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Comprehensive Mapping of Volatile Organic Compounds in Fruits

Tutor

Dr. Fulvio Mattivi

Department of Food Quality and Nutrition, Fondazione Edmund Mach

Advisor

Prof. Vladimir Shulaev

Department of Biological Sciences, University of North Texas

Ph.D. Thesis of

Manoj Shahaji Ghaste

Department of Food Quality and Nutrition

Fondazione Edmund Mach

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This thesis is lovingly dedicated to my Mother. Her support, encouragement, belief and constant love have sustained me throughout my life.

Declaration

I, Manoj Shahaji Ghaste confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Thesis abstract

Volatile organic compounds (VOCs) are the key aroma producers in fruits and sensory quality of fruits is widely determined by qualitative and quantitative composition of VOCs. The aroma of grape is a complex of hundreds of VOCs belonging to different chemical classes like alcohols, esters, acids, terpenes, aldehydes, furanones, pyrazines, isoprenoids and many more. VOCs play important role as they determine the flavor of grapes and wine made from it. The objective of this thesis is to study of VOCs through development of different mass spectrometry based analytical methodologies and its applications for the comprehensive investigation and construction of database of the VOCs in grapes.

First part of the study was dedicated to generation of a database of grape VOCs through the screening of multiple grape varieties (n=124) representing different species, color and origin. The experiment was carried out using headspace solid-phase microextraction (HS-SPME) and gas chromatography mass spectrometry (GC-MS) based approach and according to metabolomics protocols. A customized dataset of reference standards (>350) was generated and, an automated pipeline for data analysis was created in collaboration with data management group of the institute. The results showed annotation of "level 1" of 117 VOCs in grape. The established database in this experiment will represent the significant portion of the future Grape Metabolome database.

The second part of the study was dedicated to study the differential behavior of volatile organic compounds and their glycosylated precursors qualitatively and semi quantitatively. Volatile secondary metabolites also exist in the form of nonvolatile and odorless glycosylated precursors in grape and studies have confirmed that concentration of these precursors can be much higher than its free counterparts. The elevated concentrations of volatiles in glycosylated forms can significantly affect the wine aroma because of possible chemical modifications throughout the process of fermentation and wine ageing. In addition, the investigation of the biosynthesis and accumulation of VOCs in the fruit tissues requires the consideration of both the free and bound forms.

To study the phenomenon an experiment was carried using solid phase extraction (SPE) of the free and glycosylated precursors; with enzymatic hydrolysis aglycone part of the precursors was released followed by subsequent GC-MS analysis. Over 10 different selected grape varieties were analyzed. Sixty-six significant different aroma compounds in grapes (pre and post hydrolysis) were identified. Identification was done based on several parameters like retention time, retention index and MS spectral database. The multivariate statistical analysis by two-way hierarchical clustering with heat map visualization showed distribution of the compounds within different varieties before and after hydrolysis.

In the third part of the study, we performed experiments dedicated to training and applications of atmospheric pressure gas chromatography mass spectrometry (APGC-MS). The experiment was carried out at the Department of Biological Sciences, University of North Texas, under the supervision of Prof. Vladimir Shulaev. We have established the metabolomics protocol for the analysis of fruit volatiles using APGC-MS with an optimized GC and MS conditions and created novel library of the fruit volatile compounds using APGC-MS system. Six different grape varieties were analyzed as a case study and experimental results showed APGC-MS as a valuable solution for metabolomics analysis. The data processing and statistical evaluation was done using XCMS and Progenesis QI$^{©}$ software. Moreover, observations based on injections of pure reference standards showed high abundance of molecular ions with minimal fragmentation at low collision energy that is typically missing in traditional vacuum source GC-MS. Moreover, the use of elevated collision energy data resulted in a spectrum similar to the traditional EI data.

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1. Introduction

Plants possess tremendous capacity to synthesize, store and release large number of volatile organic compounds (VOCs). These compounds are produced as secondary metabolites by plants and play many important functions in its life cycle. VOCs are low molecular weight compounds (MW<300) with low boiling points and shows large structural diversity (figure 1). Plants synthesizes these compounds through a variety of biosynthetic routes, predominantly from amino and fatty acids, terpene biosynthetic pathways and carotenoid cleavage (Mathieu, Terrier, Procureur, Bigey, & Günata, 2005; Pichersky, Noel, & Dudareva, 2006). Based on the basic skeleton produced through these pathways the diverse classes of volatiles are further synthesized via modification reactions like acylation, methylation, oxidation/reduction etc. (El Hadi, Zhang, Wu, Zhou, & Tao, 2013). Many volatile organic compounds are synthesized in fruits depending upon its genetic and other characters (Aprea et al., 2011; Degenhardt, Köllner, & Gershenzon, 2009; Emanuelli et al., 2010; Jiang & Zhang, 2010; Myles et al., 2011; Pacifico et al., 2011; Anthony L. Robinson et al., 2013).

According to the official definition given by European commission "Volatile organic compound (VOC) is any organic compound having an initial boiling point less than or equal to 250° C measured at a standard atmospheric pressure of 101.3 kPa" (Official Journal of the European Union, 2004).

Monoterpene is one of the important class of fruit VOCs, especially grapes, this class contains some of the most aroma active compounds like citronellol, nerol, geraniol, alpha terpineol and linalool with aroma threshold ranging 100-500 µg/L (J. Marais, 1983). In general, monoterpenes contains 10-carbon backbone structure synthesized from the common precursor geranyl diphosphate and catalyzed by enzymes catalyzes called monoterpene synthases e.g. S-linalool synthase, geraniol synthase and (R)-limonene synthase (Chen, Tholl, Bohlmann, & Pichersky, 2011; Lund & Bohlmann, 2006).

Sesquiterpene also significantly represents the terpenic-fraction of grape volatiles e.g. beta-caryophyllene, humulene, farnesene, farnesol and cadinene are considered important in grapes (Coelho, Rocha, Delgadillo, & Coimbra, 2006). Formation of sesquiterpene starts

from farnesyl diphosphate and enzymes sesquiterpene synthase. The synthesis works similarly to carbocationic based reaction mechanisms catalyzed by monoterpene synthases. However, the larger carbon skeleton of farnesyl diphosphate (FPP) and the presence of three, instead of two, double bonds greatly increase structural diversity of the products (Degenhardt et al., 2009).
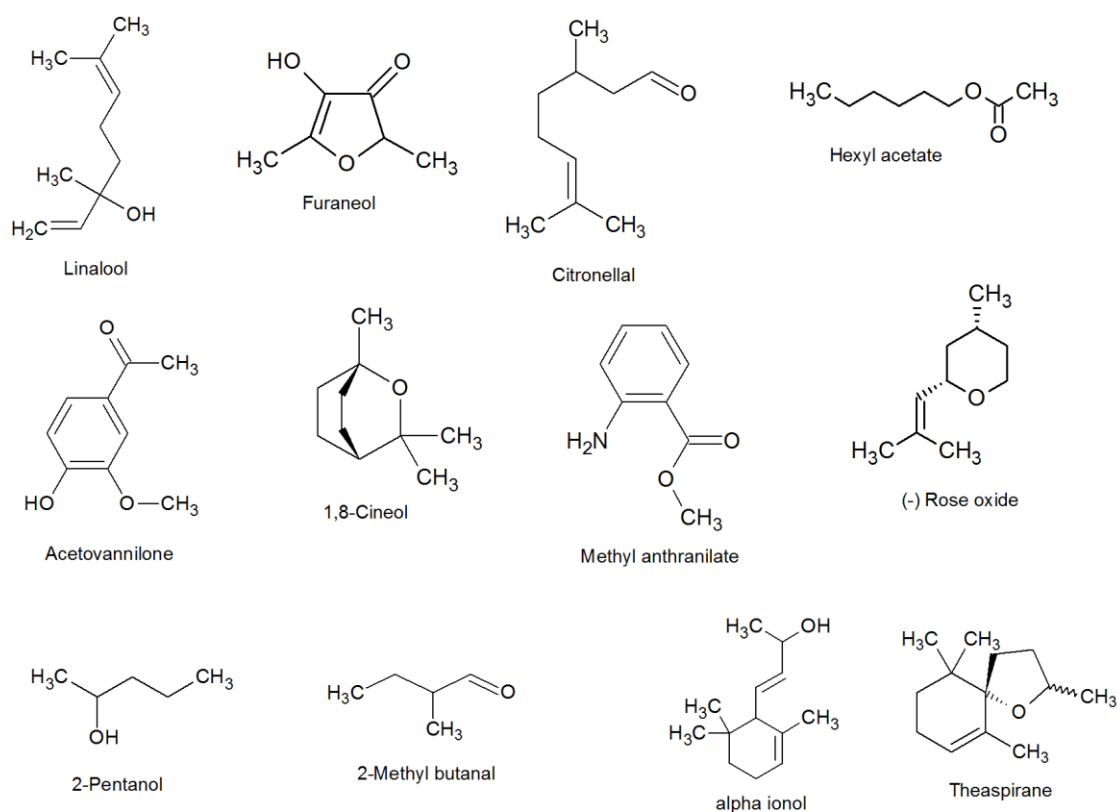
Figure 1 Chemical structures of some plant derived VOCs

C13-norisoprenoids beta-damascenone, beta-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) are also considered as potent aroma producers in both red and white wines (J. Marais, 1983; Skouroumounist & Winterhalter, 1994; Maurizio Ugliano & Moio, 2008; Winterhalter, Sefton, & Williams, 1990). The primary biosynthesis of these compounds takes place with the breakdown of carotenoids with carotenoid cleavage dioxygenase (CCD) catalysis (Günata, 2013).

Different VOC mediates many biotic interactions of the plants with other plants, insects and microorganisms, such as attracting pollinators and seed dispersers, defense against pest and pathogen by attracting other predator species and interspecific signaling in plants (figure 2). These kind of interactions are widely studied aspects in chemical ecology (Buttery, Ling, & Wellso, 1982; Gershenzon, 2007; Shulaev, Silverman, & Raskin, 1997). Several specific examples of plant VOC functions include role of methyl salicylate as airborne signaling molecule of tobacco mosaic virus infection in tobacco plant which activates the disease resistance and the expression of defense-related genes in neighboring plants and in the healthy tissues of the infected plant (Shulaev et al., 1997).
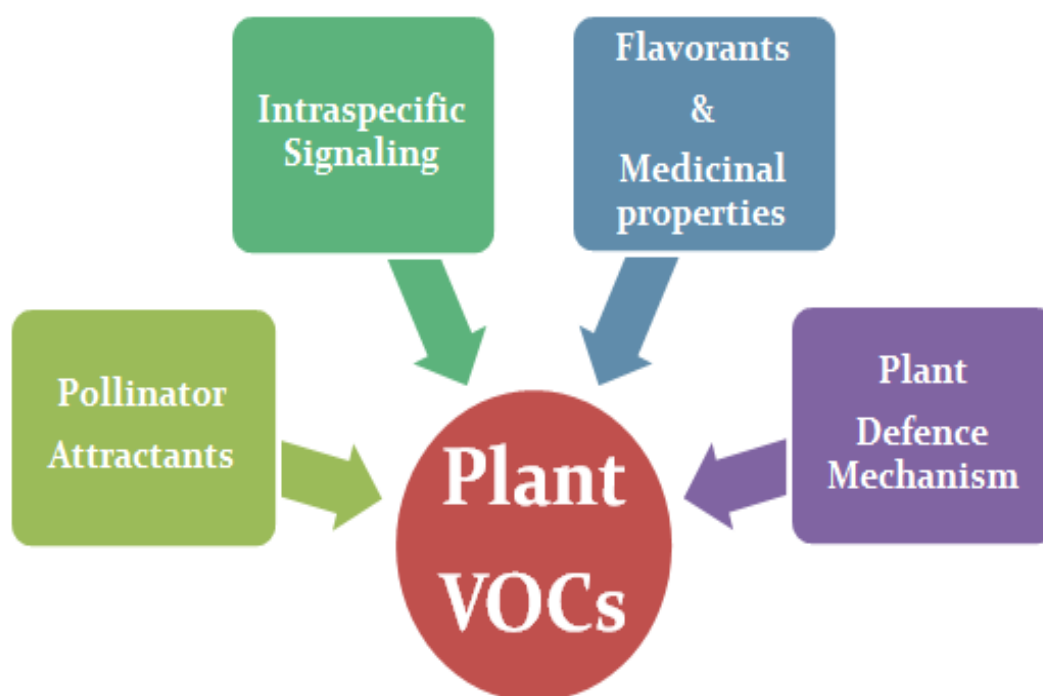
Figure 2 multiple roles of plant VOCs

Studies by Takabayashi et al 1994 demonstrated that volatile terpenes linalool, farnesene, ocimene, 4,8-dimethyl-1,3,7-nonatriene, 4,8,12-trimethyl-1,3,7,11-tridecatetraene were released by different plants in response to herbivore attack. Particularly in grapevine the compounds (E)-beta-caryophyllene, (E)-beta-farnesene and (E)-4,8-dimethyl-1,3,7-nonatriene were reported as attractants of grapevine moth *Lobesia botrana* (Tasin, Bäckman, Bengtsson, Ioriatti, & Witzgall, 2006). Antimicrobial activity of plant volatiles is also

reported, volatile compounds like hexanal, 2-E-hexenal, 3-Z-hexenal were known to show antifungal activity against a number of fungi and were suggested as an alternative approach to synthetic fungicides (Rowan, 2011; Jun Song & Bangerth, 1996).

Many of the VOCs are important as scents to the humans because of their detection or sense by the human olfactory system at trace levels. High volatility and low molecular weights makes them readily diffusible into the gas phase and therefore detected by human sensory system. The human genome encoded many test receptors and several hundred olfactory receptors involved in recognition of specific foods and their compositions, but the impact of a chemical on flavor perception is determined by both its concentration and the odor threshold (Goff & Klee, 2006). The foodborne stimulus space has co-evolved with, and roughly match our circa 400 olfactory receptors as best natural agonists (Dunkel et al., 2014) (table 1). (Buck & Axel, 1991) described the odorants as "volatile chemical compounds that are carried by inhaled air to the olfactory epithelium located in the nasal cavities of the human nose". Some of the key aroma compounds in fruits and their odor description are described in table 1.

Some VOCs are also known to produce the off-flavor or undesirable aroma in food and beverages. Compounds 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole, 1-octen-3-one, (+)-fenchone, ethyl acetate, vinyl-4-guaiacol, indole-3-acetic acid, tryptophan were some of those known to be generate off-flavors in wine (Boutou & Chatonnet, 2007; F. Mattivi, Vrhovšek, & Versini, 1999; Roland, Vialaret, Razungles, Rigou, & Schneider, 2010). Production of stale-flavor is also one of the problem faced by of recent food packaging industry, and different studies are currently being carried out to understand the possible production mechanism of the precursors of stale-flavors and possible ways to eliminate them from the process (Patel, Prajapati, & Balakrishnan, 2014; Perkins, Zerdin, Rooney, D'Arcy, & Deeth, 2007).

Due to their sensory properties and increasing demand in different industries, many plants derived VOCs are produced synthetically at large scale; Food, beverage, cosmetics, perfume and pharmaceuticals are some of the key industries amongst its potential consumers. International Fragrance Association (http://www.ifraorg.org) is an official self-regulatory

organization of the fragrance industry worldwide. It was founded in 1973 and is based in Geneva, Switzerland. In 2011, a survey conducted by the organization listed around 3059 chemicals used in the flavor and fragrance industry worldwide. This survey was estimated to represent about 90% of world's production volume of fragrances. Some of the common industrially using flavor compounds are geraniol, linalool, citronellol, limonene etc.

Table 1 Important Plant/Fruit derived VOCs and their odor description.

No	Name of the compound	Odor descriptor
1	Linalool	Citrus, orange, floral, terpene, waxy and rose,
2	Geraniol	Floral, sweet, rose, fruity and citronella-like with a citrus nuance,
3	Citronellol	Floral, rosy, sweet, citrus with green fatty terpene nuances
4	Limonene	Sweet, citrus and peel
5	Hexanoic ethyl ester	Sweet, fruity, pineapple, waxy, fatty and ester with a green banana nuance
6	Octanoic ethyl ester	Waxy, sweet, musty, pineapple and fruity with a creamy, dairy nuance
7	Vanillin	Sweet, vanilla, vanillin, creamy and phenolic
8	Furaneol	Sweet, slightly burnt brown caramellic, cotton candy with a savory nuance
9	Ethyl cinnamate	Sweet, balsamic, spice, fruity and powdery
10	beta-damascenone	Woody, sweet, fruity, earthy with green floral nuances
11	alpha Ionone	Sweet, woody, floral, violet, tropical fruity
12	Hexanol	Pungent, etherial, fruity and alcoholic, sweet with a green top note
13	Myrtenol	Camphoreous, woody, cooling, minty with a medicinal nuance
14	Methoxy pyrazine	Green pea green bell pepper green pea galbanum
15	3-Mercapto hexanol	Sulfurous, metallic and pungent with a slight spicy, green leafy, wasabi-like and vegetative note with and earthy nuance

Odor descriptions were adapted from online database of "The good scents company". (http://www.thegoodscentscompany.com)

Apart from their diverse ecological functions and sensory properties, many VOCs are significant to human health and used as medicines, this is the perhaps less studied aspect of these compounds. A commonly found fruit VOC geraniol was reported to inhibit the

ornithine decarboxylase activity, a key enzyme of polyamine biosynthesis, which is enhanced in cancer growth in humans (Carnesecchi et al., 2001). Similarly it inhibits a mevalonate biosynthesis which suppresses the growth of hepatoma and melanoma in transplanted rats and mice (Yu, Hildebrandt, & Elson, 1995). Other compounds citronellol, linalool and limonene were found to have chemoprevention and anticarcinogenic properties (Gould, 1997; Usta et al., 2009; Zhuang et al., 2009).

Complex profile of VOCs keeps changing during the life cycle of the plant. Factors like age, genetics, environmental conditions, postharvest handling, storage conditions, sunlight, irrigation, fertilization, chemical applications and other human practices can alter the qualitative and quantitative composition of the VOCs among fruit. Genetic variation is very important factor which can be responsible for differentiation of VOCs in grapes (Emanuelli et al., 2010, 2013). For example, terpenoids are abundant in *Vitis vinifera* Muscat grapes while c-13 norisoprenoids are dominant in *V. cinerea*, the Native American grapes (Results from chapter 4) (Sun, Gates, Lavin, Acree, & Sacks, 2011). Age or maturity also affects aroma of the fruit, as fully ripen fruits throw more aroma than unripe and immature fruits(May, Lange, & Wüst, 2013; Anthony L. Robinson et al., 2014; Sarry & Gunata, 2004). Aroma potential was reported to be highest in vines under mild water deficit and moderate nitrogen supply and severe water deficit limits the aroma potential in grapes (Des Gachons et al., 2005). Refrigeration induced changes in levels of 3-methylbutanal, linalool, guiacol, hexanol, *trans*-2-hexenal and *trans*-3-hexenol are reported in tomato (Díaz de León-Sánchez et al., 2009).

