was not high enough to determine an accurate count.

| Code | Origin | Age | Gender | Average O.C.V. | Max. O.C.V. | Salivary flow (g/min) | N° papillae |
|---|---|---|---|---|---|---|---|
| **C1** | CHINA | 22 | F | 84.54 ± 5.36 | 89.44 | 2.38 | 51 |
| **C2** | CHINA | 23 | F | 74.15±0.35 | 74.54 | 3.21 | NA |
| **C3** | CHINA | 24 | F | 73.10±2.49 | 75.97 | 3.90 | 75 |
| **C4** | CHINA | 23 | F | 92.02±1.49 | 93.23 | 2.82 | 48 |
| **C5** | CHINA | 23 | F | 100.17±3.19 | 102.66 | 5.95 | 76 |
| **C6** | CHINA | 28 | M | 91.83±3.79 | 95.88 | 2.79 | 82 |
| **C7** | CHINA | 22 | F | 58.23±3.20 | 59.75 | 4.19 | 61 |
| **C8** | CHINA | 24 | F | 65.27±5.10 | 70.77 | 4.93 | 52 |
| **C9** | CHINA | 24 | F | 47.88±8.73 | 56.38 | 4.48 | 36 |
| **C10** | CHINA | 23 | F | 80.03±3.62 | 81.93 | 2.68 | 55 |
| **C11** | CHINA | 23 | F | 91.34±6.53 | 96.74 | 1.97 | 17 |
| **C12** | CHINA | 24 | M | 94.97±4.39 | 98.02 | 1.73 | 56 |
| **C13** | CHINA | 22 | F | 91.53±4.41 | 96.27 | 2.27 | 91 |
| **C14** | CHINA | 23 | M | 111.00±4.95 | 113.95 | 4.79 | 44 |
| **C15** | CHINA | 22 | M | 72.61±2.52 | 111.18 | 2.73 | 94 |
| **E1** | EUROPE | 23 | M | 120.97±3.66 | 126.73 | 3.47 | 52 |
| **E2** | EUROPE | 24 | M | 114.41±3.20 | 117.42 | 2.67 | 40 |
| **E3** | EUROPE | 22 | F | 66.37±3.19 | 70.02 | 2.53 | 31 |
| **E4** | EUROPE | 23 | M | 92.57±2.44 | 98.63 | 5.62 | 60 |
| **E5** | EUROPE | 25 | M | 98.43±2.44 | 100.04 | 3.98 | 21 |
| **E6** | EUROPE | 25 | M | 134.97±2.28 | 136.58 | 5.65 | 58 |
| **E7** | EUROPE | 24 | M | 92.30±11.80 | 102.71 | 3.23 | 72 |
| **E8** | EUROPE | 25 | F | 79.18±1.43 | 80.83 | 3.12 | 68 |
| **E9** | EUROPE | 23 | F | 74.13±3.62 | 78.24 | 7.14 | 51 |
| **E10** | EUROPE | 24 | F | 85.08±2.72 | 86.86 | 3.26 | 41 |
| **E11** | EUROPE | 23 | M | 89.81±5.02 | 93.36 | 3.18 | NA |
| **E12** | EUROPE | 24 | F | 72.08±3.79 | 74.28 | 4.03 | 39 |
| **E13** | EUROPE | 22 | M | 91.83±5.45 | 106.36 | 4.61 | 84 |
| **E14** | EUROPE | 23 | M | 97.08±4.50 | 102.00 | 9.18 | 56 |
| **E15** | EUROPE | 26 | M | 90.7±2.34 | 93.29 | 1.77 | 62 |

**Table S7.2:** Mass peaks list obtained by PTR-MS analysis. Mass peaks in bold identify compounds possibly related to aroma compounds contained in mint essential oil. * highlights mass peaks associated to aroma compounds emitted by humans.

| Measured mass (m/z) | Theoretical mass | Chemical Formula | Tentative identification | Reference |
|---|---|---|---|---|
| 41.038 | 41.0391 | C3H5+ | Alkyl fragm alcohol | (S Yener et al., 2014) |
| 43.017 | 43.0184 | C2H3O+ | Common fragment | (S Yener et al., 2014) |
| 44.996* | 44.9971 | CO2H+ | Carbon dioxide | (Moser et al., 2005) |
| 45.033 | 45.0340 | C2H4OH+ | Acetaldehyde | (Heenan et al., 2012; Pedrotti et al., n.d.) |
| 60.052* | 60.0525 | C2[13]CH6OH+ | 2-propanone isotope | (Heenan et al., 2012; Moser et al., 2005) |
| **67.054** | 67.0547 | C5H7+ | Terpene fragment | (Sine Yener et al., 2015) |
| 69.069* | 69.0704 | C5H8H+ | Isoprene | (Moser et al., 2005) |
| 75.044 | 75.0440 | C3H6OH+ | Methyl-acetate/acetol | (Charles et al., 2015a) |
| 77.059 | 77.0597 | C3H8O2H+ | | |
| 79.054 | 79.0542 | C6H7+ | Benzene | (Vita et al., 2015) |
| **81.068** | 81.0699 | C6H9+ | Fragment of menthyl acetate, 1-8 cineole and monterpenes | (Heenan et al., 2012; Masi et al., 2017) |
| 82.067 | 82.0651 | C5H7NH+ | 1-methylpyrrole | |
| **83.085** | 83.086 | C6H11+ | Fragment of menthyl acetate / fragment of menthol | |
| **93.069** | 93.0699 | C7H9+ | Fragment of monoterpenes | (Masi et al., 2017; Tani, Hayward, & Hewitt, 2003) |
| **95.086** | 95.0855 | C7H11+ | Fragment of monoterpenes | (Masi et al., 2017; Tani, Hayward, & Hewitt, 2003) |
| 97.101 | 97.1012 | C7H13+ | Alkyl fragment | (Aprea et al., 2015) |
| 108.967 | | | | |
| **109.101** | 109.1011 | C8H13+ | Terpene fragment | (Masi et al., 2017) |
| 112.096 | | | Unknown fragment | |
| **123.118** | 123.1168 | C9H15+ | Sesquiterpene fragments | (Masi et al., 2017) |
| **137.134** | 137.1324 | C10H16H+ | Mix of mononoterpenes | (Maleknia et al., 2007; Masi et al., 2017; Tani et al., 2003) |

| | | | | |
|---|---|---|---|---|
| **139.148** | 139.1487 | C10H19+ (dehydration) | (-) Menthol / fragment of menthyl acetate | (Davidson et al., 1999b; Heenan et al., 2012; Španěl & Smith, 1998) |
| **151.113** | 151.1123 | C10H14OH+ | Menthofuran | (Kumar et al., 2014; Masi et al., 2017) |
| **153.130** | 153.1274 | C10H16OH+ | Carveol | (Maleknia et al., 2007) |
| **155.141** | 155.1436 | C10H18OH+ | 1-8 cineole / menthone | (Heenan et al., 2012; Masi et al., 2017; Steeghs, 2004) |

**Table S7.3:** Welch's t-test p values. For each time interval Chinese and European and the total of males and females are compared for the aroma release of peak m/z 95.086. * shows time bins that reported a p.value<.01 for the origin effect while ^ indicates the one that showed a significant difference for the gender variable.

| Time intervals | Mean Chinese [ppb] | Mean European [ppb] | Mean Female [ppb] | Mean Male [ppb] |
|---|---|---|---|---|
| **0-20 sec** | 2.73 ± 1.49 | 3.32 ± 2.38 | 3 ± 2.19 | 3.04 ± 1.79 |
| **20-40sec** | 6.73 ± 4 | 6.06 ± 3 | 6.66 ± 3.7 | 6.14 ± 3.4 |
| **40-60sec** | 7.58 ± 3.95 | 6.4 ± 3.61 | 7.91 ± 4.24 | 6.11 ± 3.13 |
| **60-80sec** | 8.24 ± 4.4 | 6.74 ± 3.8 | 7.87 ± 4.69 | 7.14 ± 3.59 |
| **80-100sec*** | 8.04 ± 4.01 | 5.85 ± 2.69 | 7.88 ± 3.92 | 6.06 ± 2.98 |
| **100-120sec*** | 8.11 ± 4.68 | 5.71 ± 2.87 | 7.51 ± 4.02 | 6.36 ± 4.06 |
| **120-140sec*** | 9.39 ± 3.84 | 6.57 ± 3.6 | 8.75 ± 3.95 | 7.26 ± 3.89 |
| **140-160sec*** | 8.84 ± 4.95 | 5.55 ± 2.45 | 7.92 ± 3.91 | 6.53 ± 4.47 |
| **160-180sec*** | 8.47 ± 3.97 | 5.58 ± 2.5 | 7.65 ± 3.59 | 6.46 ± 3.58 |
| **180-200sec*** | 9.48 ± 5.56 | 6.32 ± 3.51 | 8.81 ± 4.81 | 7.05 ± 4.89 |
| **200-220sec*** | 8.69 ± 4.32 | 5.69 ± 2.51 | 8.21 ± 4.37 | 6.24 ± 2.97 |
| **220-240sec*** | 7.5 ± 4.18 | 5.31 ± 2.57 | 7.43 ± 4.22 | 5.44 ± 2.65 |
| **240-260sec*** | 7.9 ± 3.94 | 5.53 ± 2.17 | 7.51 ± 3.93 | 5.97 ± 2.59 |
| **260-280sec*** | 8.05 ± 4.9 | 5.56 ± 2.2 | 7.65 ± 4.68 | 6.01 ± 3.03 |
| **280-300sec*** | 7.92 ± 3.86 | 5.45 ± 2.45 | 7.38 ± 3.86 | 6.04 ± 2.91 |
| **300-320sec*^** | 9.87 ± 5.01 | 5.97 ± 2.72 | 9.4 ± 4.92 | 6.53 ± 3.5 |
| **320-340sec*^** | 8.79 ± 4.34 | 5.38 ± 2.46 | 8.42 ± 4.44 | 5.83 ± 2.83 |
| **340-360sec*** | 8.17 ± 4.5 | 5.58 ± 2.9 | 7.8 ± 4.53 | 6.01 ± 3.2 |
| **360-380sec*^** | 8.2 ± 4.6 | 4.63 ± 2.36 | 7.66 ± 4.74 | 5.25 ± 2.86 |
| **380-400sec*** | 8.53 ± 5.01 | 5.2 ± 2.42 | 7.84 ± 4.63 | 5.97 ± 3.71 |
| **400-420sec*** | 8.61 ± 4.63 | 5.49 ± 2.44 | 8 ± 4.46 | 6.17 ± 3.33 |
| **420-440sec*^** | 9.3 ± 4.3 | 6.52 ± 4.42 | 9.64 ± 4.62 | 6.26 ± 3.86 |
| | **Gum out** | | | |
| **440-460sec*** | 6.19 ± 3.16 | 3.94 ± 2.85 | 5.68 ± 3.28 | 4.49 ± 3.05 |
| **460-480sec** | 2.72 ± 1.02 | 2.05 ± 1.59 | 2.36 ± 1.07 | 2.42 ± 1.61 |
| **480-500sec** | 2.08 ± 1.14 | 1.63 ± 0.72 | 1.9 ± 1.18 | 1.82 ± 0.73 |

**Table S7.4:** Welch's t-test p values. For each time interval Chinese and European and the total of males and females are compared for the aroma release of peak m/z 139.148. * shows time bins that reported a p.value<.01 for the origin effect while ^ indicates the one that showed a significant difference for the gender variable.

| Time intervals | Mean Chinese [ppb] | Mean European [ppb] | Mean Female [ppb] | Mean Male [ppb] |
|---|---|---|---|---|
| **0-20 sec** | 0.22 ± 0.1 | 0.27 ± 0.19 | 0.22 ± 0.12 | 0.27 ± 0.17 |
| **20-40sec** | 0.61 ± 0.34 | 0.62 ± 0.31 | 0.63 ± 0.35 | 0.61 ± 0.3 |
| **40-60sec** | 0.81 ± 0.37 | 0.77 ± 0.34 | 0.84 ± 0.37 | 0.74 ± 0.34 |
| **60-80sec** | 0.91 ± 0.44 | 0.8 ± 0.36 | 0.9 ± 0.47 | 0.82 ± 0.34 |
| **80-100sec** | 0.96 ± 0.45 | 0.81 ± 0.35 | 0.96 ± 0.46 | 0.81 ± 0.34 |
| **100-120sec** | 0.97 ± 0.45 | 0.76 ± 0.33 | 0.92 ± 0.42 | 0.81 ± 0.38 |
| **120-140sec*** | 1.11 ± 0.43 | 0.83 ± 0.38 | 1.05 ± 0.42 | 0.89 ± 0.42 |
| **140-160sec*** | 1.07 ± 0.47 | 0.77 ± 0.33 | 1 ± 0.45 | 0.85 ± 0.41 |
| **160-180sec*** | 1.06 ± 0.43 | 0.8 ± 0.36 | 0.98 ± 0.39 | 0.88 ± 0.44 |
| **180-200sec*** | 1.11 ± 0.52 | 0.84 ± 0.34 | 1.07 ± 0.5 | 0.89 ± 0.41 |
| **200-220sec*** | 1.11 ± 0.49 | 0.84 ± 0.34 | 1.06 ± 0.5 | 0.9 ± 0.36 |
| **220-240sec** | 1.02 ± 0.48 | 0.78 ± 0.37 | 0.99 ± 0.48 | 0.83 ± 0.39 |
| **240-260sec** | 1.04 ± 0.5 | 0.79 ± 0.29 | 1.01 ± 0.5 | 0.82 ± 0.33 |
| **260-280sec** | 1.05 ± 0.52 | 0.8 ± 0.31 | 1 ± 0.51 | 0.86 ± 0.35 |
| **280-300sec*** | 1.05 ± 0.47 | 0.8 ± 0.33 | 1 ± 0.48 | 0.85 ± 0.36 |
| **300-320sec*** | 1.19 ± 0.51 | 0.84 ± 0.34 | 1.14 ± 0.52 | 0.89 ± 0.37 |
| **320-340sec*** | 1.21 ± 0.54 | 0.84 ± 0.38 | 1.17 ± 0.57 | 0.89 ± 0.38 |
| **340-360sec*** | 1.15 ± 0.55 | 0.8 ± 0.38 | 1.08 ± 0.57 | 0.88 ± 0.41 |
| **360-380sec*** | 1.09 ± 0.56 | 0.76 ± 0.4 | 1.06 ± 0.6 | 0.79 ± 0.37 |
| **380-400sec*** | 1.17 ± 0.61 | 0.78 ± 0.36 | 1.12 ± 0.62 | 0.84 ± 0.4 |
| **400-420sec*** | 1.12 ± 0.52 | 0.81 ± 0.35 | 1.09 ± 0.54 | 0.85 ± 0.36 |
| **420-440sec*^** | 1.22 ± 0.46 | 0.86 ± 0.4 | 1.22 ± 0.47 | 0.87 ± 0.39 |
| | **Gum out** | | | |
| **440-460sec*^** | 1.1 ± 0.49 | 0.75 ± 0.34 | 1.07 ± 0.5 | 0.79 ± 0.36 |
| **460-480sec** | 0.61 ± 0.25 | 0.48 ± 0.32 | 0.57 ± 0.31 | 0.52 ± 0.27 |
| **480-500sec** | 0.46 ± 0.23 | 0.36 ± 0.2 | 0.42 ± 0.23 | 0.41 ± 0.22 |

**Table S7.5:** Welch's t-test p values. For each time interval Chinese and European and the total of males and females are compared for the aroma release of peak m/z 151.113. * shows time bins that reported a p.value<.01 for the origin effect while ^ indicates the one that showed a significant difference for the gender variable.