Constant development and advancement of the analytical tools like gas chromatography, mass spectrometry as well as rapid sample extraction and enrichment methods are providing new comprehensions to the field of plant VOCs analysis and characterization. Moreover, with the advent of advanced metabolomics tools it is possible to perform the comprehensive studies covering large number of metabolites. The holistic approach for the analysis, annotation and comprehensive databases of the VOCs will assist the future need to understand numerous biological interactions, also in the quality assurance of the food products, medicine and upcoming research in associated fields.

2. Methodology in the analysis of fruit VOCs

2.1. Gas chromatography (GC) and mass spectrometry (MS)

Gas chromatography-mass spectrometry (GC-MS) is the most commonly and widely used technique for the analysis of VOCs. GC-MS is the most compatible technique for the analysis of VOCs due to their properties like high vapor pressure, volatility, small molecular weights, relatively low-polarity and good thermal stability. A GC-MS (figure 3) is the combination of a gas chromatograph (GC) which is involved in separation of the chemicals and a mass spectrometer (MS) which further ionizes and detects the chemicals according to their mass to charge (m/z) ratio.

GC technique is mainly used for the analysis of volatile compounds from different biological and environmental matrices; GC also can be used with different types of detector based upon applications. Detectors like those that flame ionization detector (FID) which is a sort of "universal detector" mainly used for the volatile hydrocarbons analysis in many industries. Electron capture detector (ECD) is a GC detector that is mainly used for the trace level analysis of organochlorine compounds (pesticides, dioxins, PCBs) due to its sensitivity and selectivity. Nitrogen phosphorous detector (NPD) is utilized for the selective analysis of volatile compounds containing nitrogen and phosphorous. GC with specific and Non-MS detectors could provide only separation, quantitation, and not the characterization of the molecule. The use of pure reference standards is mandatory in those cases. The GC analysis was later enhanced by its combination with mass spectrometry, as it complemented the analysis with the m/z information of the compound fragments. Ever since its discovery, almost more than a century, mass spectrometry (MS) becomes one of the fundamental research tools with applications covering many fields of biology, chemistry, pharmaceutical and medical sciences.

Typical GC-MS system comprises gas chromatograph hyphenated to mass spectrometer (figure 3) which provides the superb separation ability of GC with simultaneous detection of the compounds giving information about its molecular mass. When the sample is injected into the GC inlet, where it is volatilized and a characteristic portion is carried onto the column by the constant stream of carrier gas. Different chemicals present in the sample are then separated based on the different strengths of interaction of the compounds with the

stationary phase (other factors like boiling point of compound, column length, column temperature, carrier gas flow rate and the polarity of analyte and stationary phase can also affect the separation). Each separated sample component then elute from the column into the mass spectrometer through the heated transfer line. The mass spectrometer is made up of three essential units, i.e. ion source, analyzer and detector system (requires high vacuum, $\sim 10^{-6}$ to 10^{-8} mm of mercury). Once the separated components from GC enter into the ion source, they are ionized based on ionization source and polarity selection. Further, the mass analyzer resolves the ions into their characteristics mass components according to their mass-to-charge (m/z) ratio and finally they are sent into the detector system for the ion detection and recording the relative abundance of each of the resolved ionic species. The signals are then amplified and sent to the data system where the chromatogram is electronically constructed.
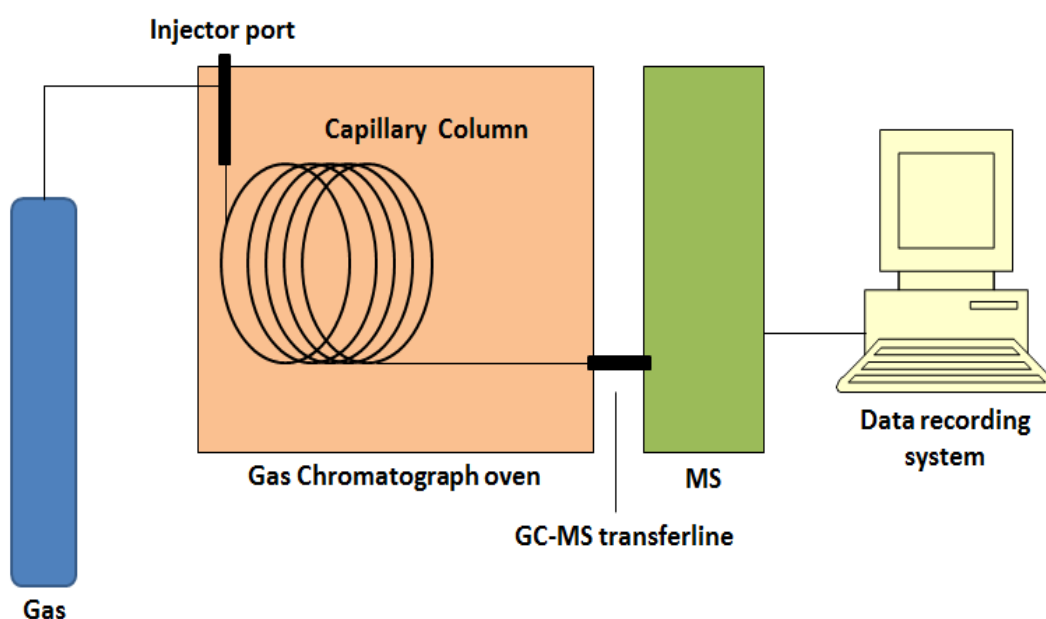
Figure 3 Schematic of GC-MS

2.2. Ionization techniques

Ionization is necessary in any MS technique in order to allow the subsequent detection of the ions generated according to their m/z ratio. The ionization techniques used in the study are as follows,

2.2.1. Electron ionization (EI)

Electron ionization or EI is the most common ionization method used in the GC-MS analysis. In EI source, (figure 4) electrons are generated by thermionic emission by heating a wired filament with high energy of 70 eV and by exposing a sample to these high-energy electrons. This is referred as "hard" ionization technique, the energy of the electrons interacting with the molecule of interest is generally much higher than in the chemical bonds of the molecule. The high energy breaks bonds in a well characterized, multiple ways (figure 5). The result is predictable, identifiable fragments from which we perform molecular identification. Abstraction of only an electron from the outer shell yields a radical cation in the positive mode ($M^{\cdot+}$) and a rich spectrum of fragments (Balogh, 2009).
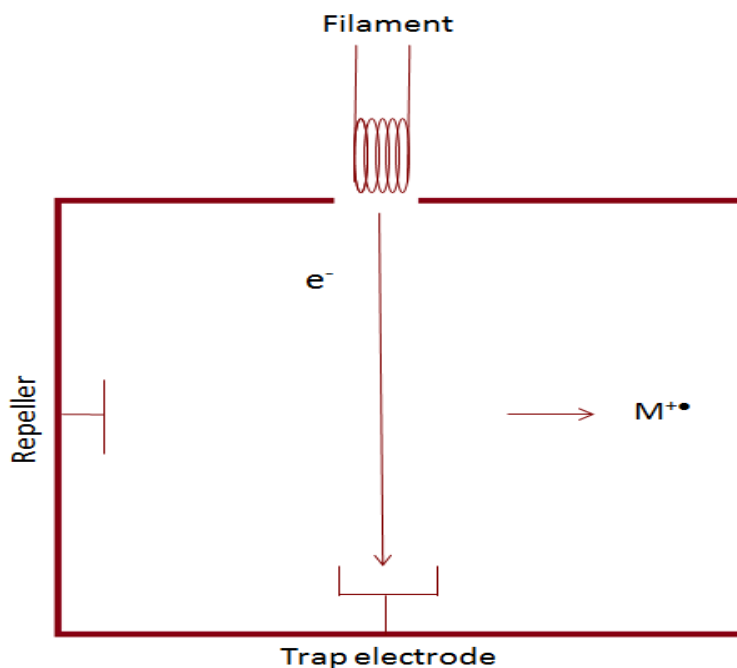
Figure 4 Schematic of EI ion source and
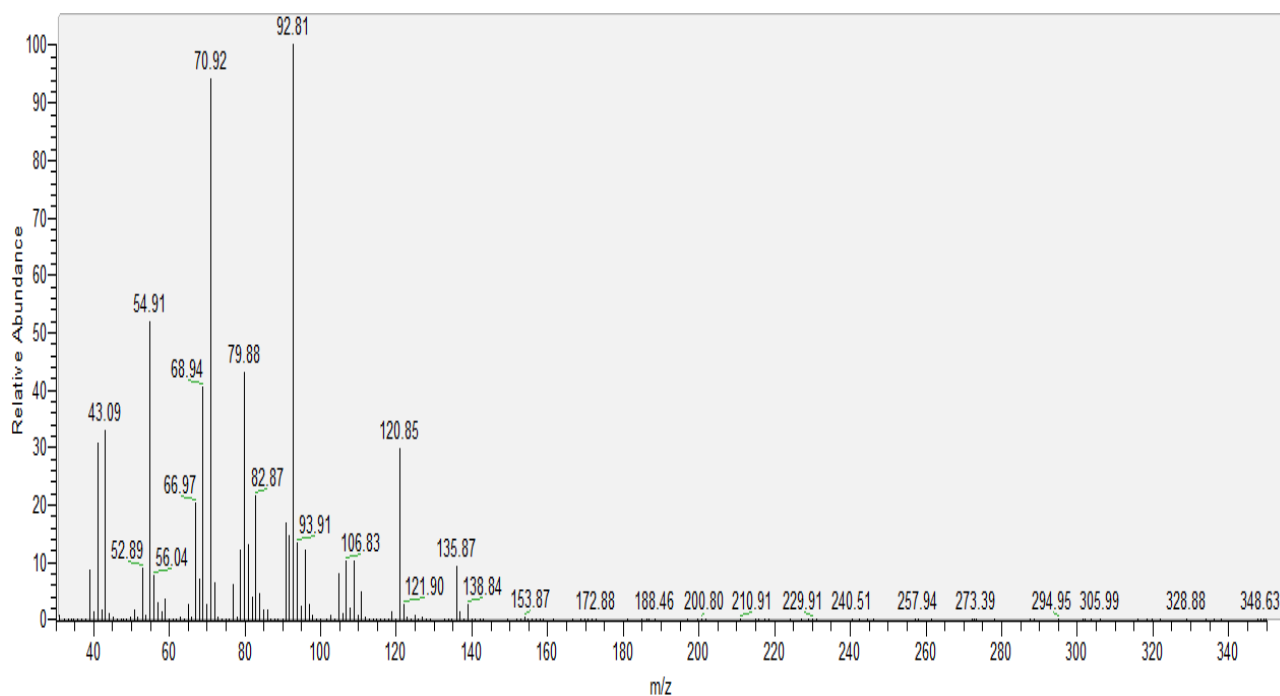
Figure 5 EI spectra of linalool from reference standard analysis

The EI spectrum (figure 5) generated by one-instrument looks much like a spectrum of the same compound from another EI instrument and because of this standardized ionization condition (70eV) method many commercial libraries are available for identification of compounds.

2.2.2. Atmospheric pressure gas chromatography mass spectrometry (APGC-MS)

Atmospheric pressure chemical ionization or APCI is the emerging method in GC-MS analysis. It is relatively soft ionization process when compared with traditional EI technique and offers a significant reduction in fragmentation. It provides more information on molecular ion (which is usually gets by knocking an electron off an organic molecule to form a positive ion and usually represented by M^+) and provides clean spectra similarly as CI does. Moreover, it does not require reagent gases like methane, ammonia and isobutene and can be simply used with nitrogen. The Waters Corporation has recently introduced this technology coupled to gas chromatography under the trade name APGC-MS. The experiment conducted using this novel technology and results will be further discussed in the fourth chapter of the thesis.

APGC ionization uses nitrogen as make-up gas that flows through the GC interface ($\approx$ 350 mL/min) and forms plasma with the help of corona discharge needle (2 μA) in the source. The plasma ionizes analytes entering into the source (figure 6). In case of charge transfer, the plasma reacts directly with analyte molecules and forming $M^{+\bullet}$. Alternatively, ionization can take place indirectly through proton transfer reactions by introducing some moisture in the system. The figure 7 also shows proton source as water but methanol or other protonic solvent can be used. It is possible to select between proton transfer and charge transfer in APGC by shifting source conditions depending on the chemistry of the target analytes.
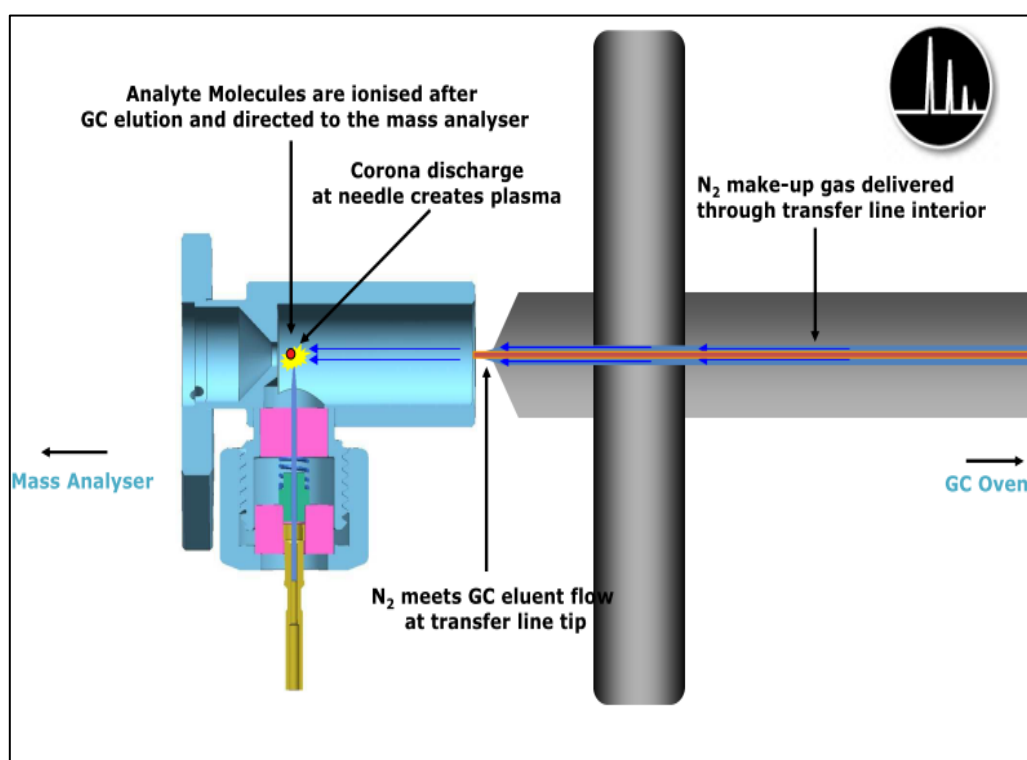
Figure 6 Schematic of APGC source components
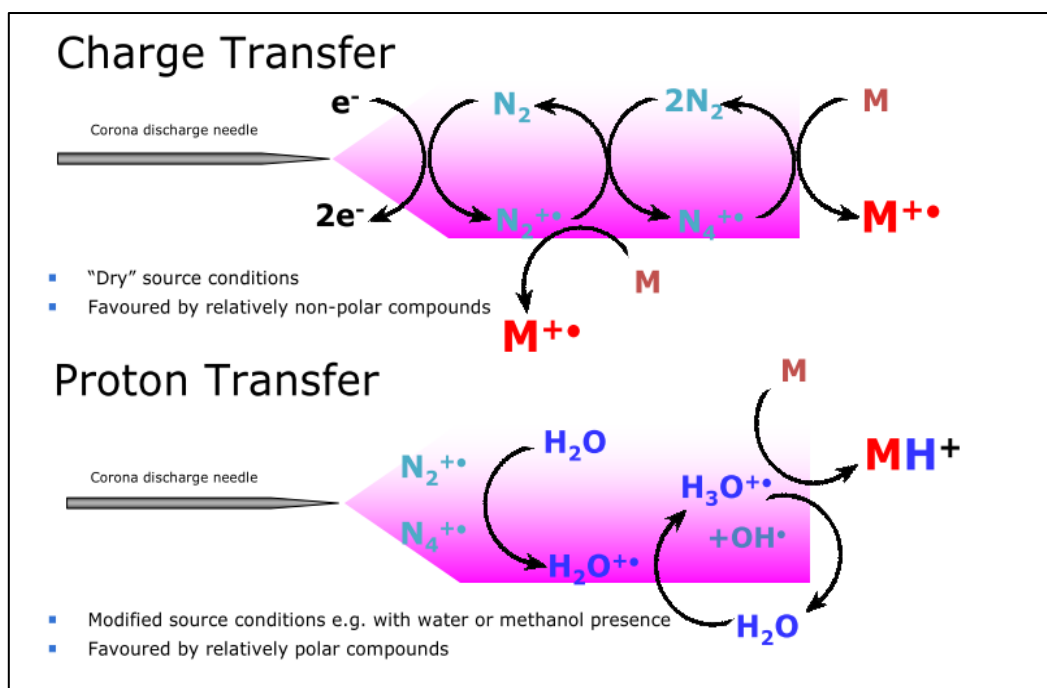
Figure 7 APGC ionization mechanism

(Figures 6 and 7 are reproduced by kind permission of Waters Corporation)

Another ionization technique often used in GC-MS is chemical ionization (CI); a soft ionization method (like APGC) generates fewer fragments and cleaner spectra of the molecules comparatively to EI. In a typical CI experiment ions are generated through the analyte collision with ions of reagent gas present (mostly used reagent gas are methane, ammonia and isobutane). Different type of the ionizations can be achieved using CI, the primary ion formation happens through the charge transfer reaction from plasma to analyte molecule which gives $M^{+\bullet}$, likewise protonation, hydride abstraction can also be possible in the CI (Balogh, 2009).

2.3. GC-MS data analysis and annotation

Data generation in GC-MS experiment depends on its size of the experiment. I.e. Typical GC-MS metabolomics experiment can produce large amounts data and thus turning the data into the results is a big challenge. Usual GC-MS data processing includes peak peaking, compound identification and quantification and most of the vendor provided software's

could perform these tasks. Xcalibur© 2.2 (Thermofisher Scientific) and Masslynx© version 4.1 (Waters Corporation) were extensively used for data processing (chapter 3, 4 & 5). Also many GC-MS brands equipped with EI ionization method provide the NIST Mass Spectral Search Program© (http://www.nist.gov/srd/nist1a.cfm) as a default software component. NIST MS search provides extensive collection of spectra of reference compounds and many different library options, the current version of NIST MS database have ≈276248 reference spectra in its main EI MS library. It is specially used for the in-silico comparison of unknown spectra with library spectra and eventually for compound identification; furthermore this library database can be linked with other software for the annotation of large number of sample sets.