| Time intervals | Mean Chinese [ppb] | Mean European [ppb] | Mean Female [ppb] | Mean Male [ppb] |
|---|---|---|---|---|
| **0-20 sec** | 2.84 ± 2.15 | 3.09 ± 3.05 | 3.32 ± 3.24 | 2.61 ± 1.8 |
| **20-40sec** | 6.16 ± 4.18 | 4.69 ± 3.03 | 6.03 ± 3.79 | 4.85 ± 3.59 |
| **40-60sec^** | 6.27 ± 3.98 | 4.26 ± 3.57 | 6.45 ± 4.25 | 4.14 ± 3.16 |
| **60-80sec** | 6.44 ± 3.93 | 4.36 ± 3.39 | 5.89 ± 4.01 | 4.95 ± 3.57 |
| **80-100sec*^** | 5.76 ± 3.27 | 3.42 ± 1.85 | 5.47 ± 3.01 | 3.76 ± 2.55 |
| **100-120sec*** | 5.71 ± 4.03 | 3.43 ± 2.47 | 5.14 ± 3.35 | 4.04 ± 3.65 |
| **120-140sec*** | 6.3 ± 3.26 | 4.24 ± 3.3 | 5.91 ± 3.31 | 4.67 ± 3.46 |
| **140-160sec*** | 6.2 ± 4.45 | 3.11 ± 1.92 | 5.29 ± 3.03 | 4.08 ± 4.31 |
| **160-180sec*** | 5.58 ± 3.49 | 2.96 ± 1.73 | 4.84 ± 3.04 | 3.75 ± 2.99 |
| **180-200sec*** | 6.56 ± 4.89 | 3.8 ± 3.58 | 5.82 ± 3.86 | 4.59 ± 4.99 |
| **200-220sec*** | 5.41 ± 3.31 | 2.99 ± 1.89 | 4.94 ± 3.24 | 3.5 ± 2.48 |
| **220-240sec*^** | 4.56 ± 3.15 | 2.66 ± 1.61 | 4.45 ± 3.01 | 2.81 ± 2.01 |
| **240-260sec*** | 5.2 ± 3.4 | 2.77 ± 1.38 | 4.79 ± 3.36 | 3.23 ± 2.04 |
| **260-280sec*** | 4.97 ± 3.69 | 2.91 ± 1.7 | 4.64 ± 3.36 | 3.28 ± 2.58 |
| **280-300sec*** | 4.66 ± 2.76 | 2.67 ± 1.49 | 4.1 ± 2.52 | 3.26 ± 2.29 |
| **300-320sec*** | 6.49 ± 4.33 | 3.18 ± 2.41 | 6 ± 3.92 | 3.75 ± 3.53 |
| **320-340sec*^** | 5.28 ± 3.24 | 2.65 ± 1.54 | 4.95 ± 3.08 | 3.04 ± 2.27 |
| **340-360sec*** | 4.95 ± 3.35 | 2.83 ± 2.04 | 4.54 ± 3.14 | 3.28 ± 2.67 |
| **360-380sec*^** | 5.22 ± 3.57 | 2.19 ± 1.37 | 4.75 ± 3.58 | 2.72 ± 2.15 |
| **380-400sec*** | 5.3 ± 3.97 | 2.67 ± 1.7 | 4.82 ± 3.64 | 3.21 ± 2.8 |
| **400-420sec*** | 5.19 ± 3.42 | 2.73 ± 1.68 | 4.67 ± 3.11 | 3.3 ± 2.66 |
| **420-440sec*^** | 5.89 ± 3.56 | 3.72 ± 3.88 | 6.39 ± 4.05 | 3.28 ± 2.98 |
| | **Gum out** | | | |
| **440-460sec*** | 3.39 ± 2.6 | 1.7 ± 2.19 | 3.06 ± 2.72 | 2.07 ± 2.28 |
| **460-480sec** | 0.96 ± 0.71 | 0.66 ± 0.88 | 0.82 ± 0.72 | 0.8 ± 0.89 |
| **480-500sec** | 0.77 ± 1.04 | 0.42 ± 0.22 | 0.72 ± 1.06 | 0.48 ± 0.25 |

Table S7.6: Welch's t-test p values. For each time interval Chinese and European and the total of males and females are compared for the aroma release of peak m/z 155.113. * shows time bins that reported a p.value<.01 for the origin effect while ^ indicates the one that showed a significant difference for the gender variable.

| Time intervals | Mean Chinese [ppb] | Mean European [ppb] | Mean Female [ppb] | Mean Male [ppb] |
|---|---|---|---|---|
| **0-20 sec** | 7.59 ± 6.22 | 9.95 ± 7.97 | 9.51 ± 10.54 | 8.02 ± 7.11 |
| **20-40sec** | 23.79 ± 19.78 | 21.63 ± 13.68 | 24.28 ± 18.7 | 21.22 ± 15.18 |
| **40-60sec^** | 28.01 ± 17.58 | 23.29 ± 15.17 | 30.66 ± 18.66 | 20.84 ± 12.51 |
| **60-80sec** | 31.45 ± 20.66 | 25.92 ± 17.46 | 30.94 ± 21.69 | 26.55 ± 16.46 |
| **80-100sec** | 31.01 ± 18.94 | 22.49 ± 13.6 | 31.7 ± 18.96 | 22.04 ± 13.36 |
| **100-120sec** | 30.36 ± 20.03 | 21.2 ± 13.38 | 29.12 ± 18.04 | 22.64 ± 16.73 |
| **120-140sec** | 37.12 ± 18.63 | 26.26 ± 20.16 | 35.49 ± 20.39 | 28.12 ± 19.24 |
| **140-160sec*** | 33.83 ± 22.96 | 21.85 ± 13.79 | 31.42 ± 19.09 | 24.51 ± 20.15 |
| **160-180sec*** | 31.48 ± 17.04 | 20.86 ± 13.66 | 29.2 ± 15.79 | 23.35 ± 16.4 |
| **180-200sec*** | 36.83 ± 25.42 | 24.36 ± 16.07 | 35.43 ± 22.82 | 26.05 ± 20.61 |
| **200-220sec*** | 33.33 ± 21.04 | 22.42 ± 13.64 | 32.43 ± 21.06 | 23.58 ± 14.57 |
| **220-240sec** | 27.82 ± 18.54 | 19.7 ± 13.83 | 28.23 ± 19.37 | 19.51 ± 12.62 |
| **240-260sec*** | 29.83 ± 19.26 | 20.38 ± 11.28 | 29.26 ± 19.82 | 21.18 ± 11.16 |
| **260-280sec** | 30.26 ± 23.46 | 20.84 ± 11.98 | 29.25 ± 21.98 | 22.06 ± 15.45 |
| **280-300sec*** | 29.14 ± 17.13 | 19.73 ± 11.5 | 28.22 ± 18.04 | 20.87 ± 11.07 |
| **300-320sec*^** | 38.11 ± 23.33 | 22.99 ± 15.23 | 37.64 ± 23.85 | 23.84 ± 15.35 |
| **320-340sec*** | 32.76 ± 19.81 | 21.88 ± 16.85 | 32.85 ± 21.51 | 22.07 ± 14.82 |
| **340-360sec** | 31.19 ± 20.41 | 21.31 ± 16.55 | 31.11 ± 22.3 | 21.63 ± 14.24 |
| **360-380sec*^** | 30.55 ± 22.48 | 16.85 ± 13.8 | 30.14 ± 23.86 | 17.6 ± 12.34 |
| **380-400sec*** | 33.45 ± 23.8 | 19.36 ± 14.05 | 31.96 ± 23.7 | 21.17 ± 15.91 |
| **400-420sec*** | 33.06 ± 21.5 | 20.53 ± 12.48 | 31.81 ± 21.35 | 22.07 ± 14.19 |
| **420-440sec^** | 38.12 ± 23.43 | 26.29 ± 22.03 | 41.77 ± 25.39 | 23.05 ± 16.95 |
| | **Gum out** | | | |
| **440-460sec*** | 23.31 ± 16.45 | 14.01 ± 13.36 | 22.32 ± 17.11 | 15.21 ± 13.33 |
| **460-480sec** | 8.12 ± 4.73 | 5.49 ± 7.45 | 7.01 ± 4.88 | 6.64 ± 7.53 |
| **480-500sec** | 5.62 ± 5.24 | 3.81 ± 2.85 | 5.11 ± 5.23 | 4.34 ± 3.17 |

# 8

# General discussion

## Show me its volatile profile and I will tell you its quality

The quality of raw materials is one of the most important factors which influences the total quality of produced food: high quality products can be produced only from high quality raw materials. Quality control of different raw materials has been evaluated through applications of PTR-MS technology in this thesis in relation to perceived food quality and flavour in particular (Figure 8.1). In the first chapters (Chapter 2-5), a rapid PTR-ToF-MS analysis was used to evaluate different quality aspects of anhydrous milk fat (AMF), lactose free (LF) milk and raw hazelnuts samples through their VOCs fingerprints. Changes in VOCs release during shelf life were investigated, together with the possibility to identify quality biomarkers for both food matrixes and to predict the sensory class established by industrial sensory evaluation. In the last chapters (Chapter 6-7), other aspects of food quality were investigated. In Chapter 6, PTR-QiToF-Ms was coupled to a dynamic sensory method to assess flavour perception and release of composite foods by systematically varying solid carrier food (bread *vs* potatoes), their texture (hard *vs* soft) and condiment fat content and viscosity. The same methodology was used in Chapter 7 to investigate consumers' characteristics like gender, ethnicity and physiological parameters on flavour release and perception of mint chewing gum. The main results and interpretations of this thesis are summarized and discussed in section 8.1 and in Figure 8.1. Then, methodological considerations (section 8.2), suggestions for future research (section 8.3) and main conclusions (section 8.4) are provided.

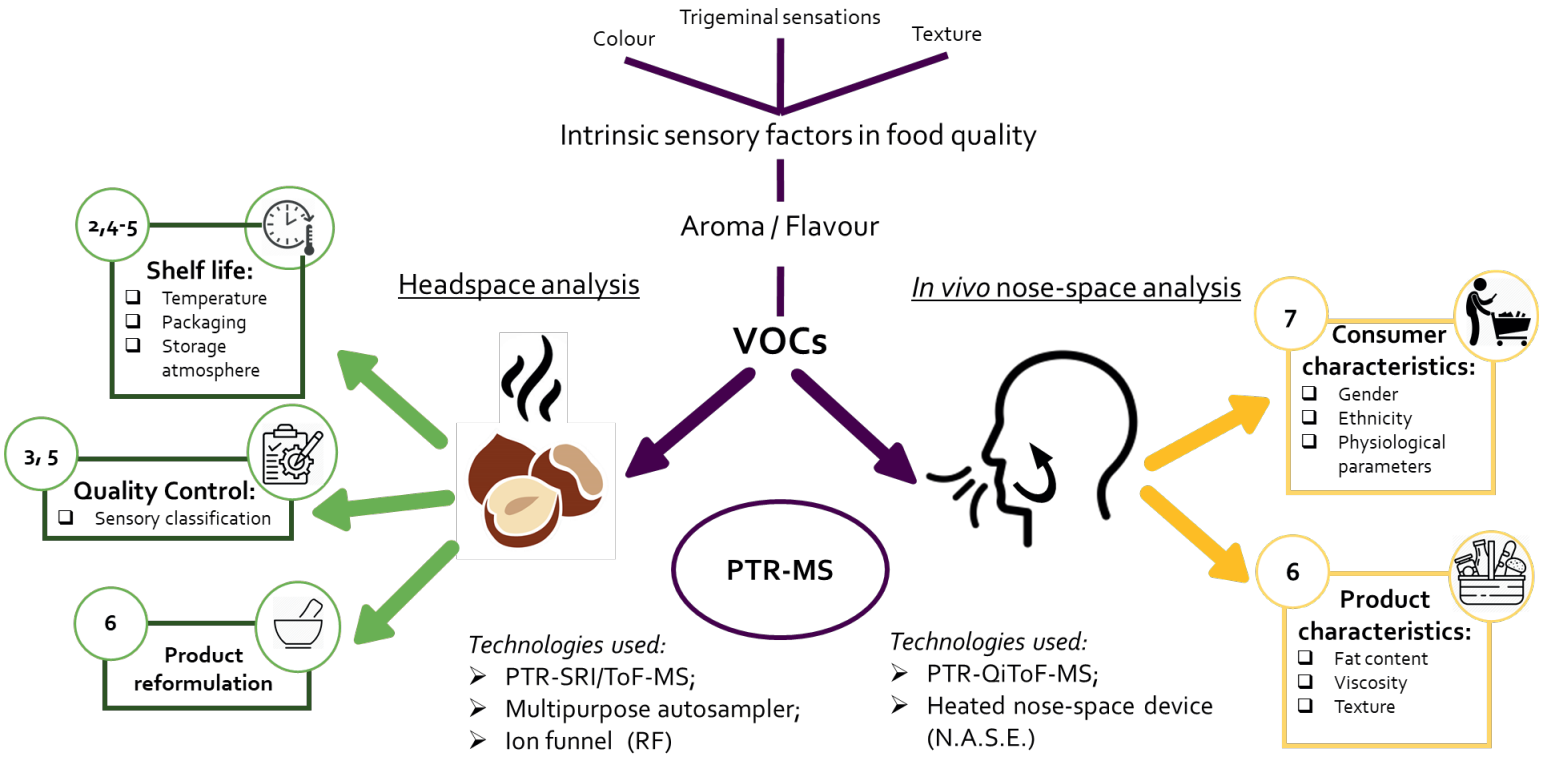



***Figure 8.1:*** *Schematic overview representing the VOCs importance in determining aroma and flavour of food products which is a fundamental part of food quality. This relation was investigated by combining both headspace and in vivo nose-space analysis to understand (i) how changes in food volatilome can give indications about raw materials quality and (ii) the impact of different consumer and product characteristics on aroma release and perception.*

## 8.1 Discussion and interpretations of main results

### 8.1.1 | VOCs fingerprint approach for shelf life monitoring

Shelf life is defined as the time during which the food product will (i) remain safe; (ii) be certain to retain desired sensory, chemical, physical, and microbiological characteristics; (iii) comply with any label declaration of nutritional data; and (iv) be acceptable to the consumer (1). Shelf life of a food product is an essential variable in the food quality function and is therefore of upmost importance to monitor and model it; not only it is important for keeping product acceptable for consumer, but as well it is a key sustainability challenge to reduce both food waste (at the consumer level) and losses (at the other levels of the food supply chain).

The results of this thesis confirm that VOCs are a useful tool for monitoring food products shelf life (Chapters 2, 4 and 5). Considering the food matrixes analysed in this thesis (AMF, UTH LFM, hazelnuts) lipid oxidation is one of the main metabolic processes that can decrease shelf life and impair sensory quality by producing oxidative rancidity (2). Different factors can affect lipid oxidation rate such as lipids composition, temperature which is known to increase exponentially oxidation rate, and exposure to light and oxygen that can induce photo-oxidation and propagate oxidation reactions respectively. While different instrumental analysis are available for monitoring lipid oxidation and oxidation stability like Rancimat, peroxide and acid values, VOCs fingerprinting provides in rapid times information not only about secondary oxidation metabolites, but as well about compounds responsible for product aroma and so perceived quality (3). GC-MS has been extensively applied in previous shelf life studies on both dairies (4–7) and raw hazelnuts (8,9). The results of this thesis (Chapter 2-5) indicate that PTR-ToF-MS fingerprinting is as well a valid sensitive strategy to collect information about VOCs evolution during shelf life at different conditions and to monitor degradation processes which can compromise food quality. Despite its specificity limitation in providing unambiguous identification when compared to GC-MS, PTR-ToF-MS has the advantages of providing a quantification of a much larger spectrum of VOCs with minimal sample preparation and in a much faster way. These advantages were used to evaluate the performance of packaging in preserving quality of AMF during storage for 240 days at 4°C (Chapter 2). AMF is known for its stability and long shelf life when compared to butter (10). Usually, manufactures indicate a shelf life up to 24 months at 4°C, 24 months at -18°C and 10 days at 45°C, the temperature at which AMF is melted for usage in confectionary products. Our approach showed that differences in VOCs emissions between the fresh standard and the stored sample were visible already after 4 months of storage at 4°C and, during storage, these differences increased.