In case of metabolomics experiments, where large sample data sets needs fast processing with some specific tasks to perform like retention time alignment, data normalization, statistical evaluation etc. Performing the specialized functions is many times not possible by using vendor specific software only. Many online tools/software are available like, XCMS (Benton, Wong, Trauger, & Siuzdak, 2008; Smith, Want, O'Maille, Abagyan, Siuzdak, et al., 2006; Tautenhahn, Patti, Rinehart, & Siuzdak, 2012) (https://xcmsonline.scripps.edu), MZmine (http://mzmine.sourceforge.net), MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst), Spectconnect (http://spectconnect.mit.edu) and MET-IDEA (Broeckling et al. 2006) offers these specialized tasks. AMDIS (http://www.amdis.net) is one of the software used for de-convolution of GC-MS data. Further statistical methods are also important to understand variation between the data. Two methods i.e. unsupervised principal component analysis (PCA) and super-vised partial least squares-discriminant analysis (PLS-DA) are widely used in metabolomics studies (Arapitsas, Speri, Angeli, Perenzoni, & Mattivi, 2014; Hendriks et al., 2011; Shulaev, Cortes, Miller, & Mittler, 2008). PCA is often used to show the most important factors of variation defining the data set of study and for the quality control of the experiment. A supervised method aims to get useful information from the dataset with an assumed hypothesis, enabling prediction of the relationship of the analytical samples in different study groups (Hu & Xu, 2013).

2.4. Sample preparation techniques

2.4.1. Solid-phase microextraction (SPME)

Solid phase microextraction (SPME) is a sample extraction and enrichment technique for the chemicals. It was developed in early 90s (Pawliszyn, Pawliszyn, & Pawliszyn, 1997; Pawliszyn, 1999, 2012) to address the need for fast, solvent free and applicable in the field sample preparation method. Presently it is a well-developed technology, suitable for large experiments since it allows the automatization of the analytical protocols, with wide range of applications in food chemistry and other relevant areas and offers extraction with minimum or no matrix effects of the sample. A typical SPME assembly consists of polymeric SPME fiber placed inside the hollow needle and plunger for the movement of fiber (figure 8)
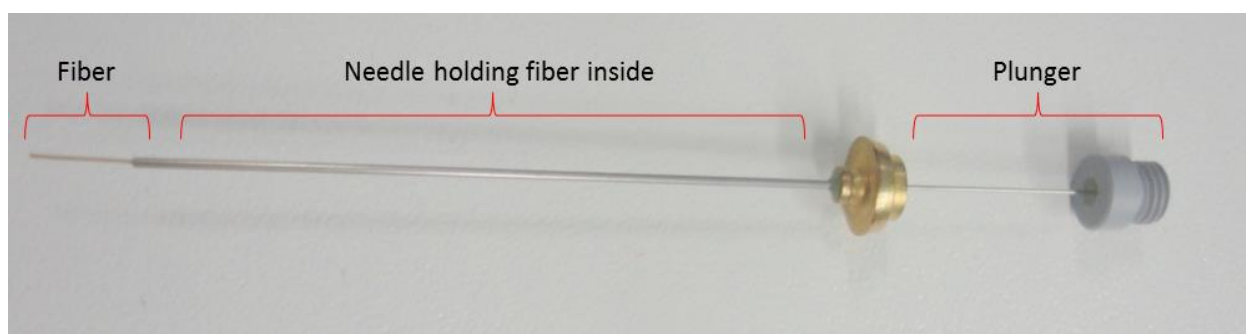
Figure 8 SPME assembly with fiber

SPME is based on the partition equilibrium of target analytes between a polymeric stationary phase (coated fused silica fiber) and the sample matrix. In order to extract analytes SPME does not require organic solvents. The transport of analytes from the matrix into the coating begins when the coated fiber has been placed in contact with the sample. The equilibrium conditions can be described as,

$$n = \frac{K_f V_f V_s C_O}{K_{fs} V_f + V_s} \quad \underline{\quad} \quad \text{(Pawliszyn 1999)}$$

where, n is the amount extracted by the coating, $K_{fS.}$ is a fiber coating/sample matrix distribution constant, V_f is the fiber coating volume, V_S is the sample volume, $C°$ is the initial concentration of a given analyte in the sample. The microextraction process is complete when the analytes concentration has reached distribution equilibrium between the sample matrix and the fiber coating.

We have used headspace solid-phase microextraction (HS-SPME) for grape volatiles extraction in the experiment reported in chapter 3. A typical HS-SPME experiment consists of a two main steps; first is adsorption and second is desorption. Volatiles in the sample are released into the headspace of the vial by heating the sample to the desired temperature and later they are adsorbed on SPME fiber (figure 9). After adsorption, the fiber is inserted onto the heated GC inlet where pre-adsorbed volatiles are released directly into the chromatographic column, and subsequently separated by GC. Several types of different fibers are now available on the market depending on the nature of analyte; many applications of SPME were reported in the field of fruit chemistry and especially for fruit aroma analysis. Volatiles in grape and wine are extensively studied by using SPME over the years (Fedrizzi et al., 2012; Nasi, Ferranti, Amato, & Chianese, 2008; Ong & Acree, 1999; Risticevic, Deell, & Pawliszyn, 2012; Sánchez-Palomo, Díaz-Maroto, & Pérez-Coello, 2005; Yang et al., 2009).
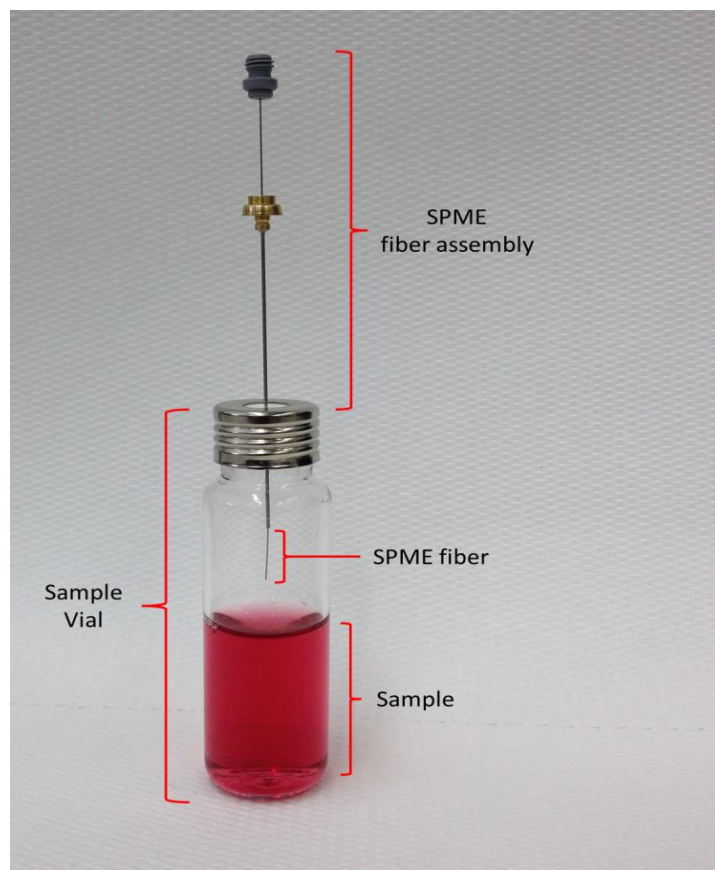
Figure 9 HS-SPME sampling mechanism

2.4.2. Solid phase extraction (SPE)
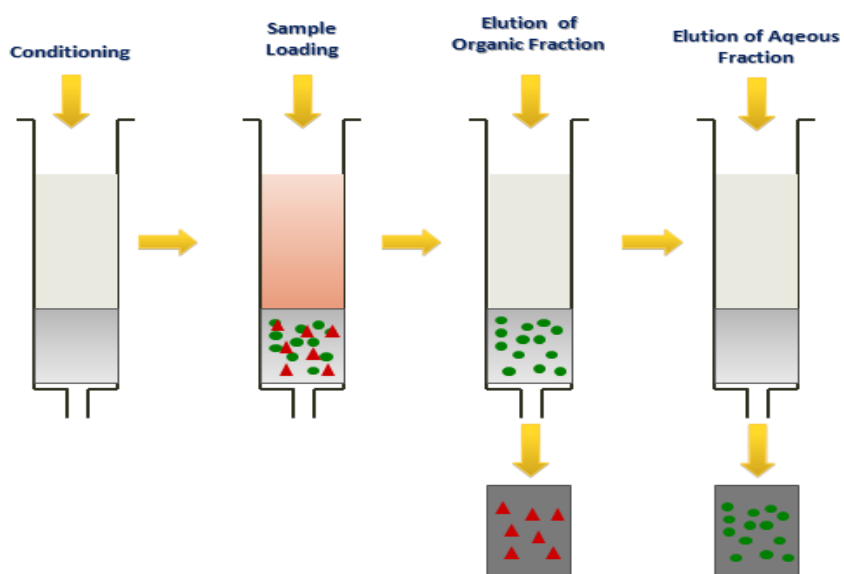
Figure 10 Solid phase extraction experiment

Solid phase extraction (SPE) is a popular sample preparation technique very similar to classical column chromatography method. It is mainly used for the sample purification and/or for the extraction of compounds. A typical syringe shaped SPE cartridge contains a sorbent particles and chromatographic phase packed in it. SPE prominently deals with liquid samples; a typical SPE workflow (figure 10) includes steps like conditioning, sample loading and extraction. In conditioning step, the chromatographic particles in the cartridge were activated by solvents (done by the methanol followed by water in our experiment) it also removes small air in the cartridges that makes further steps easier. Next step is sample loading, where liquid sample is loaded onto the cartridge and passed through by applying vacuum or pressure. In figure 10 sample loading is displayed by orange colored cartridge where active analytes in red and green color are being trapped in the chromatographic phase. In next step the set of non-polar analytes (red color) is eluted with choice of organic solvent and then rest of polar metabolites (green) were eluted with polar solvent.

In the experiment (chapter 4), we have used Isolute ENV+ (1 g, 6 mL) cartridges for the extraction of free and glycosidically conjugated volatiles from the grapes. Many studies (Baek & Cadwallader, 1999; Boido et al., 2003; Metafa & Economou, 2012; Vrhovsek et al., 2014) reported the use and applications of SPE in grape aroma analysis. This technique is also used for sample simplification, matrix effect reduction, fractionation and trace components concentration.

3. Comprehensive mapping of volatile organic compounds in grapes

3.1. Introduction

Grape is one of the oldest fruit to be cultivated by the humankind and today it is amongst most extensively produced and consumed fruit in the world. Currently about ≈20970, cultivars registered globally (*Vitis* international variety catalogue, http://www.vivc.de). In the year 2013 Grape ranked 3rd highest produced fruit worldwide following to apple and banana with 77 million tonnes production and about 7.1 million Ha area under cultivation. Moreover, in the same year totally 27.4 million tonnes of grape wine was produced worldwide (FAOSTAT 2013, http://faostat3.fao.org). Increasing consumption of grape and wine over the years places grape as an economically important fruit and the development of new grape varieties will play a very crucial role in the growing need of the consumers of grape and wine. Moreover, new varieties with consistent production of balanced and flavorful berries are always been an interest to the community of grape growers and oenologists. Prior information on the quality of grape (genetic background, polyphenols, sugar, disease resistance and aroma profile) can make great impact on the breeding of new grape varieties. Furthermore, additional information on these quality parameters can aid grape growers in optimal selection of harvest plans and other agricultural practices.

VOCs in grape are one of the important factors that determine the aroma based varietal characteristics and are crucial part the flavor of grape and its other processed products like wine, raisin etc. For many years VOCs been extensively studied through different studies where hundreds of compounds in grape and wine were identified (Anthony L. Robinson et al., 2013, 2014; Schreier, Drawert, & Junker, 1976). Synthesis of volatile organic compounds in grape occur through different biosynthetic pathways which are mainly depends on its genetic characters. In addition to grape derived compounds, many others are introduced through the process of vinification (including pressing, fermentation, ageing) in the case of wine. The complex profile of these compounds gives unique characteristic aroma to grape and its wine.

Metabolomics is an emerging field in the biology and chemistry that offers a valuable tool for the study of multiple classes of plant secondary metabolites on large scale. Studies in recent years have demonstrated different metabolomics approaches to understand different

molecular mechanisms in the plant (Cramer et al., 2007; Figueiredo et al., 2008; Flamini et al., 2013; Schauer, Zamir, & Fernie, 2005; Jianqiang Song, Shellie, Wang, & Qian, 2012). Similarly, several studies were also reported using different metabolomics profiling methods in grapes (Figueiredo et al., 2008; Gil et al., 2013; Pacifico et al., 2011; Son et al., 2009). Figueiredo et al. 2008 described transcriptional and metabolomics profiling of grape for understanding possible innate resistance against pathogenic fungi. Recent study by Vrhovsek and co-workers (Vrhovsek et al., 2014) proposed a targeted metabolomics profiling for the quantitation of multiple volatiles in grape.

At present genomes of the different fruits like grape (Grimplet et al., 2012; Jaillon et al., 2007; Velasco et al., 2007), apple (Velasco et al., 2010) and strawberry (Shulaev et al., 2011) are available and continuous advancement in the field of genomics could reveal many more fruit genomes in the near future and as suggested and cited by Oksman-Caldentey et al. (Oksman-Caldentey & Saito, 2005)

*Spectacular advances in plant **metabolomics** offer new possibilities together with the aid of systems biology, to explore the extraordinary complexity of the plant biochemical capacity. State-of-the art genomics tools can be combined with **metabolic profiling** to identify key genes that could be engineered for the production of improved crop plants*

Metabolic profiling of the volatile aroma compounds in large selection of grape genotypes through the state-of-the-art methodology was performed in this experiment. The database of compounds identified in selected grape varieties was created, which will be further combined to the grape metabolome database of the.

3.2. Material and methods

3.2.1. Sample collection

Diverse collection of grape genotypes representing different species, colors and genetic characters was selected for the experiment (table 3). The Fondazione Edmund Mach, San

Michelle all'Adige (TN) Italy is one of the leading institutes in the world in the field of grape and wine research and holds broad range of ampelographic collection of grapes. All the grape varieties included in this chapter were obtained from the same collection. Healthy grape berries were sampled in four consecutive vintages (2007-2010) at technological maturity, where technological maturity is defined as the content of soluble solids in the must corresponding to 18° Brix. The berries were collected from different vines and different bunches of each vine. Approximately 500 g of berries from three different wines were collected. Table 1 indicates the list of the grape varieties included in the study.

Girelli F3 (30, 53, 66, 104, Pn x Me) and IASMA ECO 3 varieties in the study were crossings of Muscat Ottonel and Malvasia di Candia; 41B is a cross of Chasselas and *Vitis berlandieri;* Kober 5 BB is a cross of *Vitis berlandieri planchon* and *Vitis riparia michaux;* Isabella is a cross of *Vitis labrusca* and *Vitis vinifera*.

Table 2 List of the grape varieties included in the study (Chapter 3)

	Variety	CODE	Variety number (*http://www.vivc.de/*)	Colour	Vintage Year				Species
					2007	2008	2009	2010	
1	Aglianico	AGL	121	RED	I	I	I	I	*Vitis vinifera*
2	Aleatico	ALE	259	RED	I	I	I	I	*Vitis vinifera*
3	Alicante bouchet	ALB	304	RED	I	I	I	I	*Vitis vinifera*
4	Ancncellotta	ANC	447	RED	I	I	I	I	*Vitis vinifera*
5	Inzolia	INZ	492	White	I	I	I	I	*Vitis vinifera*
6	Barbera	BAR	974	RED	I	I	I	I	*Vitis vinifera*
7	Grignolino	GRI	1283	RED	I	I	I	I	*Vitis vinifera*
8	Vernaccia trentina	VEN	1329	White	I	I	I	I	*Vitis vinifera*
9	Franconia	FRN	1459	RED	I	I	I	I	*Vitis vinifera*
10	Sangiovese	SAN	1709	RED	I	I	I	I	*Vitis vinifera*
11	Cabernet franc	CAF	1927	RED	I	I	I	I	*Vitis vinifera*
12	Cabernet sauvignon	CAS	1929	RED	I	I	I	I	*Vitis vinifera*
13	Nero d'Avola	NED	1986	RED	I	I	I	I	*Vitis vinifera*
14	Carmenere	CAR	2109	RED	I	I	I	I	*Vitis vinifera*
15	Tannat	TAN	2257	RED	I	I	I	I	*Vitis vinifera*
16	Cataratto	CAT	2341	White	I	I	I	I	*Vitis vinifera*
17	Cesanese	CES	2398	RED	I	I	I	I	*Vitis vinifera*
18	Chardonnay	CHA	2455	White	NI	I	I	I	*Vitis vinifera*
19	Ciliegiolo	CIG	2660	RED	I	I	I	I	*Vitis vinifera*
20	Corvina	COR	2863	RED	I	I	NI	I	*Vitis vinifera*
21	Croatina	CRO	3251	RED	I	I	I	I	*Vitis vinifera*
22	Riesling	RIE	10077	White	I	I	I	I	*Vitis vinifera*
23	Dolcetto	DOL	3626	RED	I	I	NI	I	*Vitis vinifera*
24	Fiano	FIA	4124	White	I	I	I	I	*Vitis vinifera*
25	Enantio	ENA	4171	RED	I	I	I	I	*Vitis vinifera*
26	Frappato	FRP	4225	RED	NI	I	I	I	*Vitis vinifera*

27	Gaglioppo	GAL	4306	RED	I	I	I	I	*Vitis vinifera*
28	Garganega	GAR	4419	White	I	I	I	I	*Vitis vinifera*
29	Cannonau	CAN	4461	RED	I	I	I	I	*Vitis vinifera*
30	Bovale Sardo	BOV	4935	RED	I	I	I	I	*Vitis vinifera*
31	Grechetto	GRH	4966	White	I	I	I	I	*Vitis vinifera*
32	Greco de tufo	GRT	4970	White	I	I	I	I	*Vitis vinifera*
33	Groppello Gentile	GRO	5078	RED	I	I	I	I	*Vitis vinifera*
34	Italia	ITA	5582	White	NI	I	I	I	*Vitis vinifera*
35	Lagrein	LGR	6666	RED	I	I	I	I	*Vitis vinifera*
36	Lambrusco olive	LAO	6698	RED	NI	I	I	I	*Vitis vinifera*
37	Lambrusco Salamino	LAS	6701	RED	I	I	I	I	*Vitis vinifera*
38	Malvasia Puntinata	MAP	7256	White	I	I	I	I	*Vitis vinifera*
39	Malvasia nera di lecce	MNL	7273	RED	I	I	I	I	*Vitis vinifera*
40	Incrocio Manzoni	INM	7360	White	NI	I	I	I	*Vitis vinifera*
41	Marsanne	MAR	7434	White	I	I	I	I	*Vitis vinifera*
42	Marzemino	MAZ	7463	RED	I	I	I	I	*Vitis vinifera*
43	Merlot	MER	7657	RED	I	I	I	I	*Vitis vinifera*
44	Molinara	MOL	7899	Pink	I	I	I	I	*Vitis vinifera*
45	Montagna	MON	7937	White	I	I	I	I	*Vitis vinifera*
46	Primitivo	PRI	7949	RED	I	I	I	I	*Vitis vinifera*
47	Moscato Rosa	MOR	8057	Pink	NI	I	I	I	*Vitis vinifera*
48	Moscato ottonel	MOO	8243	White	I	I	I	I	*Vitis vinifera*
49	Muscat Rouge de Madere	MRM	8249	Pink	I	I	I	I	*Vitis vinifera*
50	Muskat vostochny	B19	8298	RED	NI	NI	I	I	*Vitis vinifera*
51	Nebbiolo	NBB	8417	RED	I	I	I	I	*Vitis vinifera*
52	Negroamaro	NEG	8456	RED	I	I	I	I	*Vitis vinifera*
53	Nosiola	NOS	8606	White	I	I	I	I	*Vitis vinifera*
54	Ortrugo	ORT	8813	White	I	I	I	I	*Vitis vinifera*
55	Visentiona	VIS	9057	RED	I	I	I	I	*Vitis vinifera*
56	Perla di Csaba	PER	9166	White	I	I	NI	I	*Vitis vinifera*
57	Pedirosso	B37	9239	RED	NI	NI	I	I	*Vitis vinifera*
58	Pignoletto	PIG	9254	White	I	I	I	I	*Vitis vinifera*
59	Pinot gris	PNG	9275	Pink	I	I	I	I	*Vitis vinifera*

60	Pinot noir	PNN	9279	RED	I	I	I	I	*Vitis vinifera*
61	Pinotage	PIN	9286	RED	I	I	NI	I	*Vitis vinifera*
62	Primitivo di giola	PRG	9703	RED	I	I	I	I	*Vitis vinifera*
63	Prosecco	PRO	9741	White	I	I	I	I	*Vitis vinifera*
64	Raboso del plave	RAB	9864	RED	I	I	I	I	*Vitis vinifera*
65	Rebo	REB	9961	RED	I	I	I	I	*Vitis vinifera*
66	Refosco	REF	9987	RED	I	I	I	I	*Vitis vinifera*
67	Ribolla gialla	RIB	10054	White	I	I	I	I	*Vitis vinifera*
68	Rondinella	RON	10189	RED	I	I	I	I	*Vitis vinifera*
69	Roussanne	ROU	10258	White	I	I	I	I	*Vitis vinifera*
70	Sagrantino	SAG	10457	RED	I	I	I	I	*Vitis vinifera*
71	Saint Laurent	SAL	10470	RED	I	I	I	I	*Vitis vinifera*
72	Montepulciano	MOT	10680	RED	I	I	I	I	*Vitis vinifera*
73	Saperavi	SAP	10708	RED	NI	NI	NI	I	*Vitis vinifera*
74	Sauvignon blanc	SAU	10790	White	NI	I	I	I	*Vitis vinifera*
75	Schiava grossa	SCG	10823	RED	I	I	I	I	*Vitis vinifera*
76	Schiava lombarda	SCL	10825	RED	I	I	I	I	*Vitis vinifera*
77	Schioppettino	SCH	10830	RED	I	I	I	I	*Vitis vinifera*
78	Syrah	SYR	11748	RED	I	I	I	I	*Vitis vinifera*
79	Tarrango	TAR	12267	RED	I	I	I	I	*Vitis vinifera*
80	Tempranillo	TEM	12350	RED	I	I	I	I	*Vitis vinifera*
81	Teroldego	TER	12371	RED	I	I	I	I	*Vitis vinifera*
82	Gewürztraminer	GWT	12609	Pink	I	I	I	I	*Vitis vinifera*
83	Uva di troila	UVT	12819	RED	I	I	I	I	*Vitis vinifera*
84	Peverella	PEV	12963	White	I	I	I	I	*Vitis vinifera*
85	Verdicchio marche	VEM	12963	White	I	I	I	I	*Vitis vinifera*
86	Verduzzo friulano	VEF	12976	White	I	I	I	I	*Vitis vinifera*
87	Verduzzo Trevigiano	VET	12977	White	NI	I	I	I	*Vitis vinifera*
88	Viogner	VIO	13106	White	I	I	I	I	*Vitis vinifera*
89	Xarello	XAR	13270	White	I	NI	I	I	*Vitis vinifera*
90	Xinomavro	XIN	13284	RED	NI	I	I	I	*Vitis vinifera*
91	Zweigelt	ZWE	13484	RED	I	I	I	I	*Vitis vinifera*
92	Pinot tete de negre	PNT	15091	RED	I	I	I	I	*Vitis vinifera*

93	Kozma palne muskotaly	KPM	15732	White	I	I	I	I	*Vitis vinifera*
94	Lagarino bianco	LAG	20366	RED	I	I	I	I	*Vitis vinifera*
95	Verdealbara	VER	22363	White	I	I	I	I	*Vitis vinifera*
96	Casetta	CAE	23015	RED	I	I	I	I	*Vitis vinifera*
97	Malvasia bianca di candia	MAC	23555	White	I	I	I	I	*Vitis vinifera*
98	Biancaccia	BAC	40336	White	I	I	I	I	*Vitis vinifera*
99	Bianera	BAN	40338	White	I	I	I	I	*Vitis vinifera*
100	Rosetta di montagna	ROS	40906	Pink	I	I	I	I	*Vitis vinifera*
101	Valderbara	VAL	40920	RED	I	NI	I	I	*Vitis vinifera*
102	Vernaccia del Cavalot	VEC	40924	White	I	I	I	I	*Vitis vinifera*
103	Vattara O biancazza	VTT	ITA362-2447	White	I	NI	I	I	*Vitis vinifera*
104	Gewürztraminer giaroni	GWG	12609	Pink	NI	NI	I	NI	*Vitis vinifera*
105	Vernazzola	VEA	P11#2453	White	I	I	I	I	*Vitis vinifera*
106	Vitis Andersoni	VAN	13491	RED	I	I	I	I	non Vinifera
107	Vitis arizonica texas	VAT	13493	RED	I	I	I	NI	non Vinifera
108	Vitis californica	VCA	13506	RED	I	I	I	I	non Vinifera
109	Vitis cinerea	VCI	13515	RED	I	I	I	I	non Vinifera
110	Vitis slavini	VSL	13596	RED	I	NI	NI	NI	non Vinifera
111	Vitis champini	VCH	16423	RED	I	I	I	I	non Vinifera
112	Vitis riparia	VRI	NA	RED	I	NI	NI	NI	non Vinifera
113	Isabella	ISA	5560	RED	NI	I	I	I	Interspecific crossing
114	Kober 5 BB	KBB	6313	RED	I	I	I	I	Interspecific crossing
115	Millardet et grasset 41 B	41B	7736	RED	I	I	I	I	Interspecific crossing
116	Nero	NER	14013	RED	I	I	I	I	Interspecific crossing
117	Nera dei baisi	NEB		RED	I	I	I	I	Interspecific crossing
118	Girelli Pn x Me	GPM	NA	RED	NI	I	I	I	Interspecific crossing
119	Girelli F3-P104	G04	NA	White	I	I	I	I	Interspecific crossing
120	Girelli F3-P30	G30	NA	White	NI	I	I	I	Interspecific crossing
121	IASMA ECO 3	G51	NA	White	I	I	I	I	Interspecific crossing
122	Girelli F3-P63	G63	NA	White	NI	I	I	I	Interspecific crossing
123	Girelli F3-P66	G66	NA	White	NI	I	I	I	Interspecific crossing
124	Girelli F3-P73	G73	NA	White	NI	I	I	I	Interspecific crossing

*I-Included varieties, *NI-Not included varieties, Accession numbers for Vernazzola & Vattara O biancazza are obtained from http://www.eu-vitis.de/

3.2.2. Grape powders

The grape berries were ground using analytical mill (IKA A11) under liquid nitrogen and stored at -80°C. For pooled sample, berries of selected grapes varieties (Gewürztraminer, Moscato Ottonel, Moscato Rosa, Riesling, Malvasia bianca di Candia, Cabernet sauvignon, Merlot, Sangiovese and Pinot gris) were mixed and powdered together and also stored at -80°C.

3.2.3. Chemicals and reagents

Magnesium sulfate, ascorbic acid and citric acid were purchased from by Sigma-Adrich (Milan, Italy). The water used was purified in a MilliQ device (Millipore, Bedford, MA; USA). D7-benzyl alcohol, d3-linalool and d11-ethyl hexanoate were purchased from Chemical Research 2000 (Rome, Italy).

3.2.4. Internal standards

A mixture of deuterated grape volatile compounds containing benzyl alcohol-d7 (24.7 mg/L), linalool-d3 (14.5 mg/L) and ethyl hexanoate-D11 (50 mg/L) was used as internal standards in the experiment. The retention times of internal standards are as follows, ethyl hexanoate-D11 (9.74 min), linalool-d3 (16.89min) and benzyl alcohol-d7 (22.86 min). Also a mixture of 18 pure standard grape aroma compounds was used in batch analysis to monitor instrumental stability.

3.2.5. Sample preparation

For the analysis, water (7 mL) and ascorbic acid (15 mg), citric acid (15 mg) and sodium azide (50 mL of a 1000 mg/L solution) were added as preservatives in the vial containing 4 g of sample grape powder and MgSO4 (2 g). All preservatives were added to avoid any microbiological/enzymatic reactions during storage of the sample at 4°C (Fedrizzi et al., 2012). An internal standard (50 µL) was also added to each sample. Four different samples

were prepared containing real sample, QC sample, Blank and Std. mixture (table 2) for the batch analysis.

Table 3 Sample types in the batch experiment

Ingredient	Volume	Sample type			
		Real Sample	QC Sample	Blank	Std. Mix
Sample Powder	4 g	Yes	--	--	--
Pooled/QC powder	4 g	--	Yes	--	--
Water	7 mL	Yes	Yes	Yes	Yes
Ascorbic acid	15 mg	Yes	Yes	Yes	Yes
Citric acid	15 mg	Yes	Yes	Yes	Yes
Sodium azide	50 μL	Yes	Yes	Yes	Yes
MgSO4	2 g	Yes	Yes	Yes	Yes
Internal standard	50 μL	Yes	Yes	Yes	Yes
Standard mixture	50 μL	--	--	--	Yes

3.2.6. Headspace solid phase microextraction (HS-SMPE)

DVB/CAR/PDMS, 2 cm 50/30μm (Supelco, Sigma-Aldrich, Milan, Italy) SPME fibers were used (Fedrizzi et al., 2012)for the extraction of volatiles. The volatiles were extracted for 40 min at 60°C with constant stirring at 450 rpm and then the analytes from fiber were desorbed into GC inlet at 250°C for 2 min in splitless mode. Once sample was prepared, the sample vial was kept in the PAL combi-xt (CTC, Zwingen, Switzerland) autosampler with controlled temperature of 4°C. The standing time of the samples in the autosampler was maintained not to exceed limit of 8 hours to prevent the further chemical reactions in the sampling vial.

3.2.7. GC-MS analysis

The GC-MS analysis was performed by using Thermo Trace GC Ultra gas chromatograph coupled to a Thermo Quantum XLS mass spectrometer (Thermo Scientific, Milan, Italy). After SPME extraction, the analytes were desorbed from fiber onto the GC with inlet

temperature of 250°C in splitless mode with a narrow liner (0.75mm id, Thermo, Milan, Italy). Chromatographic separation was achieved by ZB-WAX 30 m long polar column with 0.25 mm inner diameter and film thickness of 0.25 µm (Phenomenex, Castel Maggiore, Italy). GC oven temperature was programed from 40°C with hold of 4 min and then ramped with 6°C/min up to 250°C with final hold of 5 min. Helium was used as carrier gas in constant flow mode with the rate of 1.2 mL/min at. Mass spectrometer was operated in positive mode electron ionization (70eV) with full scan mode at a scan range of 30-350 Dalton. MS transfer line and ion source was set at 250°C. The instrument was operated and controlled by Xcalibur 2.1.0 software. Figure 11 shows the total ion chromatogram (TIC) of one of the grape samples analyzed under above conditions.
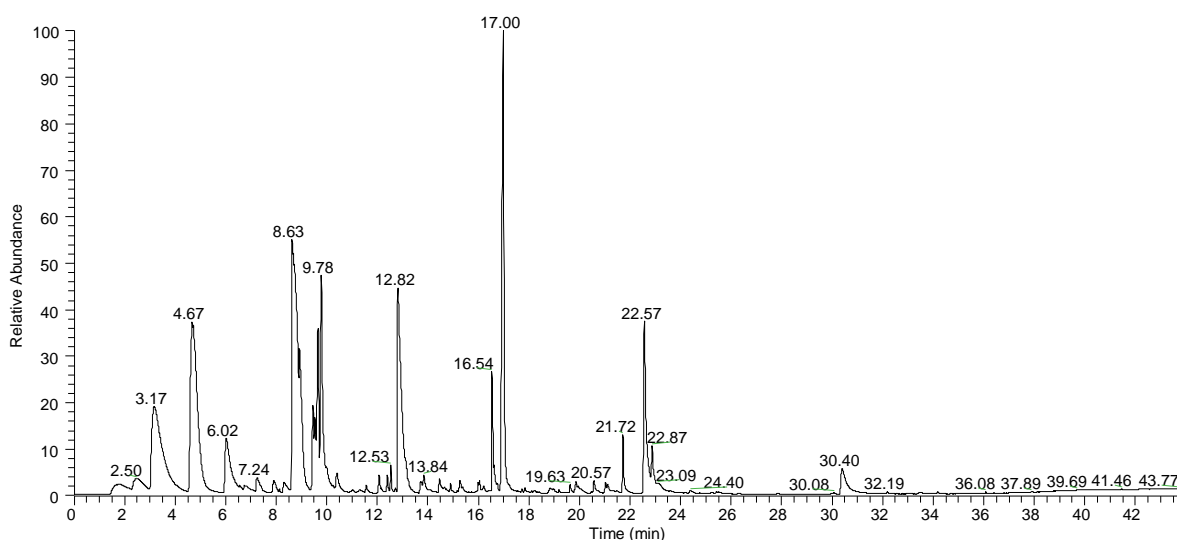
Figure 11 TIC of Kozma Palne Muscotaly

3.2.8. In-house database for the volatile organic compounds

For the annotation, an in-house database of the volatile organic compounds in grape was created at the institute. Several pure reference standards of the grape volatiles (≥350) were analyzed (in mixture or individually) by maintaining the same instrumental parameters as for samples. Different parameters for each compound like name, retention time and CAS number were recorded to create a multidimensional dataset of the compounds. Instrument generated raw files of the standards analysis were converted into the computable document files (CDF) for the further data mining. Later the information of each compound and

corresponding CDF files were used to generate pseudospectra of the compounds. The pseudospectra were generated based on manually identified retention time of the pure compounds and further validated with Wiley/NIST 08 database; finally, they are stored in the in-house library database. This database is further used for the compound annotation using MetaMS pipeline (Wehrens, Weingart, & Mattivi, 2014).

3.2.9. Annotation and quantitation

The compound annotation and identification was done exclusively using MetaMS pipeline (Wehrens et al., 2014). If two orthogonal properties, pseudospectra and retention time of the standard database compound matches with pseudospectra and retention time of the sample then it is considered as the level 1 identification, i.e. complete annotation. The quantification of the compounds is achieved by comparing the ion intensities of the pseudospectra in the experimental data to the ion intensities of the pseudospectra in the standard database. In order to obtain reliable and robust quantifications as many ions as possible are considered within one pseudospectra. The relative intensities values of the pseudospectra in the real samples to the patterns in the in-house database were obtained by using least-trimmed-squares regression (Wehrens et al., 2014).

3.2.10. Data analysis

To obtain an more illustrative results for the each varieties, average of intensity values of four vintages were considered for the statistical analysis (Boido et al., 2003). Multivariate statistical analysis was performed on log transformed data (Farneti et al., 2015; Tarr et al., 2013), figure 13 & 14 were generated using SIMCA P+ (version 12.0, Metrics) and figure 16 was generated using R 3.0.2 internal statistical functions and the Package *diversitree* (version 0.9-4)(Farneti et al., 2015; Fitzjohn, 2012). Bubble graph (figure 15) was generated using plotly online data analysis tool (https://plot.ly/).

3.3. Results

Using the automated pipeline, we have successfully identified total 117 compounds at level 1 collectively in the all selected varieties and vintages. In order to provide the most robust results, the automatic identification was also verified manually in not less than 3 independent samples here. The list of all the compounds is given here in table 4.