The same approach was applied in Chapter 4 to explore changes in VOCs fingerprints of UHT lactose-free milk during storage at 20°C over a 150 days period. As for AMF (Chapter 2), most mass peaks increased during shelf life, especially the ones related to methyl-ketones like 2-pentanone ($m/z$ = 87.081) and 2-heptanone ($m/z$ = 115.112) due to decarboxylation of β-keto fatty acids or the β-oxidation of free saturated

fatty acids (11). In both milk and AMF, methanethiol, known for contributing to the "cooked flavour" of dairy matrixes (12) decreased during storage as consequence of its oxidation to form dimethyl disulfide and dimethyl trisulfide (13).

In both the experiments (Chapter 2 and 4), the rapidity of the analysis allowed to include more variables in the experimental design as for example batch-to-batch variability. For both AMF and UTH LFM milk differences in the batches were highlighted in the early stage of shelf life. The introduction of this factor, which can greatly enlarge the number of samples to analyse, allows for a better representation of the huge variability that agroindustry needs to cope with along the supply chain.

The analysis rapidity allowed as well to follow VOCs evolution during a test in accelerated conditions where AMF samples were kept at 50°C up to 11 days in glass vials (Chapter 2). Considering that AMF is 99.8% dairy fat and despite its low water content, changes in VOCs are mainly due to lipid oxidation (even if lipolysis may also occur). The accelerated test at 50 °C, not only confirmed the presence of some VOCs previously observed in the headspace of heated butter (14), but it also allowed to explore concentration increase of some oxidation markers such as nonanal and hexanal. These two compounds, produced by oxidation of oleic and linoleic acid, are known to cause flavour defects in dairy products when increasing in concentrations during storage (4,15). Hexanal formation in dairy products was also observed in other two recent PTR-MS studies. While Asaduzzaman and co-workers observed hexanal evolution in milk samples spiked with copper ions during storage at 4°C (15), Beauchamp and colleagues followed in real-time the formation of hexanal and others VOCs induced by milk photo-oxidation (16). The possibility to follow VOCs time evolution in real time opens new interesting scenarios in food science. Not only it will allow to characterize formation of aroma compounds responsible for off-flavours during food degradation processes like oxidation and mould formation, but as well it may be used to investigate formation of key aroma compounds during food processing.

Finally, in Chapter 5, the same approach detected some mass peaks that gave information about harvest year and about the effect of controlled atmosphere ( see Table 5.3 and Figure 5.4). Even if shelf life was not the focus of the experiment the identified pool of mass peaks is a suitable candidate for assessing sample age/freshness that should be further investigated.

In summary, PTR-ToF-MS fingerprinting was shown to be a valid technique for collecting information about VOCs evolution during shelf life at different conditions (*e.g.* packaging, temperature, batch variability, controlled atmosphere) since it was possible to monitor degradation processes which can compromise food quality such as lipid oxidation.



### 8.1.2 | VOCs fingerprint approach to predict raw materials quality and support sensory evaluation

Geographical origin, botanical variety, livestock diet, post-harvest managing, storage, transportation conditions and manufacturing, are only some of the factors that influence the variability of

raw materials received by agroindustry. These factors can significantly affect sensory evaluation and VOCs profile of raw materials and can be transferred along the food chain until the final product. The approach presented in Chapter 3 and 5 deals with these variability elements by analysing industrial sampling of AMF and raw hazelnuts. Our results demonstrate that VOCs fingerprints obtained with PTR/SRI-ToF-MS, coupled with a multipurpose autosampler to enhance analysis standardization and computing power, can be used for supporting sensory quality control in agroindustry. Both unsupervised and supervised data mining approaches were tested to discriminate or build predictive models of sensory classes - obtained from sensory industrial evaluation - from VOCs data. While in supervised data-mining techniques (also known as *predictive*), the known information ("the labels") about the measured samples or from a training set is used to predict or classify other samples, unsupervised techniques (also known as *descriptive*) do not predict a target value or attribute but rather reveal hidden structure and relation among data.

For what concerns AMF (Chapter 3), both unsupervised and supervised data analysis were used to predict the three categories ("AGED", "OK" and "NO") in which samples were divided. The class belonging to the samples that were thermally treated at 50°C for one week was separated from the two other classes due to higher concentration of most of the VOCs. As observed, in Chapter 2, temperature has a relevant effect in increasing oxidation rates and VOCs concentrations. "NO" samples also showed an increase in VOCs associated to oxidative rancidity when compared to "YES" samples, but also presented higher concentrations of other compounds like methanol - probably depending on diet differences (17) – and aromatic hydrocarbons. The latter, could also indicate diet differences (18) but as well could be related to a contamination given by migration of these compounds from the packaging into AMF (Chapter 2). The good quality samples showed a fingerprint with lower concentrations for most of the aroma compounds. The same trend was observed also for raw hazelnuts (Chapter 5) where the good quality samples showed lower concentrations for most of the detected mass peaks in VOCs fingerprints. This is in agreement with sensory analysis results of the industrial partner where the "gold" standard for a raw material should have a flavour as neutral as possible, without any off-notes. Therefore, in our case, industrial raw materials are penalized when they present strong flavours since these could be maintained along the whole supply chain till the product is delivered to consumers.

Despite its limitation as one-dimensional analytical method in providing unambiguous compound identification, both supervised and unsupervised data mining methods on PTR-ToF-MS data showed promising results in discriminating samples classes. While, PLS-DA predictive models on AMF (Chapter 3) showed a correct classification rates of 97% (data from $NO^+$ for the "YES" *vs* "NO" comparison), unsupervised clustering on raw hazelnuts (Chapter 5) had a correct classification rate of 95% in separating conform and non-conform samples (data from $H_3O^+$). Even if food fingerprints obtained from PTR-ToF-MS approach cannot explain on their own the complex processes behind sensory perception, our data, shows that they can predict with good accuracy the industrial quality classification. The reasons behind this good

match may lay in the intrinsic characteristics of the approach which, with its high sensitivity, is able to quantify a wide range of compounds from complex aroma mixtures. While GC-MS approach is able to monitor only a limited number of compounds per round based on long extraction procedures and by column selectivity, PTR-MS, by monitoring minimal changes (ppbV) in VOCs concentration allows to discriminate food samples not only based on key aroma compounds concentrations, but by considering the whole volatilome. Therefore, this thesis suggest that the technique may be applied extensively also for static headspace measurements of volatile mixtures: while GC-MS may be performed to identify specific compounds associated with a given PTR-MS peak (19), PTR-ToF-MS fast fingerprinting could be used to screen quality of a large number of samples of both raw materials and manufactured products. This pre-screening would be beneficial for industrial sensory evaluation: only suspicious defected samples selected by the analysis will be further tested by sensory methods to understand more precisely their quality and decide their destination purpose (see Figure 8.4 and paragraph 8.3 for more details).

Another QC application that was tested in Chapter 5 was the ability of non-invasive PTR-MS fingerprinting to discriminate between raw hazelnuts lots with different levels of gustatory and visual defects. The technique sensitivity was enough to detect significant VOCs variations between samples with 10 and 20% of defected hazelnuts. This approach may be of extreme interest for QC applications: large lots of raw materials may be screened in few seconds without the need of analysing sample portions from each lot. However, the approach should be further validated on larger volumes and with a more accurate mixture range better representing the critical industrial thresholds for QC (*e.g.* 1%, 2%, 5% of defected hazelnuts).

In all these chapters were headspace (HS) analysis was performed, key odorants for each food matrix were detected. It is curious to notice that some of these compounds, which are known for being an essential part of the product aroma, were found with higher concentrations in samples stored for longer times or with a lower quality. For example, in dairies *m/z* 87.081, tentatively identified as a mixture of ketones and aldehydes (2-pentanone;2/3-methylbutanal; pentanal), was found to increase during shelf life (Chapter 2 and 4) and to have a significantly higher concentration in "NO" samples than the one in "YES" samples (Chapter 3). The same was true for the mass peak associated to 3-methyl-5-heptanone which is known to contribute to the *fruity* and *nutty* aroma of raw hazelnuts (20) (Chapter 5). This means that VOCs concentration are critical in determining perceived aroma and so product quality. Determining the relation between odorant concentrations and the overall aroma is an incredibly complicated task and no methodology have been developed yet to quantify the cut-off concentration that a key aroma constituent should have to be recognized as a good quality marker or as an off-flavour. Schieberle and co-workers developed a complex molecular sensory science approach were a combination of techniques including GC-O, aroma extraction dilution analysis (AEDA), stable dilution assays (SIDA) and calculation of OAV are used to identify and quantify concentrations of key odorants (21). These are then used to prepare artificial aroma solutions that mimic the overall aroma of the matrix under investigation and are evaluated through sensory analysis. Their

approach was applied to many food products like hazelnuts (20,22), fermented sausage (23) and honey (24). This has been a breakthrough innovation in the field of molecular sensory science, but is dependent on human evaluation which is inherently a double edge sword since an individual assessor sensitivity may induce significant variability in to perceiving particular aroma substances (25). Moreover, in most cases artificial aroma solutions are made in water or in oils and not in a real food matrix. During food consumption, not only a small percentage of food VOCs reach the olfactory epithelium, but as well the concentrations that are released depend on a variety of intrinsic and extrinsic factors like oral processing behaviours. These variables, together with sensory multimodal interactions, may be underestimated or not taken in consideration when a liquid solution is used. For this reason, measuring *in vivo* aroma release in the nose during real food consumption, coupled with dynamic sensory evaluation, is a promising approach to explore the link between VOCs concentrations in food and sensory aroma perception.

### 8.1.3 | *In vivo* nose-space analysis: product and consumer, two sides of a coin

Chapter 6 and 7 focused on coupling PTR-QiToF-MS and Time Intensity to better investigate the complex relation between aroma release and perception and the impact of product and consumer characteristic on this relation. Understanding how product's characteristics affect VOCs release it is of great interest for agroindustry because it will allow to produce food items that better match consumer expectations. For example, one common dietary trends in the Western world is the reduction of saturated fats, free sugars and salt from diets (26). While this reduction will have a positive impact in the fight against the obesity epidemic and the spread of non-communicable diseases it poses extraordinary challenges at production level. Food industries are challenged in delivering the same or even better quality products by reducing or reformulating their ingredients, which is known to have an impact on product flavour. In this perspective, measuring both aroma perception and release will provide a more complete information on the impact of product reformulation, allowing to build predictive models on specific VOCs release as function of different food properties (*e.g.* viscosity, fat and sugar contents).

In this perspective, Chapter 6 focused on product characteristics. The chapter, investigates how composite foods and their properties affect lemon perception and *in vivo* aroma release of citral and limonene of a standardize trained panel. The study was part of a broader PhD project which focused on the effects of food properties on food oral processing behavior, food intake and sensory perception of composite foods. Characterizing composite foods is gaining interest not only for the increased sensory complexity as the characteristics of one food product influences the flavour release and sensory perceptions of the other food, but also because it is more representative of the natural consumption context (27,28).

In our case, the nose-space PTR-QiToF-MS approach allowed to add the composite foods *in vivo* aroma release characterization. Particularly, when the condiment (mayonnaise) was consumed with the carriers (potato/bread), the cognitive effects play a key role in sensory perception of composite foods. The presence of an accompanying food increased the delivery of aroma compounds into the nasal cavity but did result in

lower perceived sensory intensities. Thus, single foods' flavour perception becomes less intense in the presence of accompanying foods. These findings confirmed that the relation between VOCs release and flavour perception is not linear but different factors play a role in making it complex. In the same study, the condiment (mayonnaise) properties were characterized. Increasing viscosity in mayonnaise led to a decrease in both in-nose aroma release and perception due to reduction in diffusion rate of the aroma compound and to their interaction with xanthan gum (29–31). Interaction of the hydrophobic aroma compound with xanthan gum also led to decreased aroma release and perception for mayonnaise with lower fat content. This, together with the higher number of fat droplets contained by the FF-HV mayonnaise which increased the interfacial area between oil and the saliva continuous aqueous phase, led to higher aroma release and perception (32).

In this experiment, panellists were chosen as homogenous as possible to reduce as much as possible inter-individual variability. Dutch young females were chosen and a precise consumption protocol was provided with a given chewing rhythm (1 chew per sec) and a fixed consumption time. On the contrary, in Chapter 7, to characterize consumers differences and their impact on flavour perception and release, a free mastication protocol was adopted and an ideal food model like chewing gum was chosen. Since the introduction of nose-space measurements an elevated inter-individual variation was noticed, which can be larger than product differences (33,34). The causes of this variation are manifold and have been mostly related to chemical, physiological, psychological and behavioural parameters (35–37). Physiological differences in salivary flow and pH, saliva composition in terms of proteins and microbiota, oral and nasal mucosa, are all factors that were shown to affect aroma release and flavour perception. Behavioural characters like eating rate, mastication, swallowing and breathing also play a role. Most of these factors are changing as a function of the individual. Our findings in Chapter 7 suggests that consumers' origin may affect one or more of these parameters. When compared to Europeans, Chinese panellists showed a higher in nose flavour release for aroma compounds of the chewing gum, together with a higher sweetness and flavour perception. While to a higher perception of mint flavour corresponded a higher aroma release in the nose, the aroma release difference was not explained by physiological parameters considered. Only a mild difference in salivary flow was observed which is known to have a weak negative correlation with flavour release (38). The mechanism underlying the different aroma persistence in the oral and nasal cavity of Chinese and European panellists is not yet clear but we hypothesize that differences in eating behaviour (39,40), oral microbiota (41,42), saliva (43), oral and nasal mucosa composition in terms of receptors and enzymes, driven by different gene expression and diet (44,45), may be partly responsible for these differences.

These findings show that collecting information on how consumers characteristic affect flavour perception and aroma release can play an important role when designing a new product for a specific consumer's segment and for the new emerging field of personalized product design. While aging is known to reduce food intake and different studies showed the link to different factors like a decline in sensory functions (46–

49) and in eating capability – mainly due to changes in salivary flow and composition and in mastication efficiency – (50–52), the reasons behind differences in cross-cultural flavour release and perception are not fully understood. The results of our study - that should be validated by a larger population study and with more attention on cross-cultural studies biases (53) - suggest a difference in both aroma release and perception between Caucasians and Asian panellists, independently from the gender. This information on differences in flavour perception in function of the geographical origin, together with a full understanding of the factors behind it, may be of interest for food industries interest to tailor their products for specific market areas. Particularly, playing on aroma concentrations may be an advantageous strategy to both reduce costs and conquer new markets without requiring more complex product reformulations.
On the other hand, the experimental results of the thesis suggest that, when preparing a nose-space *in vivo* experiment to characterize the flavour release and perception of a food product, special attention should be put in defining the consumption protocol. Moreover, additional participant selection criteria should be introduced to reduce inter-individual variability.

## 8.2 Methodological considerations

### 8.2.1 | Headspace measurements

When considering PTR-MS HS application for quality control purposes, sampling issues may arise due to the high number of samples normally screened by agroindustry. Despite measurement rapidity, manual sampling can be one of the process bottleneck. In this case, the introduction of an automated sampling system for PTR-MS measurements (54) greatly improves the processing power and the measurements stability allowing to perform experiments on large sample sets with an elevated number of replicates (typically 3-5). In this thesis, for all the headspace experiments (Chapter 2-5) a multipurpose sampler for GC (Gerstel, Germany) with temperature controlled trails, a purge tool and a headspace adapter using a conventional syringe-based system to enable gas phase exchanges, were used in combination with PTR-ToF-MS. This introduction allowed for a better measurement standardization with a full automation of sampling processing (54). Samples flushing with nitrogen, storage, incubation for VOCs equilibration and measurement were managed by autosampler, reducing potential sources of variation. In this way, systematic errors introduced by humans can occur only during sample preparation phases (*e.g.* melting, weighting, grinding). Flushing the headspace of each sample with nitrogen should eliminate possible differences introduced during sample preparation and as well prevent oxidation led by oxygen. More importantly, the autosampler allowed to process an extraordinary number of samples in a relatively short time, considering that measurements were performed continually (day and night) on four trails which allowed to load around 128 samples per round. In all four studies more than 3000 samples were analysed. When looking at the raw materials examined in this thesis, the number of variables that could have

influenced samples quality is enormous: manufacturer, seasonality, harvest year, post-harvest management, storage, transportation, cultivar, cow's diet, geographical origin are just some of the factors that may have influenced VOCs fingerprints. Allowing a fast screen of as many samples as possible is the way to have a more complete picture of the extraordinary variability in raw materials quality that food industries need to face. Therefore, for applications in industrial quality control, where an elevated number of samples need to be processed, it is highly recommended to couple PTR-ToF-MS technology with an autosampler.