Table 4 List of the compounds identified (Chapter 3)

No.	Name	Class	Molecular Formula	Monoisotopic mass	Retention time	ChemSpider ID
1	Acetic acid	Acid	$C_2H_4O_2$	60.02	14.87	171
2	1-Decanol	Alcohol	$C_{10}H_{22}O$	158.17	17.28	7882
3	1-Hexanol	Alcohol	$C_6H_{14}O$	102.10	12.87	7812
4	1-Nonanol	Alcohol	$C_9H_{20}O$	144.15	19.59	8574
5	1-Octanol	Alcohol	$C_8H_{18}O$	130.14	17.28	932
6	1-Octen-3-ol	Alcohol	$C_8H_{16}O$	128.12	14.91	17778
7	1-Pentanol	Alcohol	$C_5H_{12}O$	88.09	9.39	6040
8	2-Phenoxyethanol	Alcohol	$C_8H_{10}O_2$	138.07	27.39	13848467
9	2-Phenylethanol	Alcohol	$C_8H_{10}O$	122.07	23.70	5830
10	3-Methyl-1-butanol	Alcohol	$C_5H_{12}O$	88.09	9.08	29000
11	5-Hexenol	Alcohol	$C_6H_{12}O$	100.09	14.00	63156
12	6-Methyl-5-hepten-2-ol	Alcohol	$C_8H_{16}O$	128.12	15.37	19533
13	Benzyl alcohol	Alcohol	C_7H_8O	108.06	23.11	13860335
14	E-2-Hexenol	Alcohol	$C_6H_{12}O$	100.09	14.03	4476685
15	E-3-Hexen-1-ol	Alcohol	$C_6H_{12}O$	100.09	13.09	4447565
16	Z-3-Hexenol	Alcohol	$C_6H_{12}O$	100.09	13.55	21105914
17	5-Hydroxymethyl-2-	Aldehyde	$C_6H_6O_3$	126.03	32.70	207215
18	5-Methylfurfural	Aldehyde	$C_6H_6O_2$	110.04	17.60	11600
19	Benzaldehyde	Aldehyde	C_7H_6O	106.04	16.52	235
20	E-2-Heptenal	Aldehyde	$C_7H_{12}O$	112.09	12.33	4446437
21	E-2-Hexenal	Aldehyde	$C_6H_{10}O$	98.07	9.43	4444608
22	E-2-Nonenal	Aldehyde	$C_9H_{16}O$	140.12	16.82	4446456
23	E-2-Octenal	Aldehyde	$C_8H_{14}O$	126.10	14.61	4446445
24	E-2-Pentenal	Aldehyde	C_5H_8O	84.06	7.44	4516892
25	EE-2,4-Heptadienal	Aldehyde	$C_7H_{10}O$	110.07	15.87	19131
26	EE-2,4-Hexadienal	Aldehyde	C_6H_8O	96.06	13.86	553167
27	Furfural	Aldehyde	$C_5H_4O_2$	96.02	15.14	13863629
28	Heptanal	Aldehyde	$C_7H_{14}O$	114.10	8.74	7838
29	Hexanal	Aldehyde	$C_6H_{12}O$	100.09	6.09	5949
30	Phenyl acetaldehyde	Aldehyde	C_8H_8O	120.06	18.94	13876539
31	Z-2-Nonenal	Aldehyde	$C_9H_{16}O$	140.12	16.82	4510945
32	beta-Ionol	C13-Norisoprenoid	$C_{13}H_{22}O$	194.17	24.51	4523692
33	Theaspirane	C13-Norisoprenoid	$C_{13}H_{22}O$	194.17	16.23	55810

34	Sclareol_I	Diterpene	C20H36O2	308.27	30.70	143282
35	Sclareol_II	Diterpene	C20H36O2	308.27	30.91	143282
36	Allyl propionate	Ester	C6H10O2	114.07	6.70	55257
37	Benzyl benzoate	Ester	C14H12O2	212.08	34.36	13856959
38	*cis*-3-Hexenyl acetate	Ester	C8H14O2	142.10	12.12	4515742
39	Ethyl acetate	Ester	C4H8O2	88.05	2.64	8525
40	Ethyl anthranilate	Ester	C9H11NO2	165.08	29.47	21106112
41	Ethyl butyrate	Ester	C6H12O2	116.08	5.00	7475
42	Ethyl decanoate	Ester	C12H24O2	200.18	19.15	7757
43	Ethyl heptanoate	Ester	C9H18O2	158.13	12.57	7509
44	Ethyl hexanoate	Ester	C8H16O2	144.12	10.12	29005
45	Ethyl octanoate	Ester	C10H20O2	172.15	14.91	7511
46	Ethyl phenylacetate	Ester	C10H12O2	164.08	21.57	13885245
47	Ethyl salicylate	Ester	C9H10O3	166.06	21.98	21105897
48	Ethyl *trans*-4-decenoate	Ester	C12H22O2	198.16	19.65	4515095
49	Guaiacwood acetate	Ester	C17H28O2	264.21	20.25	55033
50	Heptyl formate	Ester	C8H16O2	144.12	12.37	7877
51	Hexyl acetate	Ester	C8H16O2	144.12	11.11	8568
52	i-Pentyl acetate	Ester	C7H14O2	130.10	7.24	29016
53	Isobornyl acetate	Ester	C12H20O2	196.15	17.95	6207
54	Isoeugenyl phenylacetate	Ester	C18H18O3	282.13	21.58	4814022
55	Methyl anthranilate	Ester	C8H9NO2	151.06	28.94	13858096
56	Methyl salicylate	Ester	C8H8O3	152.05	21.38	13848808
57	alpha-Asarone	Ether	C12H16O3	208.11	32.09	552532
58	2-Ethylfuran	Furan	C6H8O	96.06	3.46	17522
59	2-Pentylfuran	Furan	C9H14O	138.10	9.88	18465
60	m-Xylene	Hydrocarbon	C8H10	106.08	7.46	7641
61	o-Xylene	Hydrocarbon	C8H10	106.08	8.62	6967
62	p-Xylene	Hydrocarbon	C8H10	106.08	7.28	7521
63	1-Octen-3-one	Ketone	C8H14O	126.10	11.68	55282
64	4-Hexen-3-one	Ketone	C6H10O	98.07	9.08	4517756
65	6-Methyl-5-hepten-2-one	Ketone	C8H14O	126.10	12.59	9478
66	alpha-Pinene	Monoterpene	C10H16	136.13	4.68	389795
67	alpha-Terpinene	Monoterpene	C10H16	136.13	9.61	7182
68	alpha-Terpineol	Monoterpene	C10H18O	154.14	20.13	13850142
69	alpha-Terpinyl acetate	Monoterpene	C12H20O2	196.15	20.00	99681
70	beta-Citronellol	Monoterpene	C10H20O	156.15	21.36	92127
71	beta-Cyclocitral	Monoterpene	C10H16O	204.19	18.32	9511
72	beta-Myrcene	Monoterpene	C10H16	136.13	8.15	28993
73	Camphene	Monoterpene	C10H16	136.13	5.62	6364
74	*cis*-Geraniol	Monoterpene	C10H18O	154.14	22.59	558917
75	Citronellal	Monoterpene	C10H18O	154.14	21.22	7506
76	Eucalyptol	Monoterpene	C10H18O	154.14	9.53	2656
77	Farnesene	Monoterpene	C15H24	204.19	20.51	4444849
78	Farnesol	Monoterpene	C15H26O	222.20	20.71	392816
79	Fenchyl alcohol	Monoterpene	C10H18O	154.14	17.70	14665
80	Geranic acid	Monoterpene	C10H16O2	168.12	30.48	9595
81	Geranyl acetate	Monoterpene	C12H20O2	196.15	21.22	1266019
82	Geranyl acetone _I	Monoterpene	C13H22O	194.17	22.72	1266569

83	Geranyl acetone _II	Monoterpene	C13H22O	195.17	23.27	1266569
84	Geranyl phenylacetate	Monoterpene	C18H24O2	272.18	21.36	4517973
85	Geranyl propionate	Monoterpene	C13H22O2	210.16	22.44	4511742
86	Isopulegol	Monoterpene	C10H18O	154.14	17.47	149356
87	Limonene	Monoterpene	C10H16	136.13	9.11	20939
88	Linalool	Monoterpene	C10H18O	154.14	17.12	13849981
89	Linalool oxide_I	Monoterpene	C10H18O2	170.13	14.82	20938
90	Linalool oxide_II	Monoterpene	C10H18O3	170.13	15.44	20938
91	Linalyl acetate	Monoterpene	C12H20O2	196.15	17.36	13850082
92	Linalyl butyrate	Monoterpene	C14H24O2	224.18	19.98	56116
93	m-Cymol	Monoterpene	C10H14	134.11	10.74	10355
94	Neryl acetate	Monoterpene	C12H20O2	196.15	20.84	1266018
95	Neryl butyrate	Monoterpene	C14H24O2	224.18	23.05	4509113
96	Neryl isobutyrate	Monoterpene	C14H24O2	224.18	21.89	4517923
97	o-Cymol	Monoterpene	C10H14	134.11	11.48	10253
98	p-Cymene	Monoterpene	C10H14	134.11	10.77	7183
99	Rose oxide_I	Monoterpene	C10H18O	154.14	12.88	25927
100	Rose oxide_II	Monoterpene	C10H18O	154.14	13.16	25927
101	Sabinene hydrate	Monoterpene	C10H18O	154.14	10.35	56155
102	Terpinen-4-ol	Monoterpene	C10H18O	154.14	18.26	10756
103	Terpinolene	Monoterpene	C10H16	136.13	11.09	10979
104	*trans*-beta-Farnesene	Monoterpene	C15H24	204.19	19.65	4444850
105	*trans*-Geraniol	Monoterpene	C10H18O	154.14	21.79	13849989
106	alpha-Cedrene	Sesquiterpene	C15H24	204.19	17.59	454638
107	alpha-Humulene	Sesquiterpene	C15H24	204.19	19.95	4444853
108	alpha-Longipinene	Sesquiterpene	C15H24	204.19	15.21	454407
109	Aromadendrene	Sesquiterpene	C15H24	204.19	18.57	9270876
110	beta-Caryophyllen	Sesquiterpene	C15H24	204.19	17.78	4444848
111	beta-Humulene	Sesquiterpene	C15H24	204.19	20.21	4476730
112	gamma-Humulene	Sesquiterpene	C15H24	204.19	19.71	21170000
113	gamma-Neoclovene	Sesquiterpene	C15H24	204.19	18.41	494832
114	Guaiazulene	Sesquiterpene	C15H18	198.14	31.46	3395
115	Guaiene (all isomers)	Sesquiterpene	C15H24	204.19	18.21	16736689
116	Isolongifolene	Sesquiterpene	C15H24	204.19	17.93	92636
117	Ledene	Sesquiterpene	C15H24	204.19	20.15	9085910

Majority of the identified compounds were from the classes monoterpenes (n=40), esters (n=21), aldehyde (n=15), alcohols (n=15), sesquiterpenes (n=12) and furthermore some identifications were made from classes like hydrocarbons, ketones, furans, diterpenes, C13-norisoprenoids, ether and acid. Compound class based description with a discussion of some key compounds is presented (figure 12)
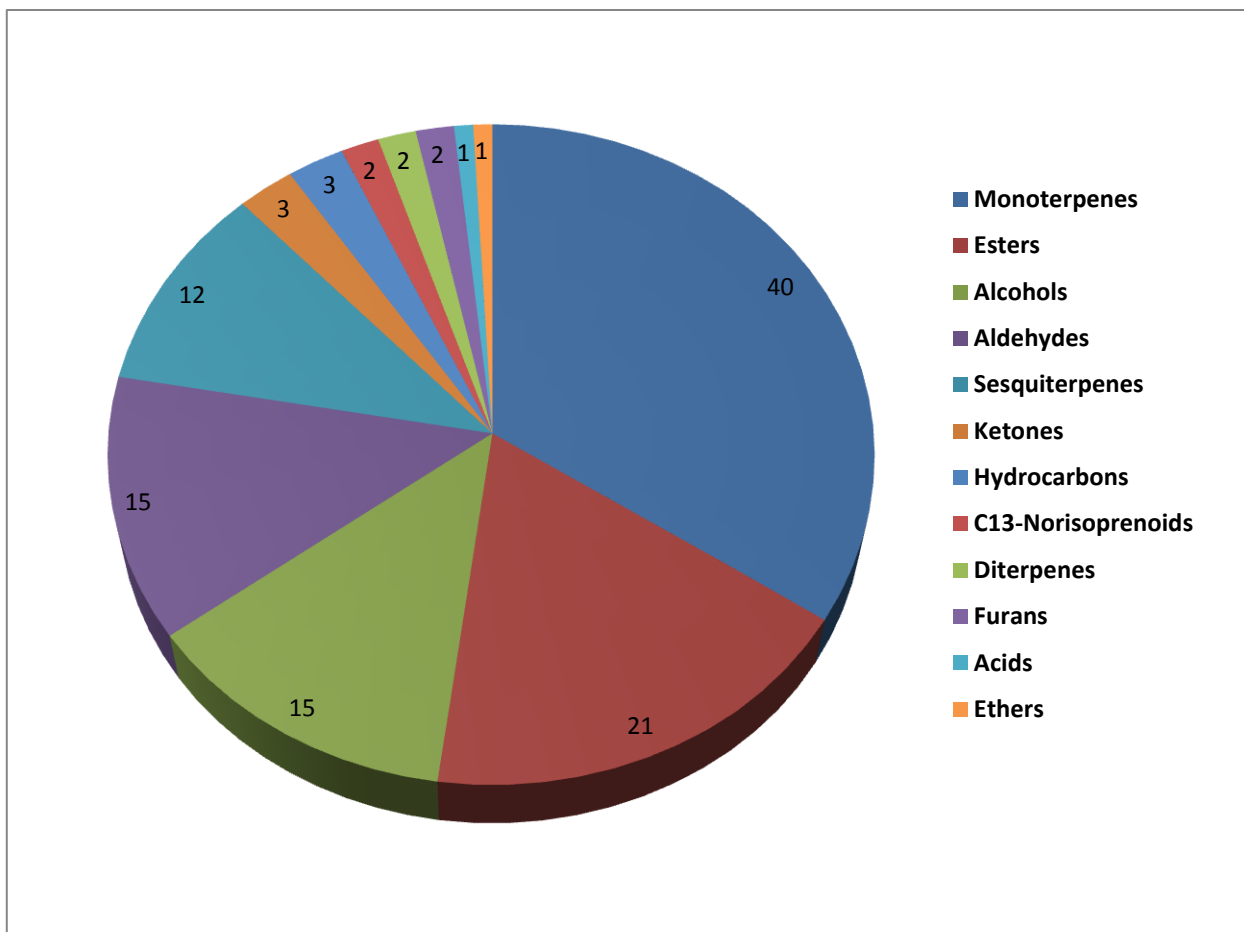
Figure 12 Chemical classes based distribution of the identified compounds

Unsupervised principal component analysis was performed. The PCA score plot (figure 13) suggested the separation of all varieties into five different groups based on their profile of volatile organic compound.

Group 1 shows grouping of 10 varieties i.e Muscat vostochny, Girelli F3-P104, Girelli F3-P30, IASMA ECO3, Girelli F3-P66, Girelli F3-P73, Gewürztraminer, Kozma palne muskotaly, Moscato ottonel and Perla di Csaba. All varieties in this group shows common presence of aroma active monoterpenes like linalool, linalool oxides, farnesol, beta-myrcene, rose oxide, geraniol, terpinolene, neryl butyrate, neryl isobutyrate and limonene. The profile of compounds displayed by this group explains the high floral aroma of these varieties and their origin, since all varieties in this group is either Muscat or its offspring (table 3).

Muscat grapes are typically known for their characteristic fruity and floral aroma originating from terpenic compounds like rose oxide, linalool oxide, linalool, a-terpineol, citronellol, nerol, geraniol, benzyl alcohol and 2-phenylethanol (Fenoll, Manso, Hellín, Ruiz, & Flores, 2009; Ribéreau-Gayon, Boidron, & Terrier, 1975; Ruiz-García, Hellín, Flores, & Fenoll, 2014). Moreover, presence of interspecific crossings G30, G04, G66, G73 and IASMA ECO3 (table 3) in this group which are progenies of aromatic cultivars Moscato ottonel and Malvasia Bianca (Emanuelli et al., 2010; Mateo & Jiménez, 2000) also supports their genetic similarities. Another aromatic variety Gewürztraminer (Girard & Fukumoto, 2002; Ong & Acree, 1999) was also included in the same group. In general, white colour (with the exception of Gewürztraminer that is pink) and high monoterpenic content observed dominantly in the varieties in this group.

In the group 2, eleven *Vitis vinifera* varieties were included i.e Alicante bouchet, Carmenere, Cesanese, Corvina, Malvasia Puntinata, Negroamaro, Primitivo, Sagrantino, Tarrango, Vernazzola and Vattara O biancazza. Varieties in this group commonly show high intensity values for the compounds farnesol, geranic acid, alpha-Pinene, citronellal and limonene. Some of the common compounds detected in this group are alcohols (1-hexanol, 1-octen-3-ol, E-2-hexenol), aldehydes (benzaldehyde, E-2-heptenal, E-2-hexenal), diterpene sclareol. Interestingly, all verities in this group showed very rich profile of all the sesquiterpenes (except alpha and gamma humulene) in the table 4 than any other group. Many sesquiterpenes found in these varieties such as alpha-cedrene, aromadendrene and isolongifolene are known for woody odor and compound like beta-caryophyllene shows spicy aroma but with very low aroma threshold. So all these variety shows very mild aromatic characteristic, only one variety in this group i.e Malvasia Puntinata showed presence of geraniol.
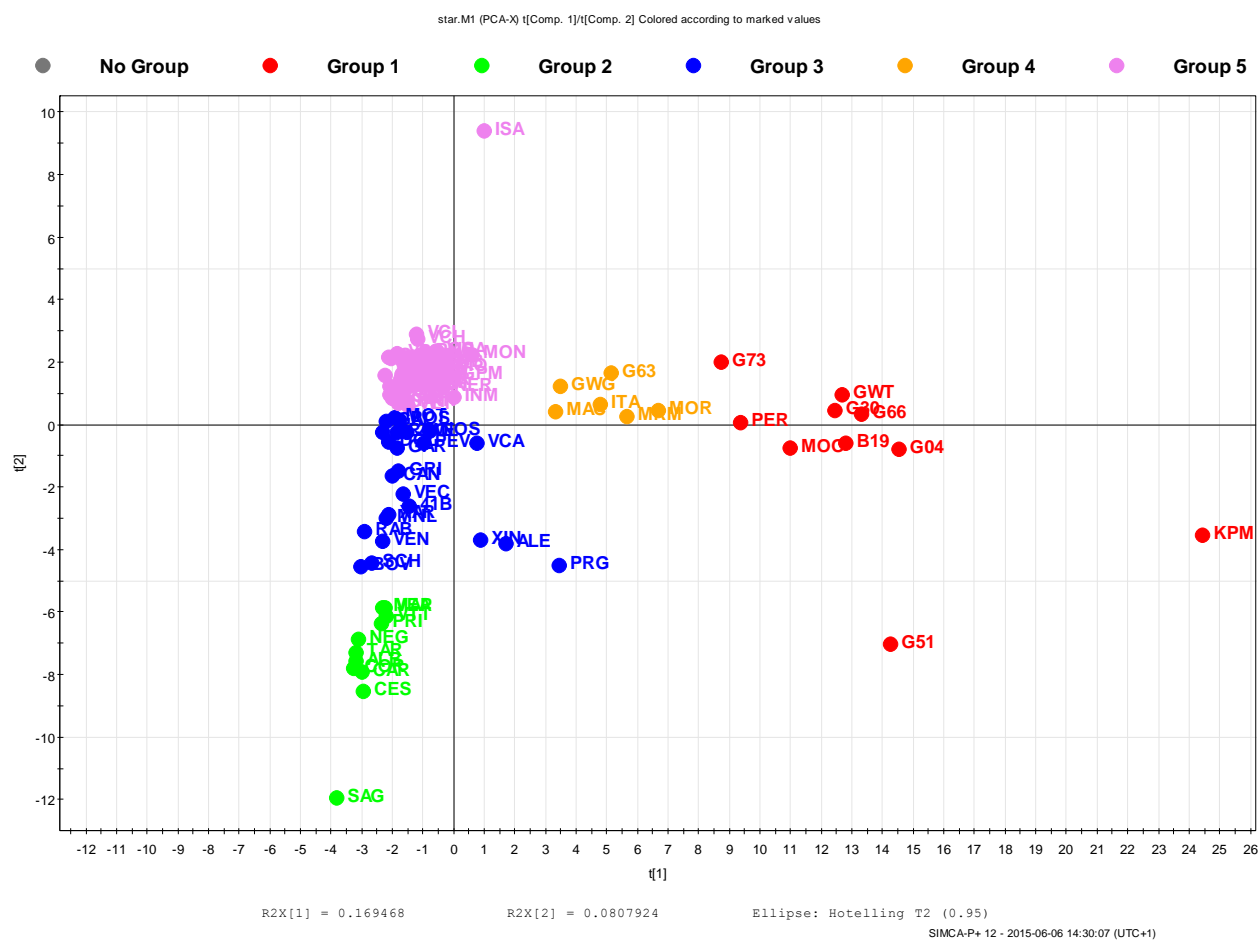
Group 3 includes 24 varieties with prominent number (15) of red *V. vinifer*a verities like Cannonau, Dolcetto, Malvasia nera di lecce and so on. Some red aromatic cultivars like Aleatico, Primitivo di giola, Xinomavro were also included in this group. All varieties in this group shows common compounds like sesquiterpenes (aromadendrene, gamma-neoclovene and guaiene), alcohols (1-octen-3-ol, E-2-hexenol), aldehydes (benzaldehyde, E-2-heptenal, E-2-hexenal), and 1-octen-3-one, ethyl anthranilate, sclareol. *Vitis californica*, a non-vinifera

cultivar with red colored berries was also included in this group. General profile of the compounds showed by the varieties in this group suggests low aroma properties of these cultivars.
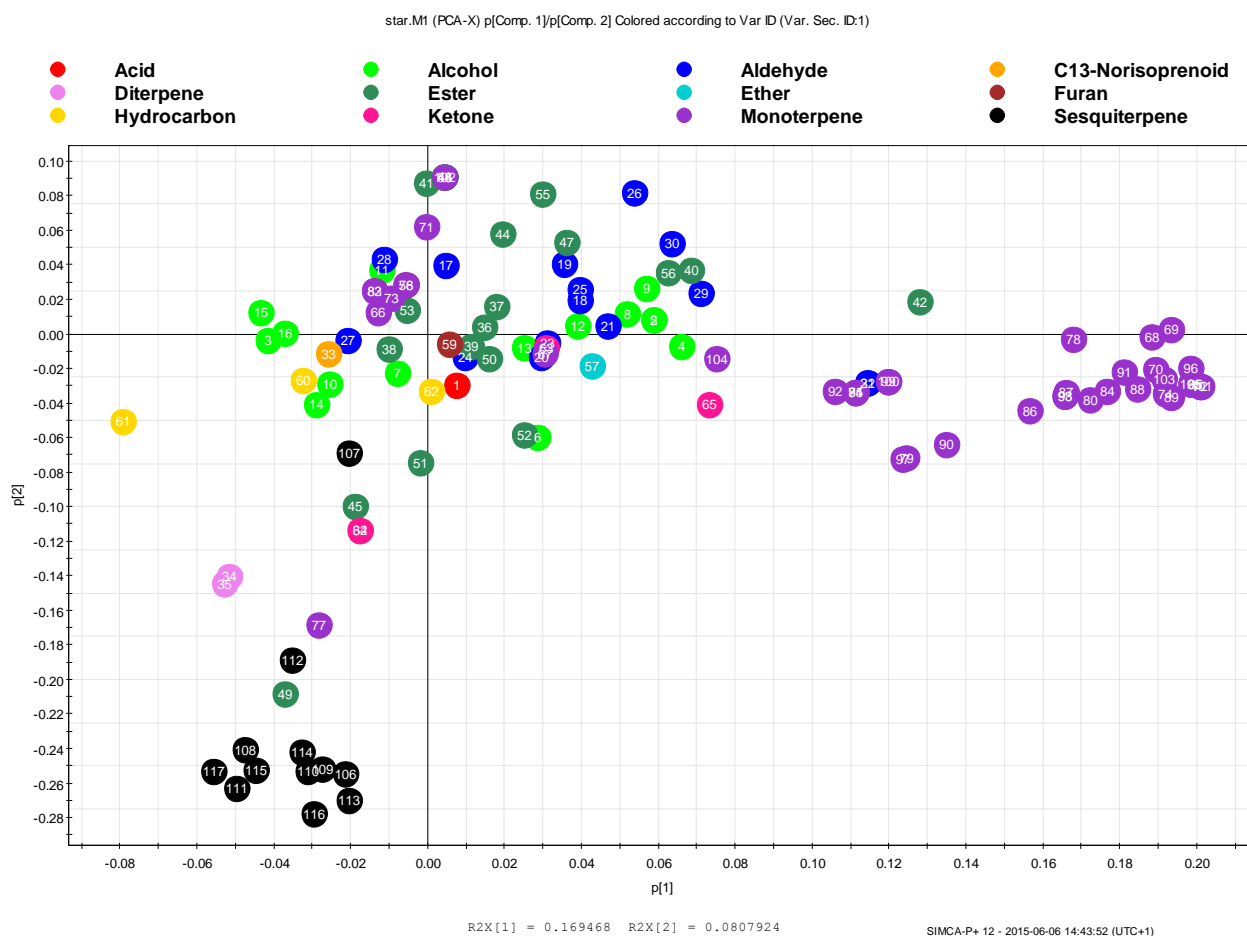
Group 4 shows six verities including five prominent aromatic cultivars Moscato Rosa, Gewürztraminer giaroni, Italia, Malvasia bianca di Candia, Muscat Rouge de Madere and interspecific crossing Girelli F3P63. All cultivars commonly shows monoterpenes (cis-geraniol, beta-citronellol, alpha-terpineol, neryl isobutyrate, trans-geraniol, rose oxide) along with ethyl anthranilate, hexanal, E-2-hexenal, 1-octen-3-ol

Group 5 comprises remaining 73 cultivars showing diverse series of colour and species of grapes. Six out of seven non-vinifera cultivars in this study were included in this group (except *Vitis californica*). This group was mainly dominated by red colored varieties i.e. out of seventy three, forty-seven red, twenty-four white and two pink colored cultivars included in this group. Commonly observed compounds in this group are alcohols (1-hexanol, 1-octen-3-ol, E-2-hexenol), aldehydes (benzaldehyde, E-2-heptenal, E-2-hexenal, hexanal), esters (ethyl anthranilate, methyl salicylate, heptyl formate) and 1-octen-3-one. This group also includes variety Isabella which is an interspecific crossing of *Vitis vinifera* and *Vitis labrusca* and which is also known as strawberry grapes due to their strong aroma similar to the strawberry (Kulakiotu, Thanassoulopoulos, & Sfakiotakis, 2004; Pacifico et al., 2011). Isabella shows presence of esters (ethyl phenylacetate, isoeugenyl phenylacetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) and aldehydes (E-2-hexenal, hexanal) prominently.

Figure 13 PCA score plot of grape varieties based on VOC identified

star.M1 (PCA-X) p[Comp. 1]/p[Comp. 2] Colored according to Var ID (Var. Sec. ID:1)

R2X[1] = 0.169468 R2X[2] = 0.0807924

SIMCA-P+ 12 - 2015-06-06 14:43:52 (UTC+1)

**Figure 14 PCA loading plot of the variables (labeled as chemical classes)**

Figure 14 illustrates the loading plot of variables (detected compounds) and distribution of the compounds, numbers inside each dot corresponds to the compounds in table 4. Monoterpenes are widely distributed throughout the graph followed by alcohols, aldehydes and esters.

Since monoterpenes are highly aroma active compounds with very lower aroma thresholds is and considered significant class of chemicals in grapes (Mateo & Jiménez, 2000), we calculated sum of the relative intensity values of all identified monoterpenes for each variety. The bubble graph of total monoterpene content (figure 15) shows first twenty varieties with higher intensity values of monoterpenes.
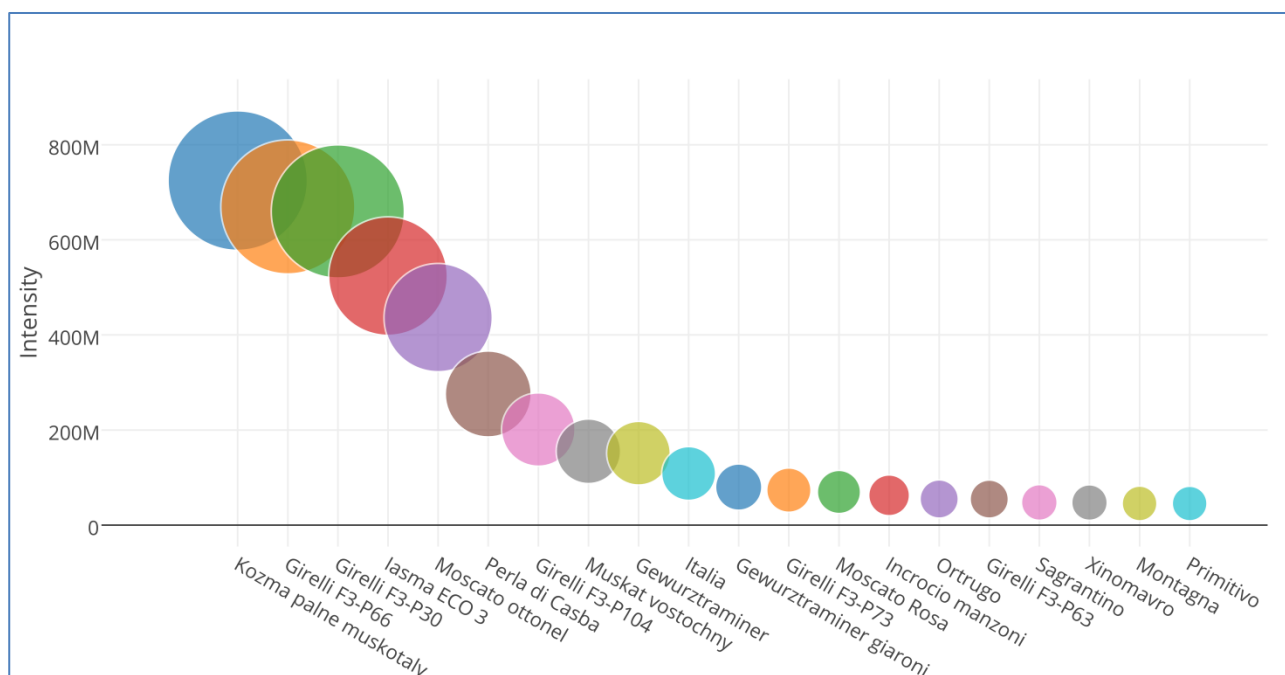
Figure 15 Bubble graph of total monoterpene content

Polar dendrogram (figure 16) based on the cluster analysis performed on log transformed data of bioactive compounds content using method reported by (Farneti et al., 2015), which further visualizes the same data with different method. The dendrogram also explains the clustering of varieties in the in the PCA.
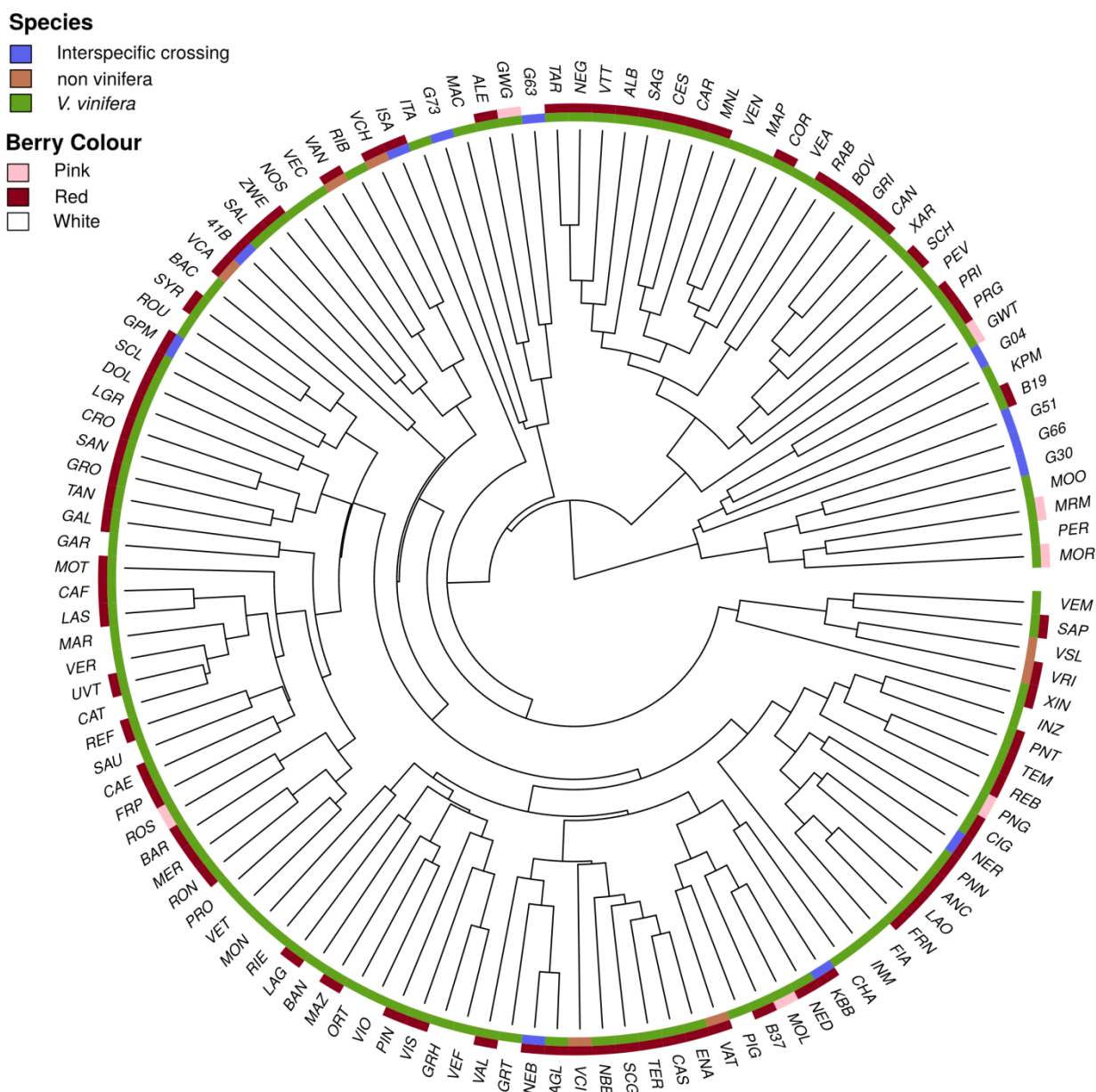
Figure 16 Polar dendrogram visualization based on the data

3.4. Discussion

Grape volatiles consist different classes of the compounds like terpenoids, norisoprenoids, aliphatic alcohols, esters, and benzenoids and so on (A. L. Robinson et al., 2013; Maurizio Ugliano & Moio, 2008). A diverse range of compounds representing different chemical classes (monoterpene, ester, aldehyde, alcohol, sesquiterpene, hydrocarbon, ketone, furan,

di-terpene, C13-norisoprenoid, ether, acid) is identified among the wide selection of grape genotypes in the present study.

Forty monoterpenes were identified in the study including compounds with high aroma thresholds like linalool and its oxides, rose oxides, geraniol, citronellol and so on. Monoterpene is a class containing some of the most aroma active compounds in the grape (Doneva-Sapceska, Dimitrovski, Milanov, & Vojnovski, 2006; Mateo & Jiménez, 2000; S G Voirin et al., 1992; Stcphane G Voirin, Baumes, Sapis, & Bayonove, 1992). Many monoterpenes were attributed to characteristic fruity and floral aroma of white wines made from muscat and non-muscat varieties like Gewürztraminer and Riesling (Mateo & Jiménez, 2000; Oksman-Caldentey & Saito, 2005; Ong & Acree, 1999; Ribéreau-Gayon et al., 1975). Concentration of these compounds above the sensory threshold is a characteristic of the so-called Muscat varieties, which gives to the ripe berries of these cultivars floral and attractive aroma, a trait widely exploited in table grapes. Studies also reported genes associated with the productions of monoterpenes in the mucsat grapes (Emanuelli et al., 2010).

Most of the varieties studied in this experiment also show 15 alcohols (including C5/C6 and aromatic) and 15 aldehydes. Alcohols like 1-pentanol, E-2-hexenol, E-3-hexen-1-ol, 2-phenoxyethanol, benzyl alcohol and aldehydes; benzaldehyde, E-2-hexenal, E-2-heptenal, E-2-pentenal were identified. Compounds like (E)-3-hexenol and its isomer (Z)-3-hexenol were considered as important analytical parameters to discriminate monovarietal Riesling wines (Oliveira, Faria, Sá, Barros, & Araújo, 2006; Rapp, Volkman, & Niebergall, 1993). C6 compounds in grape are mainly derived from grape polyunsaturated fatty acids (primarily originated from membrane lipids) linoleic and aplha-linolenic acids, through a cascade of enzymatic reactions. This biochemical pathway yields into C6 aldehydes and therefore to C6 alcohols. The presence of these compounds is known to be modulated by the condition of extraction of juice (contact with oxygen during mechanical harvest and pressing, addition of exogenous antioxidants).

Twenty-one esters were identified; ethyl esters like ethyl and methyl anthranilate, ethyl butyrate, ethyl salicylate, ethyl hexanoate, methyl salicylate etc. were identified. Volatile esters also contributes to important floral and fruity sensory properties of wines, is an

important class of grape and wine (Boss et al., 2015). Different esters are formed during fermentation including the fatty acid ethyl esters and the acetate esters, both of which contribute important fruity notes to wines (Vianna & Ebeler, 2001).