In Chapter 5 was also introduced the use of an ion funnel for the visual and sensory defects experiments and for some measurements in the blind classification experiment (data not shown). When comparing the measurements performed with the direct current (DC, normal mode) and the radio frequency (RF) of the ion funnel it was noticeable an increase in the detection sensitivity. The RF technology may then be useful when analysing food matrix that have very limited VOCs emissions and for which an increase in detection sensitivity may be crucial (55). Even if for raw hazelnuts this increase in sensitivity may not be essential, in other experiments performed with other food matrixes like skimmed milk powder (data not shown) which has limited VOCs emissions, it significantly improved the analytical information collected.

In Chapter 3 and 5 the selective reagent ionization (SRI) system was extensively applied. While $O_2^+$ ionization mode did not led to any noticeable improvement in terms of VOCs identification and predictive models performance - probably due to increased difficulties in spectra interpretation (56) - $NO^+$ ionization mode data were very useful for both targeted and untargeted approach. As seen in other studies, the possibility to separate isobar compounds (56,57), was used to better investigate ketones and aldehydes contributions to VOCs fingerprints of AMF and raw hazelnuts. Moreover, in Chapter 3 this ionization mode led to the best PLS-DA model in terms of correct classification rates. It is therefore recommended to consider implementation of this ionization mode for quality control purposes.

From the results obtained in this thesis, PTR-ToF-MS fingerprinting approaches are expected to become a very potent tool in quality control of raw materials and for evaluating changes during shelf life. However, as observed for authenticity issues (58), while a lot of effort has been directed in analytical method development, data evaluation in terms of validation and standardization is still under development (59). For PTR-ToF-MS HS measurements different steps are crucial like data extraction, mass selection and statistic evaluations where different approaches can lead to different results. While for data extraction a validated approach was adopted (60) which can partially contribute to noise reduction, for mass selection a novel approach was adopted, described in details in the experimental chapters (Chapter 2-5). Mass peaks selection was noticed to have a relevant impact on the performance of both unsupervised and supervised multivariate data analysis methods. For all the food matrixes, when performing unsupervised PCA and clustering, features selection improved samples separation. The picture was more complicated for what concern supervised PLS-DA (Table 3.3 and Table S3.3). Selecting mass peaks did not always led to better

classification performances as testified by predictive models built with $H_3O^+$ and $NO^+$ ionization modes. In these cases the adopted procedure for reducing mass peaks led to loss of relevant information for sample classes discrimination. On the other hand, the low and mid-level data fusion approaches applied in Chapter 3 did not led to a better classification rate, probably due to the opposite problem: too much noise was introduced in the model. Moreover, the model including all mass peaks from all the ionization modes, due to the elevated number of variables higher than the sample number, is vulnerable to overfitting (61). Therefore, for establishing PTR-MS technology in industrial QC programs an effort should be made to further standardize and validate strategies for generating reliable predictive models.

Finally, it should not be forgotten, that sensory evaluation plays a key role: it is the base on which we build prediction models from VOCs and as well the final evaluation by consumers which, ultimately, will determine if the product will be successful or not. For this reason, it is essential to ensure accurate and repeatable sensory evaluations of products at industrial level. It is useful to remember that, independently from the method used, in many cases, the results lack of validity may be attributable to the defective realization of the test and/or to an incorrect analysis of the information obtained. Predictive models built on ambiguous or not repeatable sensory evaluations are more or less like collecting frogs in a bucket.

Regarding the sensory methods used in this thesis, mainly difference from control and in/out methods were applied by the industrial partner. In both methods a comparison with a golden standard - a premium quality food product for that category – is performed. It is important to guarantee stability for the golden standard over time. For our approach of quality evaluation based on flavour and VOCs would be fundamental to better characterize the "flavour blueprint" defined as the combinatorial code of the entire set of odour- (and taste) active food components in their natural concentrations in the food (21).

### 8.2.2 | The need of standardization for *in vivo* nose-space measurements

#### 8.2.2.1 Instrumentation:

For the *in vivo* nose-space measurements a PTR-QiToF-MS was used in combination with a heated device. From the instrumental point of view, the addition of a quadrupole interface (Qi), like the RF mode, allows an average increase in technique sensitivity by a factor of 25 when compared to PTR-ToF-MS (62). As seen in the introduction (section 1.4), for *in vivo* monitoring of flavour release, sensitivity is a key issue due to the wide range of odour threshold values and the VOCs dilutions occurring during consumption (63). Therefore, increased sensitivity is necessary for *in vivo* applications, especially when measuring real food matrixes not spiked with aroma compounds and with relative low VOCs emissions.

As well, based on observations and results of Chapter 6 and 7, the usage of a heated device for sampling breath is advised. The device has a double advantage. From one side it improves panellists comfortability, which is essential when conducting multiple or long measurements combined with sensory tests. On the other side, the possibility to keep sampling tubes and inlets heated ensure a better transmission of all VOCs. This is of primary importance for sticky compounds as vanillin which condensate in the inlet lines by reducing

sensitivity and extending response time and memory effects (64). In this way, is possible to ensure real online evaluation also for these compounds. A similar effect can be obtained by shortening inlet lines and by directly injecting VOCs into the drift tube without passing through the multiport valve at which usually the inlet line is connected. It is good to mention that, like for other analytical techniques, different materials for the sampling lines and for the instrument components (*e.g.* ion source, drift tube) have been used since the birth of PTR-MS to reduce memory effects and absorption of "sticky" compounds on the instrument surfaces (65). The most common materials for PTR-MS parts, tubing and the heated device were Teflon and silcosteel surface treatment due to their mechanical and chemical properties. Nowadays, polyether ether ketone (PEEK) has also been introduced as tubing material and ongoing research is testing the potentialities of sulfinert coating of stainless steel tubing due to its properties that makes it one of the most inert substrate available for VOCs transfer. A new design of the heated device, including sulfinert treatment, may help in preventing VOCs accumulation in cold zones and improving VOCs transmission by reducing their interactions with materials surface. Moreover, new device designs could be developed to improve participant comfort during experimental sessions. It should not be forgotten that when performing sensory tests, real-life settings are important to closely reproduce the normal way in which we eat (66–68). In our study, a Ionicon nose-space air sampling extension (N.A.S.E.) device was used. However, the ergonomic Teflon® nosepiece device recently developed by Le-Quere and co-workers (69–71) may be a valid alternative which guarantee higher flexibility and comfort at the price of a lower sampling temperature than the N.A.S.E. device (Figure 8.2).

**A) Ergonomic Teflon® nosepiece device** **B) Ionicon nose-space air sampling extension**

***Figure 8.2:*** *Visualization of two different devices for in-nose air collection. On the left is presented the ergonomic Teflon® nosepiece device and a participant (bottom left) performing a TDS test while his breath is sampled (images courtesy of Dr. LeQuere). On the right the N.A.S.E. device is presented. At the bottom right is visible a participant at Edumund Mach Foundation laboratories while performing a TI test on chewing gum while her air is sampled and injected into PTR-MS.*

8.2.2.1 Protocols:

Another main methodological issue in nose-space measurements is protocol standardization. The growing international interest and research effort in nose-space measurement has resulted in a range of diverse sampling and analytical methods. These, united with the high inter (and intra) variability among human subjects, hamper the possibility to compare results and contribute to the lack of replication of research findings. Recently, the "Peppermint-initiative" has been launched and a benchmark study across different organizations in Europe and UK have been conducted to standardize breath experiment for a variety of different techniques (PTR-MS, GC-MS, SIFT-MS, etc.). Each working group performed the peppermint breath experiment with its own methods for sampling and analysis. This was based on sampling breath of 10 participants at regular time intervals after the ingestion of a peppermint oil food supplement capsule which produce a well- characterised perturbation in the VOC breath profile (Figure 8.3).

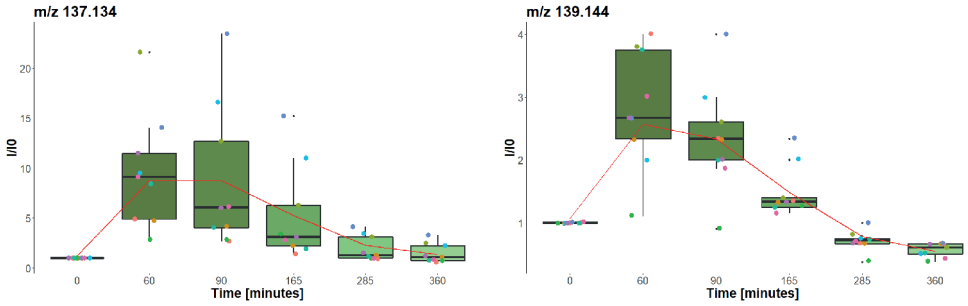



***Figure 8.3:*** *Washout profile for monoterpenes (m/z 137.134) and menthone (m/z 139.144) for the peppermint breath experiment performed by our group. The ratio between the intensity from breath at time $T_n$ (I) and the intensity at $T_o$ ($I_o$) is plotted against time. Normalized count per seconds were used. Participants are characterized using different colors while the continuous red line represents the theoretical average washout profile.*

The study intent was to provide the best available information on the respective analytical performance of different techniques and methods. Starting from this point, it will be possible to provide useful comparative evaluation of different sampling methodologies and instrumental approaches being tested. The final aim is to draft some best practices for each instrument that should be adopted when performing breath experiments. Methods standardization will allow to compare results from different laboratories and in this way, better investigating inter-individual variability (Figure 8.3).

Such common effort for benchmarking and for standardization is still lacking for nose-space measurements. Therefore, special care should be taken when designing nose-space experiments. Based on the results obtained in this thesis, the author elaborated a serious of recommendation. For studies aiming at characterizing product properties impact on aroma release and perception, additional selection criteria should be included for participants recruitment. Selecting participants from only one nationality, one gender and one specific age range may contribute to reduce variability. A normal BMI would also be preferable (44,72,73). As well, running few screening measurements for physiological parameters like oral cavity volume, salivary flow, saliva pH and breathing rate is advised. Screening for participants eating rate may also be beneficial since a standardized consumption protocol with a fixed chewing rate and, when possible, fixed swallowing times are advised. Few studies compared the effect of imposed or free oral processing protocols on aroma release. While Leclerq and Blancher found that only limited advantages were provided by imposing a chewing and swallowing patter for gelled candies (74), Aprea and co-workers observed that an imposed oral processing protocol reduced significantly the high variability connected with the individuals eating behavior (75). Recruiting participants with a similar eating rate to the one prescribed by the chewing protocol would simplify standardization procedures. In addition, it will help to reduce results variability since

eating rate and, more in general eating behaviour is known to influence both aroma release and flavour perception.

Finally, before running experimental sessions, besides the common rules usually adopted when running sensory tests (*e.g.* avoid drinking, eating and perfumes usage for 2 h before sessions), ensuring a common meal or, even better a common diet would help in uniformizing data. Despite is a topic still under investigation, previous researches, together with our results in the peppermint study where we investigated the effect of two different type of breakfasts on the washout profiles (data not shown), indicates that diet may play a role in influencing concentrations of VOCs in human breath (76–78).

On the other hand, when the aim is to investigate the effect of consumers characteristics on aroma release and perception a free oral processing protocol should be adopted to allow individual differences to emerge. As well, the use of an ideal food matrix like chewing gum and jellies or food products spiked with few aroma compounds, is advised.

A conclusive remark on methodologies should be made on sensory dynamic methods. In both Chapter 6 and 7, aroma release and sensory perception were determined simultaneously using PTR-QiToF-MS combined with Time-Intensity (TI) methodology. The advantage of TI is the opportunity to dynamically track the intensity perception of a flavour of interest. A well-known disadvantage of Time-Intensity is the potential occurrence of sensory dumping (79), as participants can score the intensity of one or two specific sensory attribute only and they are not able to report perceived changes in other sensory attributes. Therefore, for complex food matrixes like composite foods, it might be worth investigating the combination of nose-space measurements with other dynamic sensory methodologies. Temporal Dominance of Sensations (TDS) and Temporal Check All That Apply (TCATA) methods allow participants to also report other sensations throughout consumption like for example perceived changes in texture perception. TDS methodology, has been recently coupled to PTR-MS nose-space measurements, allowing to identify compounds related to temporally dominant aroma sensations in a variety of products like candies (80) alcoholic beverages (81,82) and coffee (34,83). Moreover, the combination was applied to assess the effects of bread crumb and crust structure on volatile release and aroma perception during oral processing (84) and the impact on aroma release and perception of size and hardness of fruit pieces in fat-free pear yogurts (85). This method has the advantage to allow evaluation of more attributes (86) which is essential for obtaining a better representation of the flavour dimension which is multidimensional and multimodal.

## 8.3 Future research

This thesis proofs the suitability of PTR-MS technology for different strategic tasks in agroindustry. The technique was mainly used with an untargeted approach but it was also possible to extract information about specific key aroma compounds.

The full integration of the technology into the quality measurement system of the supply chain quality management, requires further studies to consolidate and validate the findings presented in this thesis. For this, it is recommended to run studies on a larger sample-set of each raw material to better represent industrial variability. Samples should be characterized through both sensory and instrumental analysis. Sensory classification should be confirmed by PTR-MS classification and then a subset of samples should be retested in blind through sensory classification to validate PTR-MS classification (Figure 8.4 Phase I). By running a larger study, it would be possible to better characterize the golden standard "VOCs blueprint" used inside the raw materials quality control and to have a larger overview on possible samples off-flavours and defects. In this perspective, it is essential to understand how much VOCs concentrations can vary from the standard before being classified as not conform to industrial quality standards.

From a data analysis point of view, PTR-MS fingerprints need machine learning and data mining methods to extract relevant information from the huge number of spectra obtained. This thesis confirmed the robustness of PLS-DA method as supervised classification method for building prediction models for food (60,87). By training the model with a sufficient number of samples able to cover industrial quality variability, it is reasonable to expect to improve models' predictive power. Other supervised multivariate data mining methods may improve prediction power of PTR-MS fingerprints. However, food industry will benefit more from the development of an automated data analysis system. In this way, once realized the HS measurement, a dedicated software may proceed with data extraction of the whole spectrum or of just a selection of key mass peaks and directly giving the classification results to the operator. With this fast and non-invasive PTR-MS approach, it would be then possible to test a higher number of samples. Only samples discarded by this fully automatized procedure will be tested through industrial sensory evaluation to confirm the defected quality (Figure 8.4, Phase II).