Twelve sesquiterpene like beta-caryophyllen, alpha-humulene, alpha-cedrene, gamma-neoclovene, guaiene, and isolongifolene were identified in this study. Sesquiterpene is an important class in grapes that mainly consist of three isoprene units and have C_{15} in its molecular formula (mostly they are $C_{15}H_{24}$), they are mainly attributed to sweet and woody aroma. A sesquiterpene guaiene recently attracted much interest since it has been suggested as the immediate precursor of rotundone (Huang, Burrett, Sefton, & Taylor, 2014; Huang, Sefton, Sumby, Tiekink, & Taylor, 2015), which is a powerful odorant present at trace levels and responsible for the peppery aroma of some red (Fulvio Mattivi et al., 2011; Wood et al., 2008) and white wines.(Caputi et al., 2011)

Other compounds from the classes like C_{13}-norisoprenoid (beta-ionol, theaspirane), aromatic hydrocarbons (m-xylene, o-xylene, p-xylene) (Schreier et al., 1976), furans (2-pentylfuran, 2-ethylfuran), ketones (1-octen-3-one, 4-hexen-3-one, 6-methyl-5-hepten-2-one), diterpenes (sclareol), ethers (alpha-asarone) and acetic acid (Vrhovsek et al., 2014) were detected. C_{13}-norisoprenoids represents another important class of chemicals in grape aroma which are produced predominantly by carotenoid breakdown (Günata, 2013; Mendes-pinto, 2009; Skouroumounist & Winterhalter, 1994; Winterhalter et al., 1990).

Present experiment shows the complete workflow of metabolomics profiling of volatile organic compounds in grape achieved by methodological approach of analysis using HS-SPME and GC-MS. The development of customized tool for compound annotation was also reported which appeared the useful strategy and increased confidence level of the annotation, and furthermore, it can be implemented within large scale or long-term metabolomics projects. Totally, 117 VOCs were identified successfully, which represents 124 grape cultivars of different origins, colour and species over four consecutive vintages.

The database of grape VOCs was established covering comprehensive range of grape cultivars from *Vitis vinifera,* interspecific crossings, non-vinifera and wild varieties. Current

database represents repository of VOCs in selected grape cultivars and can be used as volatile pattern reference compendium for selection of clones for breeding programs or vinification projects.

Since the current experiment is a part of **Grape Metabolome** project, the current database represents the significant portion it and will be further used to complete the comprehensive picture of grape metabolites and combination of the metabolomics data with genomics data (Oksman-Caldentey & Saito, 2005) can furthermore give more insights for engineering the grape metabolic pathways.

3.5. Contributions

This experiment is part of ongoing multidisciplinary project "**Grape Metabolome**" at the Research and Innovation Centre, Fondazione Edmund Mach, San Michelle all'Adige-38010 under the management of Dr. Fulvio Mattivi. The dataset of VOCs from this experiment will be the part of upcoming grape metabolome database. The experiment described in this chapter will be my main contribution to the database and manuscript in preparation. I thank the entire team involved in this project for their kind help. I personally thank Jan Stanstrup for making the polar dendrogram diagram and biostatistics group the compound annotation.

4. Chemical composition of volatile aroma metabolites and their glycosylated counterparts uniquely differentiates individual grape cultivars

4.1. Introduction

Aroma is an important aspect of quality in grapes and one of the factors that ultimately determines the quality of the wine made from it. Study of grape aroma has been a significant subject in the grapevine research community for many years. The grape aroma is a product of complex chemistry, as compounds from different classes develop grape aroma and give specific sensory characteristics to cultivars (Ebeler & Thorngate, 2009). Variability in chemical composition or concentration significantly changes aroma of grape of different grape species, cultivar-specific aroma in grapes and wine has been addressed in some previous studies (Dourtoglou, Antonopoulos, Dourtoglou, & Lalas, 2014; Nasi et al., 2008). For example monoterpenol linalool, geraniol, nerol and α-terpineol are present in high concentrations in Muscat grapes and contribute to the floral aroma (Ribéreau-Gayon et al., 1975; Schreier et al., 1976). This sensorial character can be appreciated both in table grapes and in wine, and lead to the selection of several highly flavoured cultivars during grape domestication and post-domestication.
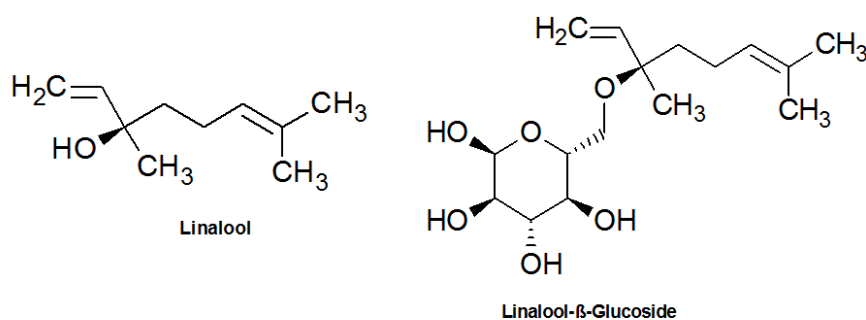
Figure 17 Linalool and its glucoside molecule

C13-norisoprenoids β-ionone, β-damascenone and vitispirane contribute to a more diverse range of aromas, while other classes lactones, alcohols, phenols, and benzenoids also make a significant contribution to the aroma of several grape cultivars (Ryona & Sacks, 2013). Many different pathways and chemical reactions are involved in the production of aroma compounds in the grape and several previous studies have identified hundreds of volatile

organic compounds in grapes (Martin, Chiang, Lund, & Bohlmann, 2012; Anthony L. Robinson et al., 2013).

Volatile aroma compounds exist in free as well as conjugated form in grapes. The conjugated part is mostly the hydrophilic, non-volatile and flavourless glycosylated molecules. Conversion of free aroma compound into glycosylated precursor occurs through the process of glycosylation, which is one of the predominant modifications in plants catalysed by a group of enzymes called glycosyltransferases (GTs). These are mainly glucoside or disaccharide or trisaccharide glycosides containing a glycosyl moiety, nevertheless for the disaccharide glycosides the glucose is further substituted with a α-L-arabinofuranosyl, α-L-rhamnopyranosyl and β-D-glucopyranosyl sugars (Boido, Fariña, & Carrau, 2013; Flamini et al., 2014; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Studies have reported that mature grapes show higher levels of glycosylated volatiles than their free counterparts; it has also been shown that glycosides of monoterpenes and C13-norisoprenoids increase post-veraison (Ryona & Sacks, 2013). The importance of these glycosylated precursors in winemaking is greatly appreciated because of mild acid and/or glycosidase catalysed hydrolysis reactions, which release free volatiles from their sugar moieties and enhance wine aroma. This is also a possible reason for the enhanced aroma profile of wine coming from neutral grapes.

Knowledge of the specific distribution of free and glycosylated volatiles in grapes is necessary for a complete understanding of varietal grape aroma and it is therefore important to consider the distribution of both free and glycosylated volatiles. Furthermore, it is also interesting to see in which glycosylated conjugates different cultivars/species tend to accumulate a specific volatile molecule. Most of the previous studies related to the analysis of glycosides in grapes were limited either to a single glycoside class or to a few grape cultivars. While the differential behaviour of aroma compounds and their precursors in different cultivars and species has never been specifically described (Gunata, Bayonove, Baumes, & Cordonnier, 1985; Maicas & Mateo, 2005; Nasi et al., 2008; Williams, Cynkar, & Francis, 1995).

Several methodologies for the extraction and analysis of free and bound compounds in grapes and wine have been reported (Fernández-González & Di Stefano, 2004; S G Voirin et al., 1992). Isolation of the glycosidic fraction in grapes was most commonly achieved using solid phase extraction (SPE) and further analysis carried out with GC-MS following acid or enzymatic hydrolysis. Some of the studies were carried out using GC-MS analysis of TMS and TFA derivatives of terpene glycosides (S G Voirin et al., 1992; Stcphane G Voirin et al., 1992) or by analysing the terpenes obtained through hydrolysis of terpene glycosides (Maicas & Mateo, 2005) using purified enzymes or commercial enzyme preparations. Intact glycosidic conjugates can also be analysed using LC-MS, NMR and IR but such an approach has been less frequently used, with a few studies being carried out on terpenes and terpene glycosides of non-aromatic grapes (Boido et al., 2013; Flamini et al., 2014; Schievano et al., 2013; Winterhalter & Skouroumounis, 1997). Some authors have proposed the use of LC/ESI-MS or MALDI-TOF-MS techniques to characterise the entire glycosylated molecule without derivatization step (Nasi et al., 2008; Schievano et al., 2013). Aim of this study was to profile the volatile aroma metabolites and their glycosidic counterparts in the ripe berries of ten selected genotypes through a comprehensive chemical profiling using GC-MS technique.

4.2. Materials and methods

4.2.1. Grape material

Ten different genotypes, including six *Vitis vinifera* cultivars, two American species and two interspecific crossing, were included in the study (table 5). All genotypes were true to type and sampled from the ampelographic collection of the Foundation Edmund Mach, San Michele all'Adige, Italy. 1 kg of healthy grapes was sampled at technical maturity, defined as a content of soluble solids in the juice corresponding to 18° (±0.5) Brix. After sampling, the berries were immediately stored at -80°C and powdered in liquid nitrogen using an analytical mill (IKA® -Werke GMbH & Co. Staufen, Germany) prior to sample preparation.

Table 5 List of the grape varieties included in the study (Chapter 4)

No	Prime name	Berry Colour	Species
1	Riesling	White	Vinifera
2	Gewürztraminer	Pink	Vinifera
3	Moscato rosa	Pink	Vinifera
4	Girelli F3P30	White	Intraspecific crossings of *Muscat Ottonel* x *Malvasia Bianca di Candia*
5	IASMA ECO3	White	
6	Girelli F3P63	White	
7	Nero	Red	Interspecific crossing of Eger 2 x Gardonyi Geza
8	Isabella	Red	Interspecific crossing of *Vitis vinifera x Vitis labrusca*
9	Arizonica Texas	Red	*Vitis arizonica*
10	*Vitis cinerea*	Red	*Vitis cinerea Engelmann*

4.2.2. Chemicals and reagents

Methanol, dichloromethane, formic acid and pentane were purchased from Sigma Aldrich (Milan, Italy). Anhydrous sodium sulphate and citric acid were purchased from Carlo Elba (Milan, Italy). The water used in the experiments was purified with a Milli-Q water purification system from Millipore (Bedford, MA, USA), SPE cartridges Isolute ENV+ (1 g, 6 mL) were obtained from Biotage (Uppsala, Sweden), a mixture of pectinases and glycosidases Rapidase AR2000© enzyme was purchased from DSM Food Specialties B.V. (Delft, Netherlands)

4.2.3. Sample preparation

Sample preparation method reported previously (Vrhovsek et al., 2014) was adopted with minor modifications for better extraction of target compounds. 30 g of grape powder, 80 mL water and 0.5 g of gluconolactone were taken and 25 μL of 1-heptanol (1257 mg/L in ethanol) was added as internal standard. The solution was then homogenized for 3 min at 20000 rpm

using an ultra-turrax homogenizer, followed by centrifuging for 5 min. at 10000 rpm at 5 °C. The supernatant obtained was then filtered through filter paper and the extract was further used for the SPE procedure.

Isolute ENV+ cartridges were conditioned with 20 mL each of methanol and milliQ water, then the grape extract was loaded and eluted through cartridges and the cartridges were washed with 20 mL of water to remove water-soluble impurities. Free volatiles were eluted with 20 mL of dichloromethane, elute was collected in a glass tube and 40 mL of pentane was added to it. This solution was dried with anhydrous Na_2SO_4 and concentrated to 200 μL using vigreux column. The glycosylated precursors were eluted with 30 mL of methanol, out of this 1 mL of methanol was provided for other researchers in the laboratory for their analysis purposes, while the rest of the fraction was evaporated to dryness by using rotary vacuum evaporator (Rotavapor RE121, BUCHI, Switzerland). Then the flask was rinsed with 10 mL of dichloromethane to remove any remaining traces of free volatile compounds.

4.2.4. Enzymatic hydrolysis of glycosidic precursors

The commercial mixture of pectinases and glycosidases, AR2000 was used for enzymatic hydrolysis of glycosylated precursors of aroma compounds. The bound fraction from step above was redissolved in 5 mL of citrate buffer at pH 5 and 200 μL of AR2000 (70 mg/mL) was added to it. This set up was kept in a 40 °C water bath for 24 hrs for the hydrolysis. After 24 hrs, 10 μL of internal standard 1-heptanol was added and free volatiles were extracted with 3 mL of pentane/dichloromethane 2:1, v/v, three times. All organic phase containing released volatiles was concentrated carefully to a volume of 200 μL for GC-MS analysis

4.2.5. GC-MS analysis of volatiles

Analysis of free volatiles was performed using a Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Milan, Italy), mounted with a PAL combi-xt autosampler (CTC, Zwingen, Switzerland). 1 μL of sample was injected in splitless mode with a splitless time of 1 min and a GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL/min, with Stabilwax® 30 m length, 0.25 mm

inner diameter and 0.25 µm thick film columns from Restek Corporation (Bellefonte, PA, USA) used for analysis. GC oven temperature was initially set at 50°C with hold for 1 min and then ramped at the rate of 2.5 °C /min to 250°C with a final hold of 10 min. The total GC runtime was 91 min. The mass spectrometer was operated in positive mode electron ionization at 70 eV and all spectra were recorded in full scan with a mass range of 40-350 Da, transfer line and source temperature set at 250°C.
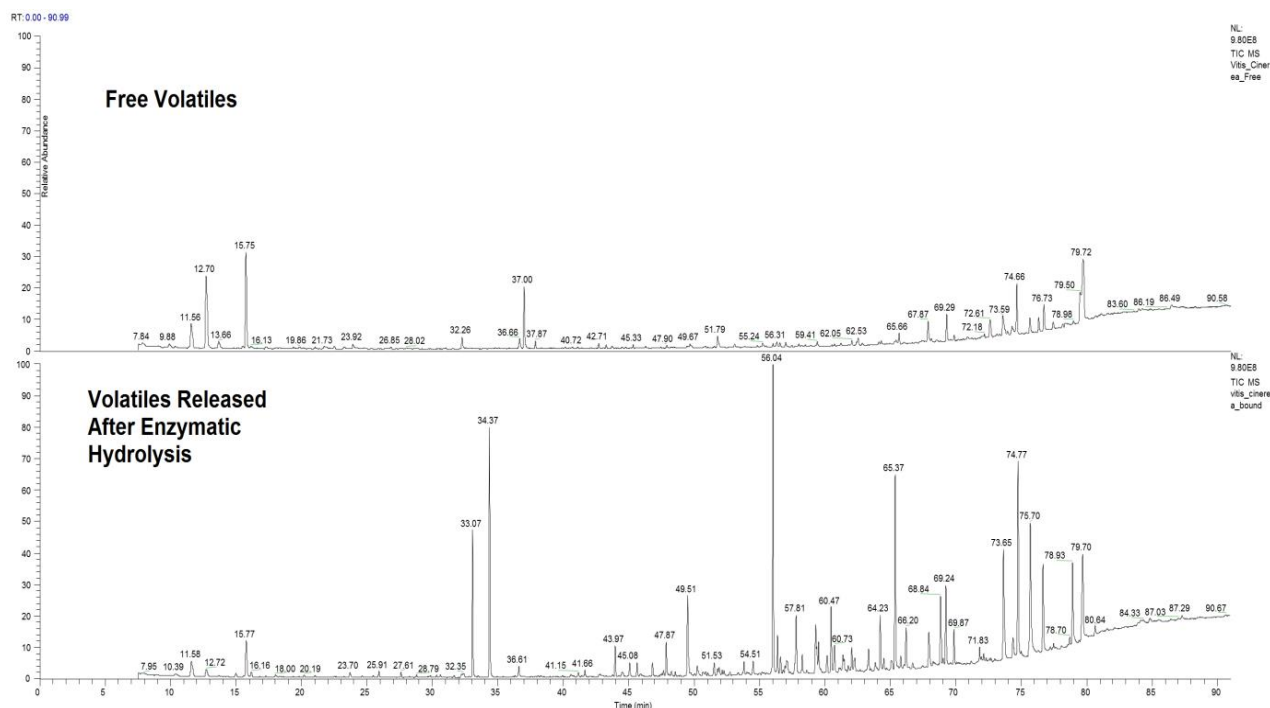
Figure 18 Difference between profile of volatile compounds in Vitis cinerea grapes before and after enzymatic hydrolysis

4.2.6. Data processing, compound identification and statistical analysis

GC-MS data processing was done with Thermo XCALIBUR™ 2.2 software. Identification of the compounds (table 6) was performed by applying assignments like reference standard analysis, retention index calculation, and by NIST MS Search Program© (version 2.0) library comparison. The response of internal standard 1-heptanol was used for normalization and to make a relative estimation of the identified compounds as commonly accepted in the analysis of aroma compounds (Azzolini et al., 2012). Cluster analysis and heatmap visualizations of the compounds detected using GC-MS (figures 19 & 20) were done by using

Pearson correlation coefficient and Ward's minimum variance method (Murtagh & Legendre, 2014) under "R" environment (http://www.r-project.org/).