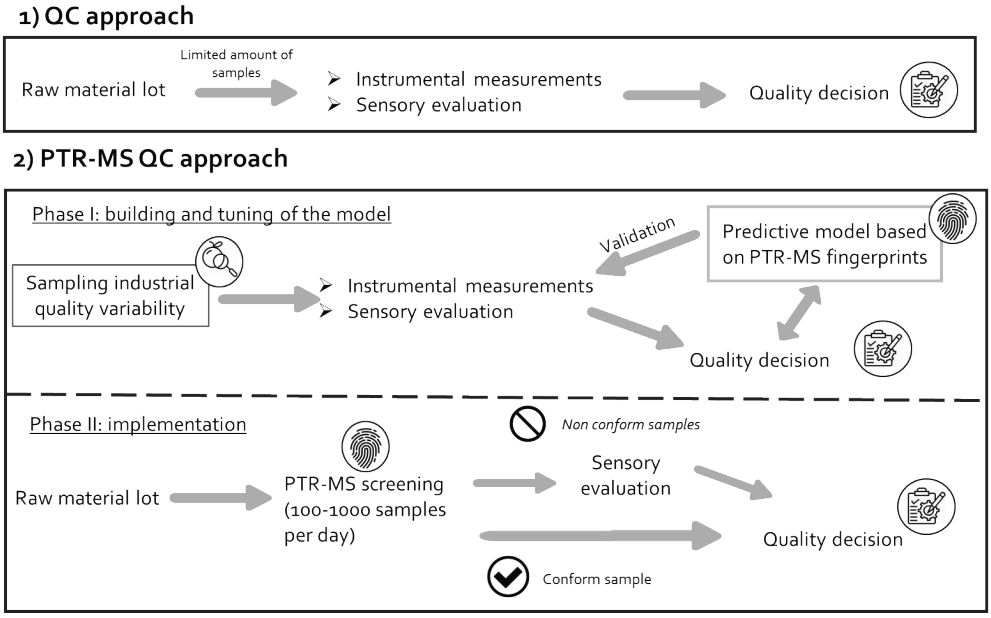



***Figure 8.4:*** *Conceptual framework for raw materials industrial quality control. A conventional QC approach (top) is compared to a PTR-MS approach (bottom). PTR-MS approach consists of a phase 1, where a predictive model based on PTR-MS VOCs fingerprints is built and trained to predict industrial sensory classification of food samples. Mass peaks selection is a fundamental step here. Other instrumental analysis, depending on the type of raw material under investigation could be included for tuning the model. Once the model is set, volatilome analysis with the help of a multipurpose autosampler can be run on large amounts of samples during phase 2. Only the samples that will be classified as non-compliant will undergo sensory evaluation to confirm defected quality.*

For what concerns shelf life evaluations, VOCs information obtained by PTR-MS should be combined with other approaches to better evaluate changes during storage or processing. For example, it would be interesting to combine the key VOCs concentration changes into the integrated approach to shelf life estimation proposed by Achour (88) where a global stability index (GSI) is calculated as a function of time. In this index, different experimental values, each with its weighting factor are combined. Including some key VOCs markers (*.e.g.* oxidation markers), by also indicating their threshold concentrations, may contribute to a more accurate shelf life calculation.

Another important application that take full advantage of the PTR-MS ability of measuring VOCs on-line with high sensitivity, is the on-line monitoring during product manufacturing. The numerous recent researches in this field where PTR-MS was applied to follow coffee roasting (89), milk lactic acid fermentation (90), beer fermentation (91) and volatilomes generated during *S. cerevisiae* growth testify that in the future the technique will be extensively applied to follow VOCs generation during food

transformation. Virtually, our approach has a wide range of possible applications. The use of PTR-ToF-MS fingerprinting could be used in a first stage to develop portable systems for data logging during transportation or storage of highly perishable products like fruit and vegetables. By characterizing the whole volatilome evolution in time in different transportation and storage conditions, it would be possible to select few critical compounds to follow with portable devices like MS-e-noses. These instruments are attractive for food industry for their portability, their relatively fast results able to provide a fingerprint of the sample and the possibility to employ cheap sensors which can be easily integrated in current production processes (92). Despite these features, there are still relatively few applications of MS-e-noses adopted in industry. This could be attributed to the drawbacks in robustness, selectivity and reproducibility of the sensors, the need for pattern recognition algorithms which can cope with the complex signal analysis (93), the tools sensitivity to operation temperatures and humidity (94) and the short sensor life-time. Technological improvements and the possibility to tune these sensors on PTR-MS data may lead to significant innovations in data logging frontiers for post-harvest quality management and monitoring quality changes during transportation and storage.

PTR-MS approach could be applied to follow in details VOCs formation during different processes like roasting, baking, cooking and fermentation. As mentioned, the method has been applied to follow VOCs evolution during coffee roasting but it could be applied to other roasting processes interesting for agroindustry. Roasting of hazelnuts have been characterized with a HS MS-nose approach, by measuring 47 analytes at fixed time intervals (95). PTR-ToF-MS approach to monitor online development of the volatilome could allow to include more variables into the experimental design like more geographical and botanical origins or more harvest years. Moreover, on-line monitoring is a powerful tool for studying dynamics of individual reaction steps like formation of VOCs from Maillard reaction during processing conditions (96–98). However, while performing on-line monitoring during cooking some challenges need to be faced. Usually, during thermal treatments, water evaporation happen which could condensate in the sampling inlet and lead to humidity problems in the drift tube. For this reason, air dilution is usually applied leading to a sensitivity reduction. In this case, the introduction of new generation PTR-MS instruments like PTR-QiToF-MS or the usage of an ion funnel may be ideal to ensure that enough sensitivity is provided to detect volatile aroma fractions.

When it comes to *in vivo* nose-space analysis, this thesis proofs the important role of PTR-MS for understanding factors shaping flavour perception and aroma release. As mentioned in the previous section, future studies should aim at standardizing as much as possible data collection, data extraction and data analysis procedures. When looking at product characterization for industrial perspective, applications of nose-space measurements coupled to sensory analysis is promising in modelling product reformulation effects on flavour perception and aroma release. With the new food trends in reducing sugar, fats and salt in processed products for improving population health, the technique could be a valid support for product

development. When looking at consumer characterization, the technique could be used to better explore inter and intra individual variability. For agroindustry, the possibility to tailor products based on consumers' features opens interesting business opportunities. Cross-cultural population studies with a much larger number of participants should be run to further validate findings on aroma release and flavour perception and investigate variability in saliva proteins, oral microbiota and oral/nasal mucosa.

## 8.4 Main conclusions

This work investigated several applications of PTR-MS in the context of food quality at agroindustry level with a focus on the sensory dimension of flavour/aroma. Considering the very promising results, PTR-ToF-MS, combined with different tools like a multipurpose autosampler, a SRI and an ion funnel, appears as an effective analytical tool for different strategic tasks in agroindustry. Given the multidimensional aspect of quality and the different factors that can affect it, it would be naive to believe that a single technique would be able to cope with such a complex challenge like predicting food quality. However, this study showed PTR-MS potentialities when coupled with other techniques for quality evaluation. First, the technique can be applied for raw materials quality control to support sensory industrial quality evaluation. VOCs fingerprints of raw materials could be obtained in a fast (approx. 1 minute) and non-destructive way and with minimal requirements in terms of sample preparation. These features make the approach able to process an important number of samples, an essential need for agroindustry quality control which works with an elevated turnover of raw materials and products. The predictive models built on PTR-MS analytical information gave promising results in term of samples classification.

Secondly, with the same approach it was possible to monitor VOCs changes during shelf life at different conditions. The effect of packaging, storage atmosphere, batch variability and temperature were tested on different raw materials. The analysis characteristics made possible to include more variable in the experimental designs and as well to monitor more closely changes largely associated to oxidation during accelerated storage conditions at 50°C.

Finally, *in vivo* nose-space measurements with a PTR-QiToF-MS, coupled to TI, were applied to investigate impact of product and consumer characteristics on aroma release and flavour perception during food consumption.

In a global context where the transition towards more sustainable and healthy food systems is essential for reaching the SDGs of the Agenda 2030, technological development can greatly contribute to push forward this transition and, at the same time, can help food chain stakeholders - - to ensure that food quality is not lost during the process from farm to fork. PTR-MS can be used for this purpose by facilitating quality monitoring of both agricultural raw materials and processed food products and help understanding the factors shaping such a complex concept as food quality.

## 8.5 References


1. Earle M, Earle R. Case Studies in Food Product Development. Case Studies in Food Product Development. 2007.
2. Velasco J, Dobarganes C, Márquez-Ruiz G. 1 - Oxidative rancidity in foods and food quality. In: Skibsted LH, Risbo J, Andersen MLBT-CD and PI of F and B, editors. Woodhead Publishing Series in Food Science, Technology and Nutrition [Internet]. Woodhead Publishing; 2010. p. 3–32. Available from: http://www.sciencedirect.com/science/article/pii/B9781845694951500010
3. Biasioli F, Yeretzian C, Gasperi F, Mark TD. PTR-MS monitoring of VOCs and BVOCs in food science and technology. Trac-Trends Anal Chem. 2011;30(7):968–77.
4. Malfia S, Piccinali P, Refiberger B, Badertscher R, Escher F, Schlichtherle-Cerny H. Determination of storage stability of butter enriched with unsaturated fatty acids/conjugated linoleic acids (UFA/CLA) using instrumental and sensory methods. Int Dairy J. 2008;18(10–11):983–93.
5. Bottiroli R, Aprea E, Betta E, Fogliano V, Gasperi F. Application of headspace solid-phase micro-extraction gas chromatography for the assessment of the volatiles profiles of ultra-high temperature hydrolysed-lactose milk during production and storage. Int Dairy J. 2020;
6. Lozano PR, Miracle ER, Krause AJ, Drake M, Cadwallader KR. Effect of cold storage and packaging material on the major aroma components of sweet cream butter. J Agric Food Chem [Internet]. 2007;55(19):7840–6. Available from: http://pubs.acs.org/doi/pdfplus/10.1021/jf071075q
7. Ajmal M, Nadeem M, Imran M, Junaid M. Lipid compositional changes and oxidation status of ultra-high temperature treated Milk. Lipids Health Dis. 2018;
8. Kinderlerer JL, Johnson S. Rancidity in hazelnuts due to volatile aliphatic aldehydes. J Sci Food Agric. 1992;
9. Cialiè Rosso M, Liberto E, Spigolon N, Fontana M, Somenzi M, Bicchi C, et al. Evolution of potent odorants within the volatile metabolome of high-quality hazelnuts (Corylus avellana L.): evaluation by comprehensive two-dimensional gas chromatography coupled with mass spectrometry. Anal Bioanal Chem. 2018;
10. Sichien M, Thienpont N, Fredrick E, Trung Le T, Van Camp J, Dewettinck K. Processing means for milk fat fractionation and production of functional compounds. In: Dairy-Derived Ingredients: Food and Nutraceutical Uses. 2009.
11. Deeth HC, Lewis MJ. High Temperature Processing of Milk and Milk Products. High Temperature Processing of Milk and Milk Products. 2017.
12. de Wit R, Nieuwenhuijse H. Kinetic modelling of the formation of sulphur-containing flavour components during heat-treatment of milk. Int Dairy J. 2008;
13. Weimer BC. Improving the flavour of cheese. Sci Technol [Internet]. 2007;601. Available from: http://books.google.com/books?id=TlJUAQAACAAJ
14. Peterson DG, Reineccius GA. Determination of the aroma impact compounds in heated sweet cream butter. Flavour Fragr J. 2003;18(4):320–4.
15. Asaduzzaman M, Biasioli F, Cosio MS, Schampicchio M. Hexanal as biomarker for milk oxidative stress induced by copper ions. J Dairy Sci [Internet]. 2017;100(3):1650–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28088419
16. Beauchamp J, Zardin E, Silcock P, Bremer PJ. Monitoring photooxidation-induced dynamic changes in the volatile composition of extended shelf life bovine milk by PTR-MS. J Mass Spectrom [Internet]. 2014;49(9):952–8. Available from: http://onlinelibrary.wiley.com/store/10.1002/jms.3430/asset/jms3430.pdf?v=1&t=j5b2bzy9&s=d893aef031c4b635db87264be6644d5634fd39b2
17. Vantcheva ZM, Pradhan K, Hemken RW. Rumen Methanol in Vivo and in Vitro. J Dairy Sci. 2010;53(10):1511–4.
18. Nozière P, Graulet B, Lucas A, Martin B, Grolier P, Doreau M. Carotenoids for ruminants: From forages to dairy

products. Animal Feed Science and Technology. 2006.
19. Joó É, Dewulf J, Demarcke M, Amelynck C, Schoon N, Müller JF, et al. Quantification of interferences in PTR-MS measurements of monoterpene emissions from Fagus sylvatica L. using simultaneous TD-GC-MS measurements. Int J Mass Spectrom. 2010;
20. Kiefl J, Schieberle P. Evaluation of process parameters governing the aroma generation in three hazelnut cultivars (Corylus avellana L.) by correlating quantitative key odorant profiling with sensory evaluation. J Agric Food Chem. 2013;61(22):5236–44.
21. Schieberle P, Hofmann T. Mapping the combinatorial code of food flavors by means of molecular sensory science approach. In: Food Flavors: Chemical, Sensory and Technological Properties. 2011.
22. Burdack-Freitag A, Schieberle P. Characterization of the key odorants in raw italian hazelnuts (Corylus avellana L. var. Tonda Romana) and roasted hazelnut paste by means of molecular sensory science. J Agric Food Chem. 2012;60(20):5057–64.
23. Söllner K, Schieberle P. Decoding the key aroma compounds of a Hungarian-type salami by molecular sensory science approaches. J Agric Food Chem. 2009;
24. Ruisinger B, Schieberle P. Characterization of the key aroma compounds in rape honey by means of the molecular sensory science concept. J Agric Food Chem. 2012;
25. Grosch W. Detection of potent odorants in foods by aroma extract dilution analysis. Trends in Food Science and Technology. 1993.
26. Recanati F, Maughan C, Pedrotti M, Dembska K, Antonelli M. Assessing the role of CAP for more sustainable and healthier food systems in Europe: A literature review. Science of the Total Environment. 2019.
27. Tang J, Larsen DS, Ferguson L, James BJ. Textural Complexity Model Foods Assessed with Instrumental and Sensory Measurements. J Texture Stud. 2017;
28. Scholten E. Composite foods: from structure to sensory perception. Food and Function. 2017.
29. Malone ME, Appelqvist IAM, Norton IT. Oral behaviour of food hydrocolloids and emulsions. Part 1. Lubrication and deposition considerations. In: Food Hydrocolloids. 2003.
30. Milas M, Rinaudo M, Knipper M, Schuppiser JL. Flow and Viscoelastic Properties of Xanthan Gum Solutions. Macromolecules. 1990;
31. Weterings M, Bodnár I, Boom RM, Beyrer M. A classification scheme for interfacial mass transfer and the kinetics of aroma release. Trends in Food Science and Technology. 2019.
32. Tarrega A, Yven C, Semon E, Mielle P, Salles C. Effect of oral physiology parameters on in-mouth aroma compound release using lipoprotein matrices: An in vitro approach. Foods. 2019;
33. Acierno V, Liu N, Alewijn M, Stieger M, van Ruth SM. Which cocoa bean traits persist when eating chocolate? Real-time nosespace analysis by PTR-QiToF-MS. Talanta. 2019;
34. Charles M, Romano A, Yener S, Barnabà M, Navarini L, Märk TD, et al. Understanding flavour perception of espresso coffee by the combination of a dynamic sensory method and in-vivo nosespace analysis. Food Res Int. 2015;69.
35. Buettner A, Beauchamp J. Chemical input - Sensory output: Diverse modes of physiology-flavour interaction. Food Qual Prefer. 2010;
36. Salles C, Chagnon MC, Feron G, Guichard E, Laboure H, Morzel M, et al. In-mouth mechanisms leading to flavor release and perception. Crit Rev Food Sci Nutr. 2011;51(1):67–90.
37. Paulus MP. The breathing conundrum - Interoceptive sensitivity and anxiety. Depress Anxiety. 2013;
38. Haahr AM, Bardow A, Thomsen CE, Jensen SB, Nauntofte B, Bakke M, et al. Release of peppermint flavour compounds from chewing gum: Effect of oral functions. Physiol Behav. 2004;82(2–3):531–40.
39. Ketel EC, de Wijk RA, de Graaf C, Stieger M. Relating oral physiology and anatomy of consumers varying in age, gender and ethnicity to food oral processing behavior. Physiol Behav. 2020;

40. Ketel EC, Aguayo-Mendoza MG, de Wijk RA, de Graaf C, Piqueras-Fiszman B, Stieger M. Age, gender, ethnicity and eating capability influence oral processing behaviour of liquid, semi-solid and solid foods differently. Food Res Int. 2019;
41. Mason MR, Nagaraja HN, Camerlengo T, Joshi V, Kumar PS. Deep Sequencing Identifies Ethnicity-Specific Bacterial Signatures in the Oral Microbiome. 2013;8(10):1–7.
42. Nasidze I, Li J, Quinque D, Tang K, Stoneking M. Global diversity in the human salivary microbiome. 2009;636–43.
43. Canon F, Neiers F, Guichard E. Saliva and flavour perception : perspectives. 2018;
44. Piombino P, Genovese A, Esposito S, Moio L, Cutolo PP, Chambery A, et al. Saliva from obese individuals suppresses the release of aroma compounds from wine. PLoS One. 2014;
45. Méjean C, Morzel M, Neyraud E, Issanchou S, Martin C, Bozonnet S, et al. Salivary composition is associated with liking and usual nutrient intake. PLoS One. 2015;10(9).
46. Kremer S, Bult JHF, Mojet J, Kroeze JHA. Food perception with age and its relationship to pleasantness. Chem Senses. 2007;
47. De Graaf C, Van Staveren W, Burema J. Psychophysical and psychohedonic functions of four common food flavours in elderly subjects. Chem Senses. 1996;
48. Kremer S, Mojet J, Kroeze JHA. Differences in perception of sweet and savoury waffles between elderly and young subjects. Food Qual Prefer. 2007;
49. Hummel T, Kobal G, Gudziol H, Mackay-Sim A. Normative data for the "Sniffin' Sticks" including tests of odor identification, odor discrimination, and olfactory thresholds: An upgrade based on a group of more than 3,000 subjects." Eur Arch Oto-Rhino-Laryngology. 2007;
50. Vandenberghe-Descamps M, Labouré H, Prot A, Septier C, Tournier C, Feron G, et al. Salivary Flow Decreases in Healthy Elderly People Independently of Dental Status and Drug Intake. J Texture Stud. 2016;
51. Fontijn-Tekamp FA, Van Der Bilt A, Abbink JH, Bosman F. Swallowing threshold and masticatory performance in dentate adults. Physiol Behav. 2004;
52. Drewnowski A, Shultz JM. Impact of aging on eating behaviors, food choices, nutrition, and health status. In: Journal of Nutrition, Health and Aging. 2001.
53. Ares G. Methodological issues in cross-cultural sensory and consumer research. Food Qual Prefer. 2018;64:253–63.
54. Capozzi V, Yener S, Khomenko I, Farneti B, Cappellin L, Gasperi F, et al. PTR-ToF-MS coupled with an automated sampling system and tailored data analysis for food studies: Bioprocess monitoring, screening and nose-space analysis. J Vis Exp. 2017;
55. Barber S, Blake RS, White IR, Monks PS, Reich F, Mullock S, et al. Increased sensitivity in proton transfer reaction mass spectrometry by incorporation of a radio frequency ion funnel. Anal Chem. 2012;
56. Yener S, Romano A, Cappellin L, Granitto PM, Aprea E, Navarini L, et al. Tracing coffee origin by direct injection headspace analysis with PTR/SRI-MS. Food Res Int. 2015;69:235–43.
57. Materić D, Lanza M, Sulzer P, Herbig J, Bruhn D, Gauci V, et al. Selective reagent ion-time of flight-mass spectrometry study of six common monoterpenes. Int J Mass Spectrom. 2017;421:40–50.
58. Riedl J, Esslinger S, Fauhl-Hassek C. Review of validation and reporting of non-targeted fingerprinting approaches for food authentication. Analytica Chimica Acta. 2015.
59. Esslinger S, Riedl J, Fauhl-Hassek C. Potential and limitations of non-targeted fingerprinting for authentication of food in official control. Food Res Int. 2014;
60. Cappellin L, Biasioli F, Granitto PM, Schuhfried E, Soukoulis C, Costa F, et al. On data analysis in PTR-TOF-MS: From raw spectra to data mining. Sensors and Actuators B-Chemical. 2011;155(1):183–90.
61. Varmuza K, Filzmoser P. Introduction to Multivariate Statistical Analysis in Chemometrics. Introduction to

Multivariate Statistical Analysis in Chemometrics. 2016.

62. Sulzer P, Hartungen E, Hanel G, Feil S, Winkler K, Mutschlechner P, et al. A Proton Transfer Reaction-Quadrupole interface Time-Of-Flight Mass Spectrometer (PTR-QiTOF): High speed due to extreme sensitivity. Int J Mass Spectrom [Internet]. 2014;368:1–5. Available from: http://www.sciencedirect.com/science/article/pii/S1387380614001584
63. Taylor AJ, Linforth RST. On-Line Monitoring of Flavour Processes. In: Food Flavour Technology: Second Edition. 2010. p. 266–95.
64. Hartungen E, Jürschik S, Jordan A, Edtbauer A, Feil S, Hanel G, et al. Proton transfer reaction-mass spectrometry: Fundamentals, recent advances and applications. In: EPJ Applied Physics. 2013.
65. Pagonis D, Krechmer JE, De Gouw J, Jimenez JL, Ziemann PJ. Effects of gas-wall partitioning in Teflon tubing and instrumentation on time-resolved measurements of gas-phase organic compounds. Atmos Meas Tech. 2017;
66. Kennedy O, Stewart-Knox B, Mitchell P, Thurnham D. The influence of context upon consumer sensory evaluation of chicken-meat quality. Br Food J. 2004;
67. Lawless HT, Heymann H. Sensory evaluation of food : principles and practices [Internet]. 2nd ed. Food science text seriesFood Sensory evaluationChimieScience des matériaux. New York: Springer; 2010. 1 online resource (xxiii, 596 pages). Available from: http://link.springer.com/10.1007/978-1-4419-6488-5
68. Jaeger SR, Hort J, Porcherot C, Ares G, Pecore S, MacFie HJH. Future directions in sensory and consumer science: Four perspectives and audience voting. Food Qual Prefer. 2017;
69. Sémon E, Arvisenet G, Guichard E, Le Quéré JL. Modified proton transfer reaction mass spectrometry (PTR-MS) operating conditions for in vitro and in vivo analysis of wine aroma. J Mass Spectrom. 2018;
70. Arvisenet G, Ballester J, Ayed C, Sémon E, Andriot I, Le Quere JL, et al. Effect of sugar and acid composition, aroma release, and assessment conditions on aroma enhancement by taste in model wines. Food Qual Prefer. 2019;
71. Schlich P, Thomas A, Visalli M, Labarre D, Semon E, Le Quéré J. Collecting and analysing in vivo aroma release and perception by pairing nosespace PTR-ToF-MS and Temporal Dominance of Sensations. In: Flavour Science: Proceedings of the XIV Weurman Flavour Research Symposium. 2015. p. 593.
72. Zijlstra N, Bukman AJ, Mars M, Stafleu A, Ruijschop RMAJ, De Graaf C. Eating behaviour and retro-nasal aroma release in normal-weight and overweight adults: A pilot study. Br J Nutr. 2011;
73. Blanchet L, Smolinska A, Baranska A, Tigchelaar E, Swertz M, Zhernakova A, et al. Factors that influence the volatile organic compound content in human breath. J Breath Res. 2017;
74. Leclercq S, Blancher G. Multimodal sensory integration during sequential eating-linking chewing activity, aroma release, and aroma perception over time. Chem Senses. 2012;37(8):689–700.
75. Aprea E, Biasioli F, Gasperi F, Mark TD, van Ruth S. In vivo monitoring of strawberry flavour release from model custards: effect of texture and oral processing. Flavour Fragr J. 2006;21(1):53–8.
76. Baranska A, Tigchelaar E, Smolinska A, Dallinga JW, Moonen EJC, Dekens JAM, et al. Profile of volatile organic compounds in exhaled breath changes as a result of gluten-free diet. J Breath Res. 2013;
77. Moser B, Bodrogi F, Eibl G, Lechner M, Rieder J, Lirk P. Mass spectrometric profile of exhaled breath - Field study by PTR-MS. Respir Physiol Neurobiol. 2005;145(2–3):295–300.
78. Ajibola OA, Smith D, Španěl P, Ferns GAA. Effects of dietary nutrients on volatile breath metabolites. J Nutr Sci. 2013;
79. Lawless HT, Heymann H. Sensory Evaluation of Food. Elementary Food Science. 2010. 600 p.
80. Déléris I, Saint-Eve A, Dakowski F, Sémon E, Le Quéré JL, Guillemin H, et al. The dynamics of aroma release during consumption of candies of different structures, and relationship with temporal perception. Food Chem. 2011;127(4):1615–24.

81. Fiches G, Saint Eve A, Jourdren S, Déléris I, Brunerie P, Souchon I. Temporality of perception during the consumption of French grape brandies with different aging times in relation with aroma compound release. Flavour Fragr J. 2016;
82. Déléris I, Saint-Eve A, Guo Y, Lieben P, Cypriani ML, Jacquet N, et al. Impact of swallowing on the dynamics of aroma release and perception during the consumption of alcoholic beverages. Chem Senses. 2011;36(8):701–13.
83. Barron D, Pineau N, Matthey-Doret W, Ali S, Sudre J, Germain JC, et al. Impact of crema on the aroma release and the in-mouth sensory perception of espresso coffee. In: Food and Function. 2012.
84. Jourdren S, Masson M, Saint-Eve A, Panouillé M, Blumenthal D, Lejeune P, et al. Effect of Bread Crumb and Crust Structure on the in Vivo Release of Volatiles and the Dynamics of Aroma Perception. J Agric Food Chem. 2017;
85. Mesurolle J, Saint-Eve A, Déléris I, Souchon I. Impact of fruit piece structure in yogurts on the dynamics of aroma release and sensory perception. Molecules. 2013;
86. Varela P, Ares G. Novel techniques in sensory characterization and consumer profiling. Novel Techniques in Sensory Characterization and Consumer Profiling. 2014.
87. Granitto PM, Biasioli F, Aprea E, Mott D, Furlanello C, Märk TD, et al. Rapid and non-destructive identification of strawberry cultivars by direct PTR-MS headspace analysis and data mining techniques. Sensors Actuators, B Chem. 2007;121(2):379–85.
88. Achour M. A new method to assess the quality degradation of food products during storage. J Food Eng. 2006;
89. Yeretzian C, Jordan A, Badoud R, Lindinger W. From the green bean to the cup of coffee: Investigating coffee roasting by on-line monitoring of volatiles. Eur Food Res Technol. 2002;
90. Soukoulis C, Aprea E, Biasioli F, Cappellin L, Schuhfried E, Märk TD, et al. Proton transfer reaction time-of-flight mass spectrometry monitoring of the evolution of volatile compounds during lactic acid fermentation of milk. Rapid Commun Mass Spectrom. 2010;
91. Richter TM, Silcock P, Algarra A, Eyres GT, Capozzi V, Bremer PJ, et al. Evaluation of PTR-ToF-MS as a tool to track the behavior of hop-derived compounds during the fermentation of beer. Food Res Int. 2018;
92. Loutfi A, Coradeschi S, Mani GK, Shankar P, Rayappan JBB. Electronic noses for food quality: A review. Journal of Food Engineering. 2015.
93. Peris M, Escuder-Gilabert L. A 21st century technique for food control: Electronic noses. Analytica Chimica Acta. 2009.
94. Marsili R. Combining Mass Spectrometry and Multivariate Analysis to Make a Reliable and Versatile Electronic Nose. In: Flavor, Fragrance, and Odor Analysis. 2001.
95. Nicolotti L, Cordero C, Bicchi C, Rubiolo P, Sgorbini B, Liberto E. Volatile profiling of high quality hazelnuts (Corylus avellana L.): Chemical indices of roasting. Food Chem. 2013;138(2–3):1723–33.
96. Sivasundaram L, Farhat I, Taylor A. On-Line Monitoring of the Maillard Reaction. In 2003.
97. Pollien P, Lindinger C, Yeretzian C, Blank I. Proton transfer reaction mass spectrometry, a tool for on-line monitoring of acrylamide formation in the headspace of Maillard reaction systems and processed food. Anal Chem. 2003;75(20):5488–94.
98. Cook DJ, Channell GA, Taylor AJ. On line monitoring of acrylamide formation. Adv Exp Med Biol. 2005;

# Appendix

## Summary

**Background and aim:** Food quality is a multidimensional concept which includes both objective and subjective factors. Among these factors, the sensory aspect is gaining more and more attention among consumers and food industry. Monitoring this aspect of quality along the food chain, and in particular the one linked to product flavour, have become essential to ensure competitiveness. In this context, food industries are searching for rapid and flexible instrumental tools to support sensory evaluations of products. Monitoring volatile organic compounds (VOCs) from farm to fork through direct injection mass spectrometry techniques seems a promising possibility. This thesis focuses on the aspects of food quality related to the sensory part of aroma by presenting different applications of proton transfer reaction mass spectrometry (PTR-MS). The technique was applied to monitor VOCs changes during products shelf life, for control and investigation of raw materials' quality and to investigate drivers of aroma release and perception during consumption.

**Material and methods:** PTR-ToF-MS was used in combination with a multipurpose autosampler for obtaining, through a rapid and non-invasive headspace analysis, VOCs fingerprint of different food matrixes like anhydrous milk fat (AMF), ultra-high temperature lactose-free milk (UHT LF milk) and raw hazelnuts. $H_3O^+$ was mainly used as precursor ion for VOCs ionization but by using a selective reagent ionization device (SRI) the possibility to use other ionization ions ($NO^+$ e $O_2^+$) was explored to obtain additional information for compound identifications and for increasing samples discrimination. As well, the introduction of an ion funnel at the end of drift tube, allowed to increase technique sensitivity by improving ions transmission from the drift tube to the mass analyser region. A similar improvement in sensitivity, was obtained when using a PTR-QiToF-MS for *in vivo* nose-space analysis. The instrument was combined with a heated device for sampling participant's breath from nostrils which has a double advantage. On one hand it improves panellists' comfortability and, on the other hand, it improves transmission of all VOCs from the device to the drift tube by reducing VOCs adsorption phenomena on tubing surfaces.
To correlate volatilome to perceived quality, sensory data were also collected. For raw materials quality control industrial sensory evaluation tests were performed, mostly through "A" – "not A" test where samples are compared to an industrial golden standard. For *in vivo* experiments, a Time Intensity method was used to follow aroma perception of panellists. Different type of recruiting criteria where included for participants. When the focus was on characterizing product reformulation in mayonnaise and the effect of combining it with different carrier foods (bread/potato) on aroma perception and release, only Dutch young females were recruited to limit inter-individual variability. For the same reason an eating protocol (fixed chewing time and swallowing) was established. When the focus was on characterizing inter-individual variability, and how gender, ethnicity and physiological parameters affect aroma perception and release, a panel constituted of

Asian-Chinese and Caucasian-Europeans (both males and females) was recruited. No fixed chewing procedure was imposed when consuming a mint chewing gum without any aroma encapsulation and sugar coating.

**Results:** The results of this thesis confirm that PTR-ToF-MS fingerprinting is a valid sensitive approach to collect information about VOCs evolution during shelf life at different conditions and to monitor degradation processes which can compromise food quality such as lipid oxidation. In Chapter 2, the proposed methodology allowed to identify the effect of different production variables on AMF volatilome. Packaging type increased AMF volatilome differences during shelf life at 4°C, while storage at 50°C decreased them due to thermal oxidation phenomena that led to more elevated intensities of aldehydes and (methyl)ketones. Production batches differences also decreased during storage at 50°C. These VOCs differences were highlighted as well in UHT LF milk in Chapter 4. Most mass peaks increased during shelf life and differences in the batches were found in early shelf life stages. In both the experiments (Chapter 2 and 4), the rapidity of the analysis allowed to include and assess simultaneously more variables.

Results from Chapter 3 and 5 demonstrate that VOCs fingerprints obtained with PTR/SRI-ToF-MS, coupled with a multipurpose autosampler to enhance analysis standardization and computing power, can be used for supporting sensory quality control in agroindustry. For both tested raw materials (AMF and raw hazelnuts), the good quality samples had lower concentrations for most of the detected mass peaks in VOCs fingerprints. This is in agreement with sensory results obtained by the industrial partner where the "gold" standard for a raw material is to have a flavour as neutral as possible, without any off-notes. Both unsupervised and supervised data mining approaches gave positive results in discriminating sensory classes. In Chapter 3, PLS-DA predictive models on AMF showed a correct classification rates of 97% in discriminating good quality samples from non-conform samples (data form $NO^+$ ionization mode) while unsupervised clustering on raw hazelnuts (Chapter 5) had a correct classification rate of 95% in separating conform and non-conform samples (data from $H_3O^+$ mode). Therefore, in an industrial quality control program, it will be possible to test the quality of a higher samples number through their volatile fingerprint obtained with fast and non-invasive PTR-MS approach. Industrial sensory evaluation will be performed only on samples discarded by the instrumental measurement to confirm the defected quality. Moreover, in Chapter 5 the approach ability to discriminate between raw hazelnuts lots with different levels of gustatory and visual defects was tested. The technique sensitivity was enough to detect significant VOCs variations between samples with 10 and 20% of defected hazelnuts. Testing for defects in large lots of raw materials in few seconds represent another improvement for industrial quality control programs.

Chapter 6 and 7 demonstrated the potentialities of coupling *in vivo* nose-space analysis with dynamic sensory analysis to better investigate the complex relation between aroma release and perception and the impact of product and consumer characteristic on this relation. In Chapter 6, the nose-space PTR-QiToF-

MS approach allowed to conclude that when consuming composite foods, decrease in sensory intensity perception was not due to a lower delivery of aroma compounds into the nasal cavity, as in-nose aroma release of condiments increased with the presence of a carrier food. Consequently, cognitive effects are likely to play a key role in sensory perception of composite foods. In a global context where agroindustry should contribute to more healthy and sustainable diets by reducing salt, sugars and fats levels in their processed food products, *in vivo* nose-space measurement coupled with sensory analysis is a valuable method for better predicting product reformulations effects.

In Chapter 7, the same approach gave indications that consumers origin may affect aroma release and perception of mint chewing gum. Differences in aroma release could be due to many different factors. Participant's origin (and diets) may be responsible for physiological differences especially in terms of saliva composition and oral/nasal microbiota. If these observations would be validated by larger population studies, they would be of great interest for the new emerging field of personalized product design and nutrition.

**Conclusion:** This thesis, investigated several applications of PTR-MS in the context of food quality at agroindustry level with a focus on the sensory dimension of aroma. When looking at food quality control programs, high throughput VOCs fingerprinting of food samples through PTR-ToF-MS coupled with a multipurpose autosampler, represents a promising approach to select raw materials that will need quality sensory evaluations. The same approach is suitable for monitoring changes during shelf life at different conditions and, more in general for on-line applications during cooking operations. *In vivo* nose-space measurements coupled to sensory dynamic methods is a valid approach to investigate impact of product and consumer characteristics on aroma release and flavour perception during food consumption. In conclusion, PTR-MS approach was proofed to be one example of technological application, which can greatly facilitate the understanding of the factors affecting food quality from agricultural raw materials to market food products.

# Riassunto

**Introduzione:** La qualità alimentare è un concetto multidimensionale che include sia fattori oggettivi che soggettivi. Tra questi fattori, l'aspetto sensoriale sta guadagnando sempre più attenzione sia per i consumatori che per l'industria agro-alimentare. Poter monitorare l'aspetto della qualità lungo la filiera, e in particolare quello legato all'aroma e al sapore del prodotto, è diventato un componente essenziale per garantire la competitività. In questo contesto, le imprese agroalimentari stanno cercando delle tecniche strumentali rapide e flessibili per supportare il controllo della qualità sensoriale dei prodotti. Il monitoraggio dei composti organici volatili (VOCs) dal campo alla tavola tramite tecniche di iniezione dirette di spettrometria di massa sembrerebbe essere una possibilità promettente. Questa tesi si focalizza sugli aspetti della qualità alimentare collegati alla dimensione sensoriale dell'aroma, presentando diverse applicazioni della tecnica di spettrometria di massa con reazione di trasferimento di protone (PTR-MS). La tecnica è stata utilizzata per monitorare i cambiamenti nei livelli dei VOCs durante la conservazione di diversi prodotti agroalimentari, per il controllo e l'investigazione della qualità di materie prime e per indagare i drivers del rilascio dei composti aromatici e della percezione durante il consumo.

**Materiali e metodi:** la tecnica PTR-ToF-MS è stata utilizzata in combinazione con un autocampionatore per ottenere, tramite un'analisi dello spazio di testa rapida e non-invasiva, la composizione in termini di VOCs di diverse matrici alimentari quali burro anidro (AMF), latte senza lattosio UHT e di nocciole crude. Lo ione $H_3O^+$ è stato utilizzato principalmente come ione precursore per la ionizzazione ma, grazie all'impiego di un device per la ionizzazione selettiva (SRI), è stata esplorata la possibilità di utilizzare altri ioni ($NO^+$ e $O_2^+$) al fine di ottenere informazioni addizionali per l'identificazione dei composti e per aumentare l'abilità di discriminazione della tecnica. Inoltre, l'introduzione di un *ion funnel* alla fine della regione di *drift*, ha permesso di incrementare la sensitività della tecnica migliorando la trasmissione degli ioni dalla regione di *drift* a quella di analisi della massa. Un simile miglioramento della sensitività è stato ottenuto utilizzando un PTR-QiTOF-MS per realizzare delle analisi *in vivo*. Lo strumento in questo caso è stato combinato con un dispositivo riscaldato per campionare il respiro dei partecipanti (via retronasale) che offre un doppio vantaggio. Da una parte migliora la comodità del pannellista e, dall'altra, migliora la trasmissione dei VOCs dal dispositivo alla regione di *drift* del PTR-MS, riducendo i fenomeni di adsorbimento e desorbimento dei VOCs sulle linee di trasmissione.

Per correlare il volatiloma con la qualità percepita sono stati raccolti anche dati sensoriali. Per le materie prime sono stati svolti test di valutazione sensoriale industriali per il controllo qualità, la maggior parte tramite test *"A"*-*"non A"* in cui i campioni venivano comparati con degli standard industriali di riferimento. Per gli esperimenti *in vivo*, il metodo *Time Intensity* è stato utilizzato per seguire in tempo reale la percezione dell'aroma, applicando diversi criteri di reclutamento dei partecipanti. Per caratterizzare l'influenza della riformulazione della maionese e della sua combinazione con diversi carrier (pane/patate) sul rilascio dei

composti aromatici e sulla percezione dell'aroma, sono state reclutate solamente giovani donne olandesi per limitare la variabilità del panel. Per la stessa ragione è stato stabilito un preciso protocollo di consumo del prodotto. Per caratterizzare la variabilità inter-individuale e come il genere, l'origine, e i parametri fisiologici influenzassero la percezione e il rilascio aromatico, è stato reclutato un panel costituito da asiatici e caucasici (sia maschi che femmine). In questo caso nessun protocollo di masticazione è stato imposto per il consumo di chewing gum alla menta.

**Risultati:** I risultati di questa tesi confermano che l'analisi rapida (*fingerpriting*) tramite PTR-ToF-MS è un approccio sensibile per raccogliere informazioni sull'evoluzione dei VOCs durante la conservazione di alimenti a diverse condizioni e per monitorare i processi di degradazione che possono compromettere la qualità alimentare come l'irrancidimento tramite ossidazione degli acidi grassi. Nel Capitolo 2, la metodologia proposta ha permesso di identificare l'effetto delle diverse variabili di produzione sul volatiloma del burro anidro. Il tipo di packaging aumenta le differenze nel volatiloma durante la conservazione a 4°C, mentre la conservazione a 50°C le diminuisce per effetto dei fenomeni di ossidazione termica che portano a intensità più elevate di aldeidi e (metil)chetoni. Le differenze tra i lotti di produzione sono pure diminuite durante la conservazione a 50°C. Queste differenze in termini di VOCs sono state evidenziate anche nel latte senza lattosio UTH nel Capitolo 4. La maggior parte dei picchi di massa trovati dall'analisi sono aumentati durante il periodo di conservazione e differenze tra i lotti sono state trovate solamente all'inizio della conservazione. In entrambi gli esperimenti (Capitolo 2 e 4), la rapidità dell'analisi ha permesso di includere e valutare più variabili simultaneamente.
I risultati dei Capitoli 3 e 5 dimostrano che le *fingerprints* dei VOCs ottenute tramite PTR/SRI-ToF-MS accoppiate ad un multicampionatore per aumentare la standardizzazione delle misure e il potere di processamento dei campioni, può essere utilizzata per supportare il controllo qualità sensoriale nell'agroindustria. Per entrambi i materiali testati (burro anidro e nocciole crude), i campioni di buona qualità hanno dimostrato avere concentrazioni più basse per la maggior parte dei picchi di massa rilevati dall'analisi. Questo dato è in accordo con i risultati dei test sensoriali industriali dove lo standard d'eccellenza di qualità per le materie prime è quello di avere un sapore il più neutro possibile, senza alcuna caratteristica sensoriale atipica. Approcci di *data mining* sia uni che multivariati hanno dato risultati promettenti nel discriminare le classi sensoriali dei prodotti testati. Nel Capitolo 3, i modelli predittivi tramite PLS-DA sul burro anidro mostrano un tasso di classificazione corretta del 97% nel distinguere i campioni di buona qualità dai campioni non conformi (dati modalità $NO^+$). I modelli di clustering non supervisionato sulle nocciole crude (Capitolo 5) hanno dato un tasso di corretta classificazione del 95% nel separare i campioni conformi da quelli non accettabili (dati modalità $H_3O^+$). In un programma di controllo qualità industriale, sarebbe quindi possibile testare la qualità di un più alto numero di campioni tramite l'analisi rapida e non invasiva dei composti volatili con l'approccio PTR-MS. La valutazione sensoriale industriale sarebbe svolta solamente sui

campioni segnalati dall'analisi strumentale per confermare il difetto. Inoltre, nel Capitolo 5, è stata testata l'abilità dell'approccio nel discriminare tra lotti di nocciole crude con diversi livelli di difetti sia visivi che gustativi. La sensibilità della tecnica si è dimostrata sufficiente per identificare variazioni significative dei VOCs tra campioni con 10% e 20% di difetto. Poter testare la difettosità di lotti consistenti di materie prime in pochi secondi rappresenta un'innovazione importante per i programmi di controllo qualità industriali.
I Capitoli 6 e 7 dimostrano le potenzialità di accoppiare l'analisi *in vivo* del respiro proveniente dalla via retronasale con metodi sensoriali dinamici per investigare in maniera più completa la relazione complessa tra rilascio dell'aroma e la percezione, e l'impatto che le caratteristiche del prodotto e del consumatore hanno su questa relazione. Nel Capitolo 6, l'analisi *in-vivo* realizzata tramite PTR-QiToF-MS ha permesso di concludere che la diminuzione dell'intensità della percezione sensoriale del condimento durante il consumo di alimenti composti (i.e. carrier + condimento), non è data da un minor rilascio di composti aromatici nelle cavità nasali visto che la presenza dei carrier ha aumentato il rilascio dei composti aromatici dei condimenti. Conseguentemente, è stato ipotizzato che gli effetti cognitivi hanno un ruolo chiave nella percezione sensoriale degli alimenti composti. In un contesto globale dove l'agroindustria deve contribuire alla transizione verso diete più sane e sostenibili anche tramite riformulazioni che prevedano riduzione di sale, zuccheri e grassi nei prodotti processati, le misure *in-vivo* del respiro della via retronasale, accoppiate all'analisi sensoriale, sono un prezioso metodo per predire meglio gli effetti di queste riformulazioni.
Nel Capitolo 7, lo stesso approccio ha fornito indicazioni sul fatto che l'origine del consumatore possa avere un effetto sia sul rilascio dei composti aromatici sia sulla percezione di chewing gum alla menta. Differenze nel rilascio aromatico possano essere state causate da diversi fattori. L'origine dei partecipanti (e le diverse diete associate) possono essere responsabili di differenze fisiologiche, specialmente in termini di composizione della saliva e di microbioma orale e nasale. Se queste osservazioni fossero validate da studi di popolazione, sarebbero di grande interesse per l'emergente campo della nutrizione personalizzata.

**Conclusioni:** Questa tesi ha investigato diverse applicazioni della tecnica PTR-MS nel contesto della qualità alimentare a livello agroindustriale, con un focus sulla dimensione sensoriale dell'aroma. Quando si considerano i programmi di controllo qualità nella filiera agroalimentare, metodi high throughput per ottenere il *fingerprinting* dei VOCs di prodotti alimentari, come la tecnica PTR-ToF-MS accoppiata con un autocampionatore, rappresentano un approccio promettente. Questo metodo può essere utilizzato per selezionare i lotti di materie prime che necessitano di ulteriori controlli di qualità tramite analisi sensoriale. Lo stesso tipo di approccio è valido per monitorare cambiamenti durante la conservazione a diverse condizioni e, più in generale, per applicazioni *on-line* durante il processamento o la cottura degli alimenti. Analisi *in vivo*, combinate con metodi sensoriali dinamici, sono un approccio valido per indagare l'impatto che le caratteristiche del soggetto e del prodotto hanno sul rilascio aromatico e la percezione dei sapori durante il consumo di alimenti. In conclusione, si è dimostrato che l'approccio PTR-MS è un esempio di

applicazione tecnologica che può facilitare la comprensione dei fattori che influenzano la qualità alimentare legati alla percezione e il monitoraggio della qualità a partire dalla materie prime agricole fino ai prodotti alimentari di mercato.

## Acknowledgments

This four years adventure would not have been possible without the help and support of a variety of special people with whom I had the honour and the pleasure of walking together. I was indeed really lucky to be guided in this complicated process but two outstanding supervisors which I will never be able to thanks enough.

**Franco**, you were a very special mentor. I appreciated from the very first moment your informality and your sense of humour. I will be forever in depth for all the scientific knowledge and professional skills you transferred to me and as well for all your patience in managing my uncertainties. You were always ready to welcome me in your office to cheer me up and to give me advise on how to look and analyse the data and to improve my ugly graphs (especially by enlarging the labels!). Not only that but in these years there was space for much more than PTR-MS science: you thought me about mushrooms (even if I know you would like to poison me from time to time), some gardening and we started a music indoctrination (on that I soon realized that my knowledge was too low for your standards) and last but not least the love for good (Trentino) food (especially tortei) and drinks (wine and grappa we like!). You were a wonderful supervisor and I hope one day you will welcome me back in Trentino.
**Vincenzo**, it have been an honour to be supervised by such a great scientist and person. Your comments and your questions on each manuscript and student's project that we have done together improved immensely both the quality of the work and my expertise in the field. I admire your dedication to work, your passion for food science and your ability to manage infinite things and a whole group by keeping an excellent scientific level. Every time I came back to FQD department it was a pleasure to meet and I was proud of the trust you put on me and on my skills since the beginning.

I like to imagine my PhD like a big tree divided between Italy, where I could reconnect with my roots and the Netherlands, where so many different branches have grown in these past years.
I would like to start by the roots, by acknowledging the support of my family to which this thesis is dedicated. Grazie a mia **mamma Marta**. Ancora mi stupisco di come sei riuscita a crescere due figli così bene da sola. La tua forza, la tua saggezza, la tua indipendenza, la tua vitalità, la tua coltivazione dei rapporti umani sono state, sono e sempre saranno d'ispirazione.
Un ringraziamento speciale anche a mio **fratello Giovanni** e il suo indispensabile supporto ed affetto. Il legame fraterno che ci lega é saldo ed indissolubile. Per primo mi hai accompagnato quassù in Olanda agli inizi del mio Master e sempre sei stato presente sia nei momenti di bisogno che ludici. Sapere che ci sei mi rassicura.
Volevo anche ringraziare **Zio Marco e Zia Maria** per avere sopportato dei vicini cosi rumorosi in tutti questi anni, grazie a **Sergio** per avermi offerto la possibilità di stare nella comoda e accogliente casa dei nonni, a

**Leo** per le grigiliate, le zombificate e per introdurmi al magico mondo delle dichiarazioni dei redditi e infine grazie alla cugina **Francesca** per i vari incontri itineranti tra Amsterdam e Italia. Un grazie anche alla **Zia Maria** per tutto l'amore e per le testimonianze di un'altra epoca.

I would like also to express my gratitude to the FEM family, including housemates and colleagues from the sensory group. **Leo**, your passion for (sensory) science, your determination and your attention to details has always inspired me. Not only you have been a wonderful colleague always ready to help and support me but as well a fantastic housemate with whom I shared my passion for good food, trash music and lot of $wag. Everyone knows that together with a Bello Figo there is always a Don Cappuci: **Riki**, thanks for sharing our anxiety about future, life and work. I know that if one day I will be tired of Babylon and (rasta)facturation you will be ready to take your backpack and go for a little trip around the world. I am glad we crossed our pathways both in the Netherlands and in San Michele.
I wish to thank **Iuliia** for her patience and to introduce me to all the secrets of PTR-MS and of data analysis in R: without you it would have been much more complicated!
Thank you **Giulia** and **Carlotta** to animate the Riserva di Pesca and for all the tasty dinners and celebration shared around Italy and the Netherlands.

Together with the FEM group I would also like to recognize the wonderful support, guidance and material I received from the Soremartec group during these years of collaboration. I had a unique chance to get to know many of you and to be surprised by your friendly working environment during our trips to Alba and your visits to FEM. Therefore I would like to express my deep gratitude to **Luca** without whom none of this it would have been possible and to **Matteo** for guiding me through the lakes of butter and the mountains of skimmed milk powder. I would like to express my appreciation as well to **Giuseppe G.** and **Giuseppe C.** for the nice collaboration and the warm welcome in Langhe with homemade Barbera and hazelnuts trees. Thanks as well to **Giacomo, Paolo, Andrea and Claire** with whom we shared so many hours of chewing.

I would also like to extend my thanks to the family **Bettinelli** for all the delicious dinners we had: you are a wonderful example in how to live a good life without too many worries.
I have to give a special thanks to my *Murga Trentinerante* family for all the smiles, parades, festivals and experiences together. Since its birth, I saw it growing strong and every time I enter a *matanza* with you is always an energy explosion. A mention also to *La Savana* and to *Spazio Teatro* with whom I had the chance to grasp the concept of (African) riddim and of the magic of the theatre world.
In these years in Italy my roots have been reinforcing not only in the Alps but as well in my hometown Verona. I am lucky to have another wonderful family of friends there. I would like to express my gratitude to my *fratellino* **Marcello**. I am always surprised and disappointed on how it is difficult to find some time to pass

together despite of the huge feelings and the pleasantness of our relation. I wish the time for our travel to discover our Argentinian roots will come soon.
A big thank you and a hug to all the friends from the Fratellanza and to the Cumpa which were always ready to welcome me back for some climbing or just chatting in front of good food and wine.

Between the roots and the branches there is a solid trunk composed by many wonderful people who have been part of both my two words. Among them I would like to big up my friend and colleague **Zok**. It was a very nice surprise to meet you in FEM again after high school and your visit in the North. Comrade of many coloured adventures and music I am glad to have you meet again and that you we are now both here in the North together. I have also to give a big up to my friend **Gabri,** with whom we shared all our pains of love and uncertainties about life which usually get better after our delicious fish dinners cheered by reggae music. Thanks to **Giacomo&Cate** for welcoming me in Wageningen, in Umbria and in Trentino: your choice of entrepreneurship is admirable and I wish you a lot of success!
There in the North I was lucky enough also to visit one of those friends that I hope it will always stay near and real. **Fenz,** let's go for more hiking or climbing around Europe together! A big thank also to **Sine** which I firstly met in FEM and is now one of my closest colleague and friend in the little village.
Recently, a new bark layer was added at this big tree, a layer with a pleasant smell of all the adventures and the places we have been through together in the last year… **Fra,** I am really glad I've met you during those days in Paris and that we were finally able to find the right time and space to let us explore ourselves! I would like to acknowledge all your precious support (both practical and mental) during the whole delivery process of this thesis: from the writing process during the lockdown till the printing at the beginning of this new trip together in the Netherlands! Without you it would not have been possible and I wish a smooth sailing for this years ahead together!

Finally comes the little special Dutch village. I arrived there the first time in 2013 for my MSc in a very difficult and confused moment of my life. Since then many new branches have been established and I lost count of the many amazing people I met.
I would like to gratefully acknowledge everyone within the **Food Quality and Design group** which always welcomed me kindly. I would firstly like to thank the students I supervised for all the their committed work, especially **Andrea** and **Kira** for their wonderful performances: without your help my PhD would not have been the same. Thanks **Corine and Kimberley** for all the support provided and for answering all my stupid questions. **Vale,** it has been a wonderful and honest friendly relation: I enjoyed so much all out moment and our thoughts and discussion about love, confusion and life's choices. You were and are one of my landmark here in Wageningen. I am very grateful to **Arianne** for the wonderful and fruitful collaboration we had. It

was a pleasure to work together and to get to know you better. I just regret we didn't link already during our studies and I hope we will stay in touch for some more science, acro and climbing together.
I am grateful to **Antonella** for welcoming me and giving me a complete overview on the whole process of closing and becoming a doctor in the Netherlands: I hope life is bringing you lot of cerveja and felicidade. Big up also to **Charlie** for her amazing power and energy: your smile is pure dynamite!
If Wageningen is a bubble, Droevendaal is a little inner bubble where time flows in a different way. I would like to thanks this unique community and all the houses that hosted me during my visits in these years (31, the Republic of 91, 95, KoffieBarrak, Weppa, 101 and 107) . I am particularly grateful to **105** which firstly welcomed in during my MSc and accepted me back for closing my PhD. Thanks as well to the Farm's folks for hosting me there and for the wonderful festivals and parties there!
Fi sure I&I give thanks to Soup Selecta aka **chef Ivo** - that recently opened his mighty baking boutique - alongside **Chef Culorgia** with whom I shared my passion for cooking and mixing dubby vibez: Jah Bless! Big up to crazy puerco **Max** and his infinite energy and passion for life, music and science. Thank to **Ale,** a new recent branch which I was very glad to establish: I am sure that in these following years we will be able to develop new brilliant theories on capitalism and food systems. Thanks to **Caremen** for sharing our (VOCs) passions.
A special dedication also to all *Los Barrakantes*: it has not always been easy but every time the energy that we gather is unique!

Finally I would like to give thanks to all the soundsystems and all the musical artists who enlighten my PhD time… even if I lost hope in getting better in DJing (in both classic and roots sense) I always enjoyed and regenerate while skanking in irie sessions.

*" A tree without roots cannot live*
*But a tree without branches shall not grow"*
*Pupajim*

## About the author

Michele Pedrotti was born in Verona (Italy) on 26 July 1991. He started his superior education with a Bachelor in Biomolecular Science and Technology at Trento University, finished in 2013 where he was student representative for two years. His Bachelor thesis was spent inside the IGEM Team UniTN 2013 where he focused in building and characterization of a bio-brick for the production of methyl salicylate for inhibit ripening of fruit during post-harvest. He then completed a MSc in Food Technology with a specialization in Sensory Science at Wageningen University (the Netherlands) with a thesis spent on investigating the effect of different stimulation of the endocannabinoid system on human sensory perception and liking. During the MSc, he did a six month internship in the Sensory and Consumer insights team at International Flavors & Fragrances (France) where he researched and improved discrimination tests practices through Thurstonian modelling. Moreover, as part of his MSc program, he followed an Erasmus semester at Copenhagen University (Denmark) where he discover the reality of dumpster diving and decided to get involved in the battle against food waste with the project Common sensing - a sensory guide to reduce food waste presented at BCFN YES! competition in 2014. From this experience he started in 2015 a collaboration with the BCFN Foundation, firstly as geographic representative (EU region) of the BCFN Alumni network and then as researcher on food waste, sustainable agriculture and the related policy and the nexus between migration and food systems.

In August 2016 he started his PhD at the department of Food Quality & Nutrition at the Edmund Mach Foundation, in the chair group of Food Quality & Design of Wageningen University and in collaboration with the industrial partner Soremartec (Ferrero). His project aimed at investigating possible applications of direct injection mass spectrometry techniques to assess food quality and the relation between volatile organic compounds and sensory perception

# List of publications:

**This thesis:**

1) "*What drives in vivo aroma release and perception of condiments consumed with carrier foods?"* van Eck A., **Pedrotti M.,** Brouwer R., Supapong A., Fogliano V., Scholten E., Biasioli F., Stieger M. Submitted at Nature Food
2) "*Quality control of raw hazelnuts by rapid and non-invasive fingerprinting of volatile compound release"* **Pedrotti M.,** Khomenko I., Genova G., Castello G., Spigolon N., Fogliano V., Biasioli F. Submitted at LWT – Food Science and Technology
3) "*The Good, the Bad and the Aged: predicting sensory quality of anhydrous milk fat by PTR/SRI-Tof-MS analysis and data mining"* **Pedrotti M.,** Khomenko I., Fontana M., Somenzi M., Falchero L., Arveda M., Cappellin L., Fogliano V., Biasioli F., **2020.** International Dairy Journal, *109*. DOI: 10.1016/j.idairyj.2020.104729
4) "*Application of PTR-TOF-MS for the quality assessment of lactose-free milk: Effect of storage time and employment of different lactase preparations"* Bottiroli R., **Pedrotti M.,** Aprea E., Biasioli F., Fogliano V., Gasperi F., **2020.** Journal of Mass Spectrometry, 2, Special Issue: 6th MS Food Day. DOI: 10.1002/jms.4505
5) "*Ethnicity, gender and physiological parameters: Their effect on in vivo flavour release and perception during chewing gum consumption*" **Pedrotti M.,** Spaccasassi A., Biasioli F., Fogliano V. **2019** Food Research International Volume 116, pp 57-70. DOI: 10.1016/j.foodres.2018.12.019
6) "*Rapid and non-invasive quality control of anhydrous milk fat by PTR-MS: the effect of storage time and packaging*", **Pedrotti M**., Khomenko I., Cappellin L., Fontana M., Somenzi M., Falchero L., Arveda M., Fogliano V., Biasioli F. **2018** Journal of Mass Spectrometry, 53(9), Special Issue: 5th MS Food Day pp. 753-76210. DOI: 1002/jms.4204

**Other publications in peer-reveiwed jorunals and books:**

1) "*Arousal influences olfactory abilities in adults with different degree of food neophobia*" Menghi L., Khomenko I., **Pedrotti M.,** Cliceri D., Aprea E., Endrizzi I., Cavazzana A., Biasioli F., Giacalone D. , Gasperi F. Submitted at Scientific Reports
2) "*A benchmarking protocol for breath analysis: the peppermint experiment.*", Henderson B., Ruszkiewicz D., Wilkinson M., Beauchamp J., Cristescu S., Fowler S., Salman D., Di Francesco F, Koppen, G., Langejuergen J., Holz O., Hadjithekli, A., Moreno S., **Pedrotti M.,** Sinues P., Slingers G., Wilde M., Lomonaco T., Zanella, D., Zenobi R., Focant J., Grassin-Delyle S., Franchina, F.A., Malásková M., Stefanuto P., Pugliese G., Mayhew C., Thomas C. L. P. **2020**. Journal of Breath Research (*Accepted for publication*).

3) *"Engage with the future": A brief summary of the 13th Pangborn Sensory Science Symposium."* **Pedrotti M., 2019**. Trends in Food Science and Technology, *93*, 259-261. DOI: 10.1016/j.tifs.2019.09.009
4) *"The new trends and challenges for sensory and consumers sciences: Outcomes from EUROSENSE 2018, a sense of taste."* **Pedrotti M., 2019**. Trends in Food Science and Technology, *83*, 274-276. DOI: 10.1016/j.tifs.2018.11.013
5) "*Linking monoterpenes and abiotic stress resistance in grapevine*" Cappellin L., Grando M.S., Zocca P., **Pedrotti M.**, Lorenzi S., Bertamini M. **2019** Bioweb of conferences – CONAVI 2018
6) *"Exploring the Migration-Food and Nutrition Security Nexus: How Aid Policies Can Maximize the Migration-Related Sustainable Development Opportunities"* Sensi R., **Pedrotti M.,** Springer Edition: Achieving the Sustainable Development Goals Through Sustainable Food Systems. Cham, Switzerland (**2019**)
7) "*Assessing the role of CAP for more sustainable and healthier food systems in Europe: A literature review"* Recanati F., Maughan C., **Pedrotti M.**, Dembska K., Antonelli M. **2019** Science of the Total Environment, 653 , pp. 908-919. DOI: 10.1016/j.scitotenv.2018.10.377
8) "*Ethylene-Producing Bacteria That Ripen Fruit*." Digiacomo F., Girelli G., Aor B., Marchioretti C., **Pedrotti M.**, Perli T., Tonon E., Valentini V., Avi D., Ferrentino G., Dorigato A., Torre P., Jousson O., Mansy S. S., D. B. Cristina. **2014** ACS Synth. Biol., 3 (12), pp 935–938 DOI: 10.1021/sb5000077.

***Conference presentations***

1) BCFN International Forum on Food and Nutrition, BCFN, Milan (IT) 2019 – *oral presentation*
2) 33rd EFFoST International Conference, WUR, Rotterdam (NL), 2019 – *oral presentation*
3) 6th MS Food Day, Società Chimica Italian Divisione di MS & Fileni, Camerino (IT), 2019 – *oral and poster presentation*
4) 13th Pangborn Sensory Science Symposium, Massey University & Firmenich & Leatherhead Food Research, Edinburgh (SCT), 2019 –*flash poster presentation*
5) 8th PTR-MS International Conference, Ionikon Analitik & Innsbruck University, Innsbruck (AT), 2019 – *oral and poster presentation*
6) EUROSENSE 2018, SISS & E3S, Verona (IT), 2018 – *poster presentation*
7) XXII International Mass Spectrometry Conference, ICS & IMSE, Florence (IT), 2018 – *oral and poster presentation*
8) 5th MS Food Day, Società Chimica Italian Divisione di MS & Coop Italia, Bologna (IT), 2017 – *poster presentation*
9) 3rd IMEKO FOODS, International Measurement Confederation & Aristothele University, Thessaloniki (GR), 2017 – *oral presentation*

10) BCFN International Forum on Food and Nutrition BCFN, Milan (IT), 2017
11) 1st International Conference on soft chemical ionisation MS and applications, FH Vorarlberg, Dornbirn (AU), 2017 – *oral and poster presentation*

# Overview of completed training activities

## Discipline specific activities

### *Courses*

Advanced Food Analysis, VLAG, Wageningen (NL), 2017

Hands-on Ionicon workshop, Ionikon Analitik, Innsbruck (AT), 2017

Sensory Perception & Food Preference: Affective drivers of food choice, VLAG, Wageningen (NL), 2018

Hands on Ionicon workshop, Ionicon Analitik, Innsbruck (AT), 2019

### *General courses*

School of scientific writing and reading, UNITN-DI, Trento (IT), 2016

VLAG PhD week, VLAG, Baarlo (NL), 2017

Applied Statistics, VLAG, Wageningen (NL), 2017

Chemometrics, VLAG, Wageningen (NL), 2017

PhD Assessment, WGS, Wageningen (NL), 2018

Career Perspective Course, WGS, Wageningen (NL), 2018

Advanced Excel course, FEM, S. Michele all'Adige (IT), 2018

### *Teaching obligations*

Supervision of MSc (12) and BSc (1) students thesis and internships (2017-2020)

FQD-31306 Predicting Food Quality (2018-2020). Wageningen University and Research, Wageningen, The Netherlands.

### *Optional activities*

VLAG PhD proposal, Trento (IT), 2016

PhD study tour to Italy, FQD-WUR/FEM, Trento & surroundings (IT), 2016

FQD colloquia, FQD, Wageningen (NL), 2016-2020

FEM Phd event, FEM, Trento (IT), 2016-2020

Symposium "Edible Insects: the value chain", VLAG/WUR, Ede (NL), 2018

BCFN Annual Retreat, BCFN, Bologna (IT), 2018

BCFN Annual Retreat, BCFN, Siena (IT), 2019

The research described in this thesis was financially supported by Soremartec Italia S.R.L. and by the graduate school VLAG. Financial support from Wageningen University for printing this thesis is gratefully acknowledged.



Cover design by KemilArt Michela Meneguzzi©
Printed by Proefschriftmaken


signal
t
MAYO