4.3. Results

Table 6 List of the compounds identified in the experiment (Chapter 4)

	Name	RT	PubChem CID	RI (calculated)	RI (literature)	Literature	Identification assignment		
	Acid								
1	Hexanoic acid	32.30	8892	1826	1847	Fukami et al., 2002	---	B	C
2	Linoleic acid	75.71	5280450	3158	---	---	---	---	C
	Alcohol								
3	n-Hexanol	11.58	8103	1342	1354	Lee et al., 2003	A	B	C
4	*trans*-3-Hexenol	12.71	5284503	1369	1378	Ruther J., 2000	A	B	C
5	2-Hexenol	13.68	5318042	1392	---	---	A		C
6	Benzyl alcohol	33.07	244	1844	1865	Fischer et al., 1987	A	B	C
7	beta-Phenyl ethanol	34.38	6054	1881	1905	Ong et al., 1999	A	B	C
8	Anisyl alcohol	47.88	7738	2240	---	---	---	---	C
9	Cinnamyl alcohol	48.10	5315892	2246	2200	Olivero et al., 1997		B	C
10	4-Methoxyphenethyl alcohol	49.51	69705	2244	---	---	---		C
11	Coniferol	76.68	1549095	3146	---	---	---		C
12	Tryptophol	80.66	10685	3333	---	---	A	B	C
	Aldehyde								
13	Benzaldehyde	18.31	240	1499	1525	Valim et al., 2003	A	B	C
14	Phenylacetaldehyde	23.26	998	1614	1609	Qian M et al., 2003	A	B	C
	Benzenoid								
15	Eugenol	43.98	3314	2131	2141	Valim et al., 2003	A	B	C
16	4-Vinylguaiacol	45.08	332	2161	2198	Baek et al., 1997	---	B	C
17	Methyl vanillate	58.28	19844	2557	2600	Selli et al., 2004	---	B	C
18	Acetovanillone	59.26	2214	2589	2685	Cullere et al., 2004	A	B	C
19	Homovanillyl alcohol	65.37	16928	2746	---	---	---	---	C
20	Homovanillic acid	69.24	1738	2867	---	---	---	---	C
	C13-Norisoprenoid								
21	3-4-Dihydro-3-oxoactinidiol I	52.73	---	2383	2418	Boido et al., 2013	---	B	---
22	3-4-Dihydro-3-oxoactinidiol II	53.71	---	2413	2458	Boido et al., 2013	---	B	---
23	3-4-Dihydro-3-oxoactinidiol III	54.05	---	2424	2479	Boido et al., 2013	---	B	---
24	3-Hydroxy-beta-damascone	56.38	5366075	2450	2563	Aubert et al., 2003	---	B	C

25	3-Oxo-alpha-ionol	59.30	5370052	2590	2651	Selli et al., 2004	---	B	C
26	4-Oxo-beta-ionol	59.51	6430464	2596	2514	Klesk et al 2004	---	B	C
27	Dihydro-3-oxo beta ionol	60.47	520295	2628	---	---	---	---	C
28	3,4-Dihydroactinidol	61.08	---	2652	---	---	---	---	C
29	Dihydro-beta-ionone	62.06	519382	2641	---	---	---	---	C
30	9-Hydroxy megastigma-4-6-dien-3-one	66.21	---	2760	---	---	---	---	C
31	Vomifoliol	73.76	5280462	2994	3167	Selli et al., 2004		B	C

Coumarin

32	Scopoletin	90.52	5280460	3679	---	---	---	---	C

Ester

33	Ethyl-beta-hydroxybutyrate	18.26	62572	1500	1483	Boulanger et al., 1999		B	C
34	Ethyl-3-hydroxyhexanoate	25.22	61293	1659	---	---	---	---	C
35	Methyl salicylate	28.67	4133	1752	1782	Anderson et al., 1987	A	B	C
36	Methyl anthranilate	46.21	8635	2193	2216	Ulrich D et al., 1997	A	B	C

Furanone

37	Furaneol	39.24	19309	2005	2039	Valim et al., 2003	A	B	C

Monoterpene

38	*trans*-Linalool oxide (furanoid)	14.97	22310	1451	1453	Ong et al., 1999	A	B	C
39	*cis*-Linalool oxide (furanoid)	16.17	11321214	1423	1423	Davies NW 1990	A	B	C
40	Linalool	19.83	6549	1535	1554	Choi H-S 2003	A	B	C
41	4-Terpineol	21.75	11230	1579	1590	López-Vázquez et al., 2010	A		C
42	1-p-Menthen-9-al I	22.00	520440	1585	1593	López-Vázquez et al., 2010	---	---	C
43	1-p-Menthen-9-al II	22.13	520440	1588	1596	López-Vázquez et al., 2010	---	---	C
44	Hotrienol	22.49	5366264	1596	1586	Engel et al., 1983	A	B	C
45	alpha-Terpineol	25.92	17100	1674	1688	Lee et al., 2005	A	B	C
46	Lilac alcohol A	26.64	526973	1691	---	---	---		C
47	*trans*-Linalool oxide (pyranoid)	27.61	26396	1743	1747	Boulanger et al., 1999	---	B	C
48	*cis*-Linalool oxide (pyranoid)	28.79	26396	1715	1720	Boulanger et al., 1999	A	B	C
49	beta-Citronellol	29.09	8842	1749	1771	Choi H. S., 2003	---	B	C
50	Lilac alcohol-B	29.52	526973	1760	---	---	A	---	C
51	Nerol	30.33	643820	1780	1753	Nishimura et al., 1995	A	B	C
52	Lilac alcohol-C	31.25	526973	1801	---	---	---	---	C
53	Geraniol	32.36	637566	1829	1850	Hognadottir et al., 2003	A	B	C
54	Exo-2-hydroxycineole	32.43	529885	1831	1723	Lee et al., 2005	---	B	C
55	Terpendiol-I	36.61	71362364	1935	1959	Aubert et al., 2003	---	B	C
56	6,7-Dihydro-7-hydroxylinalool	37.79	120154	1967	---	---	---	---	C
57	Terpendiol-II	43.27	71362364	2112	2134	Boulanger et al., 1999	---	B	C
58	Hydroxy citronellol	46.05	526767	2188	---	---	---	---	C
59	Hydroxy nerol	48.12	---	2247	---	ref. (27)	---	---	D

60	*trans*-8-Hydroxy linalool	48.30	5280678	2246	2267	Chassagne et al., 1999	---	B	C
61	*cis*-8-Hydroxy linalool	49.62	5280678	2253	2267	Chassagne et al., 1999	---	B	C
62	Geranic acid	50.34	5275520	2307	---	---	A	---	C
63	p-Menth-1-ene-7,8-diol	56.03	---	2444	---	Versini et al., 1991	---	---	C
Phenol									
64	Chavicol	50.07	68148	2304	2340	Aubert et al., 2003	---	B	C
65	3,4,5-Trimethoxyphenol	71.83	69505	2961	---	---	---	---	C
Quinone									
66	2-Methoxyhydroquinone	68.85	69988	2860	---	---	---	---	C

Identification based on, [A] By comparing mass spectra and retention times with those of pure reference standards, [B] By retention index match on a similar phase column (literature provided in the Appendix), [C] NIST mass spectral database, [D] In-house mass spectral database from previous studies done at institute

4.3.1. Distribution of free and enzymatically released volatile aroma compounds in selected grape cultivars

- **F3P30, F3P63 and IASMA ECO 3**

These are new botrytis-tolerant Italian cultivars obtained by intraspecific crossing of Muscat Ottonel and Malvasia di Candia. They are specifically designed for the production of aromatic white wines and this is the first report on their aroma profiling. In the analysis of free part, all three varieties are typically rich in monoterpene alcohols that are commonly known for flowery and fruity aroma. In F3P30 and IASMA ECO3, very high concentrations of linalool, terpendiol (I & II), *trans*-linalool oxide (furanoid & pyranoid) and *trans*-8-hydroxy linalool were detected in free the form. Surprisingly, similar compounds showed lower concentrations after hydrolysis. But F3P63 showed all the identified compounds in free form

lower than after hydrolysis (Except for terpendiol-I) also geraniol in F3P63 was found very high in released form in (1177.5 µg/kg) than in free (158.3 µg/kg).

- **Gewürztraminer**

Gewürztraminer is one of most popular aromatic grape variety and its wine is known for unique aroma, reminiscent of tropical lychee fruit due to the rich profile of monoterpenes (Martin et al., 2012; Ong & Acree, 1999; S G Voirin et al., 1992). Studies have attributed the distinct flavor of Gewürztraminer, Riesling and Muscat grapes and wines to the presence of certain monoterpenes linalool, geraniol, nerol, and linalool oxides (Schreier et al., 1976), *cis*-rose oxide (Guth, 1997), 4-vinylguaiacol (Grando, Versini, Nicolini, & Mattivi, 1993), beta-phenylethanol and phenethyl acetate for spicy and rose aromas of Gewürztraminer (Ong & Acree, 1999). In free form, this variety show not very rich profile of aroma active compounds, but in bound form shows high concentrations of geraniol and geranic acid, also as reported previously; 4-vinylguaiacol was also detected in the bound form. In comparison to the previous cultivars, Gewürztraminer was shown to be poorer in the free and bound form of all monoterpenols, while it was richer in the bound forms of C13-norisoprenoids, benzenoids and alcohols.

- **Riesling**

Riesling is a popular *Vitis vinifera* aromatic grape cultivar with an explicit flowery aroma; it is used to make dry, semi-sweet and sparkling white wines. C13-norisoprenoids and some bound monoterpenols were attributed to Riesling berries (Ryona & Sacks, 2013; Strauss, Wilson, Anderson, & Williams, 1987; Winterhalter et al., 1990). In the free part, not much significant identification were found but in bound form compounds beta-phenylethanol, benzyl alcohol, 3-keto-alpha-ionol, acetovanillone and *cis*-8-hydroxy linalool were found in significantly high concentrations.

- **Moscato Rosa**

Like any other Muscat species this cultivar is also known for its very aromatic characteristics and typical flowery aroma rich in monoterpenes (Ribéreau-Gayon et al., 1975; A. L. Robinson et al., 2013). In free form, it shows the compounds such C6 alcohols and monoterpenes linalool (124.1 µg/kg), geraniol (139.7 µg/kg), geranic acid (40.4 µg/kg) and linalool oxides. Increased concentration of geraniol (1945.8 µg/kg) and geranic acid (827.8 µg/kg) were observed in bound form similarly some of the other compounds also showed enhanced concentrations in bound form.

- **Nero**

Interspecific crossing of Eger 2 x Gardonyi Gez*a* from Eger, Hungary by the breeder Jozef Csizmazia, this is a resistant table grape cultivar with a black berry colour. In the free fraction, compounds were identified like n-hexanol (332.2 µg/kg), hexanoic acid (166.3 µg/kg), *trans*-3-hexenol (151.7 µg/kg). While in the bound fraction, benzyl alcohol (1397.2 µg/kg) and homovanillyl alcohol (1213.1 µg/kg) were identified with the highest concentrations. Monoterpenes 8-hydroxy linalool (*cis* & *trans*), geraniol, geranic acid were also observed in high concentration, this variety was also rich in C13-norisoprenoidic compounds 3-keto-alpha-ionol, vomifoliol, dihydro-3-oxo-beta-ionol and 3-hydroxy-beta-damascene.

- **Isabella**

Isabella is an interspecific crossing of *Vitis vinifera* and *Vitis labrusca*, also known as the strawberry grape because of its very prominent strawberry-like aroma. The small sweet berries are consumed as table grapes in addition to being used for the production of homemade wines, since it retained some popularity in spite of being banned from the EU market. Previous studies have shown the specific aroma properties of

Isabella (Ong & Acree, 1999; Pinho & A.Bertrand, 1995). The free form had the highest concentration of beta-phenylethanol and ethyl beta-hydroxybutyrate, some of the other compounds from classes C6 alcohols, monoterpenols and c13-norisoprenoids were also detected in lower concentrations. However, after hydrolysis many important aroma active compounds were released with very high concentrations like beta-phenylethanol, ethyl-beta-hydroxybutyrate, benzyl alcohol and alpha-terpineol. Moreover, furaneol, the key odorant in the strawberry was also found in both fractions of Isabella and which explains its strawberry-like aroma (Pinho & A.Bertrand, 1995). Overall, the bound form of the Isabella is clearly rich in aroma compounds qualitatively and quantitatively.

- **Arizonica Texas**

This is a variety of native North American wild grape species *Vitis arizonica* with very small grapes also known as the Canyon grape. The ripe grapes are edible and relatively flavorful. To our knowledge, there is no literature available on the aroma compounds of this species to date. In free form C6 alcohols *trans*-3-hexenol, n-hexanol showed high concentrations with other minor compounds detected. However, in bound form this gives rich profile of compounds releasing many aroma active chemicals. Beta-phenylethanol (7049.6 μg/kg) and benzyl alcohol (3708.6 μg/kg) were the highest concentration, also 8-hydroxy linalool (*cis* & *trans*) were found in the bound form, and scopoletin (1067.8 μg/kg) was also identified.

- *Vitis cinerea*

Another Native American red grape species with very small berries, commonly called winter grapes. Previous studies have reported odor active compounds methoxypyrazine, eugenol, 1,8-cineole and *cis*-3-hexenol in *Vitis cinerea* wines (Sun et al., 2011). Not any significant aroma contributor was identified in free fraction but complex profile of compounds, qualitatively and quantitatively, was observed after hydrolysis. Rich profile of different C13-norisoprenoids several monoterpenes were detected. Compounds like beta-phenylethanol, benzyl alcohol, homovanillyl alcohol showed higher concentrations. Overall, free aroma profile of *Vitis cinerea* appears to be poorer than the bound, which appeared more complex. Many compounds detected previously in wines of *Vitis cinerea* were also detected in the

bound form e.g. eugenol, alpha-terpineol and beta-phenylethanol. Figure 18 also shows the difference between the profiles of compounds before and after enzymatic hydrolysis.

4.3.2. Effects of the enzymatic hydrolysis of glycosylated precursors

The commercial mixture of pectinases and glycosidases, AR2000 was used for the enzymatic hydrolysis of glycosylated aroma compounds. The choice was made based upon several previous studies (Flamini et al., 2014; Martin et al., 2012; Schneider, Charrier, Moutounet, & Baumes, 2004; Vrhovsek et al., 2014; Wightman & Price, 1997). A "golden hydrolysis procedure" has still to be established, and any choice has pros and cons. We ruled out the hydrolysis with strong acids, since it is known to produce several artefacts and labile aroma compounds can be destroyed with this approach. The enzyme-based strategy with AR2000 was chosen as a milder approach. This product was developed at INRA Montpellier exactly to release the bound aroma in wines. In this way, we choose to focus on the characterization of those bound compounds that can be released by treatment with GRAS enzymes authorized (and widely used) for the wine production

In a recent study by Flamini and coworkers demonstrated the potential of AR2000 for the complete hydrolysis of grape monoterpene glycosides (Flamini et al., 2014). Nevertheless, we found that AR2000 had a non-specific effect on releasing glycosylated polyphenols (data not shown), as has also been reported in previous papers (Wightman & Price, 1997). The data suggests that the potential bound aroma released, measured using GC-MS after enzymatic hydrolysis, is only part of the overall potential bound aroma of our cultivars.

4.4. Discussion

The results obtained here provide a first robust and reliable comparison of the aroma potential of the 10 selected genotypes and support the unique differentiation of individual grape cultivars based on the chemical composition of volatile aroma metabolites and their glycosylated precursors.

The diversity of the individual grape cultivar is visualized by using the hierarchical clustering and heatmap (figure 19 & 20). The clustering of free aroma compounds (figure 19) shows varieties F3P30, F3P63 and IASMA ECO3 are grouped closely, with the high concentration of the monoterpenol linalool, terpendiol-I and II and *trans*-linalool oxide (furanoid). Gewürztraminer and Moscato Rosa are grouped together, and show high concentrations of geraniol and geranic acid and some C6 alcohols. Isabella and Nero are also positioned separately with a prominent presence of beta-phenylethanol and furaneol. Riesling, Arizonica Texas and *Vitis cinerea* also shows high concentrations of C6 alcohols and homovanillinic acid. In the clustering of glycosylated aroma compounds (figure 20) shows *Vitis cinerea,* Arizonica Texas and Nero are in same group, all these are red varieties and shows beta-phenylethanol, benzyl alcohol, homovanillinic acid and some C13-norisoprenoids. Isabella and Riesling together form a group that is particularly rich in similar compounds as above three varieties and compounds like beta-phenylethanol, ethyl-beta-hydroxybutyrate and furaneol. F3P30, IASMA ECO3, F3P63 are also in same group which shows high concentrations of bound monoterpenes. Gewürztraminer and Moscato Rosa are grouped together and which are specifically rich in nerol, geranic acid, hydroxy nerol, and beta-citronellol
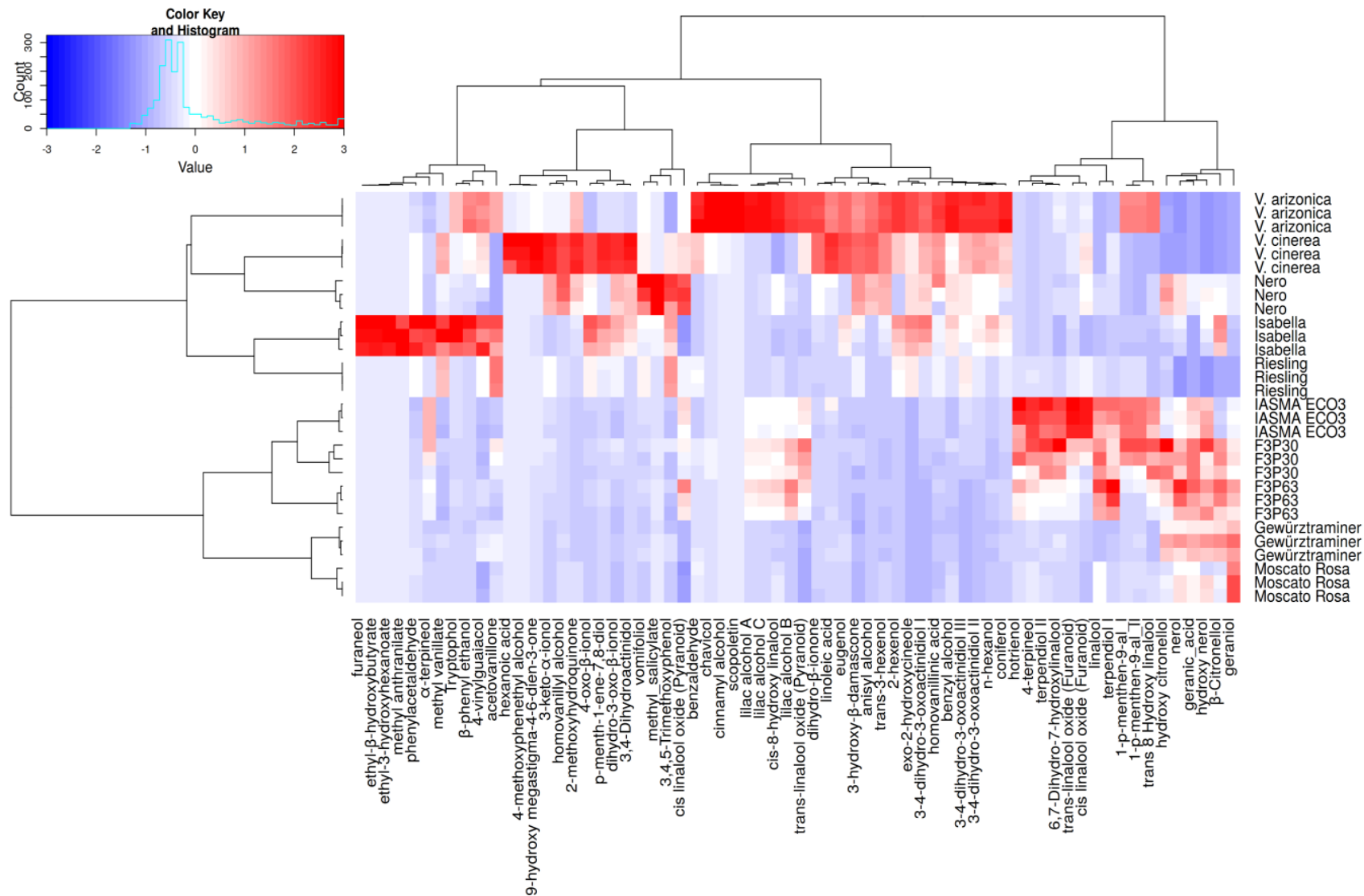
Figure 19 Hierarchical clustering and heat map visualization of glycosylated aroma compounds released after enzymatic hydrolysis
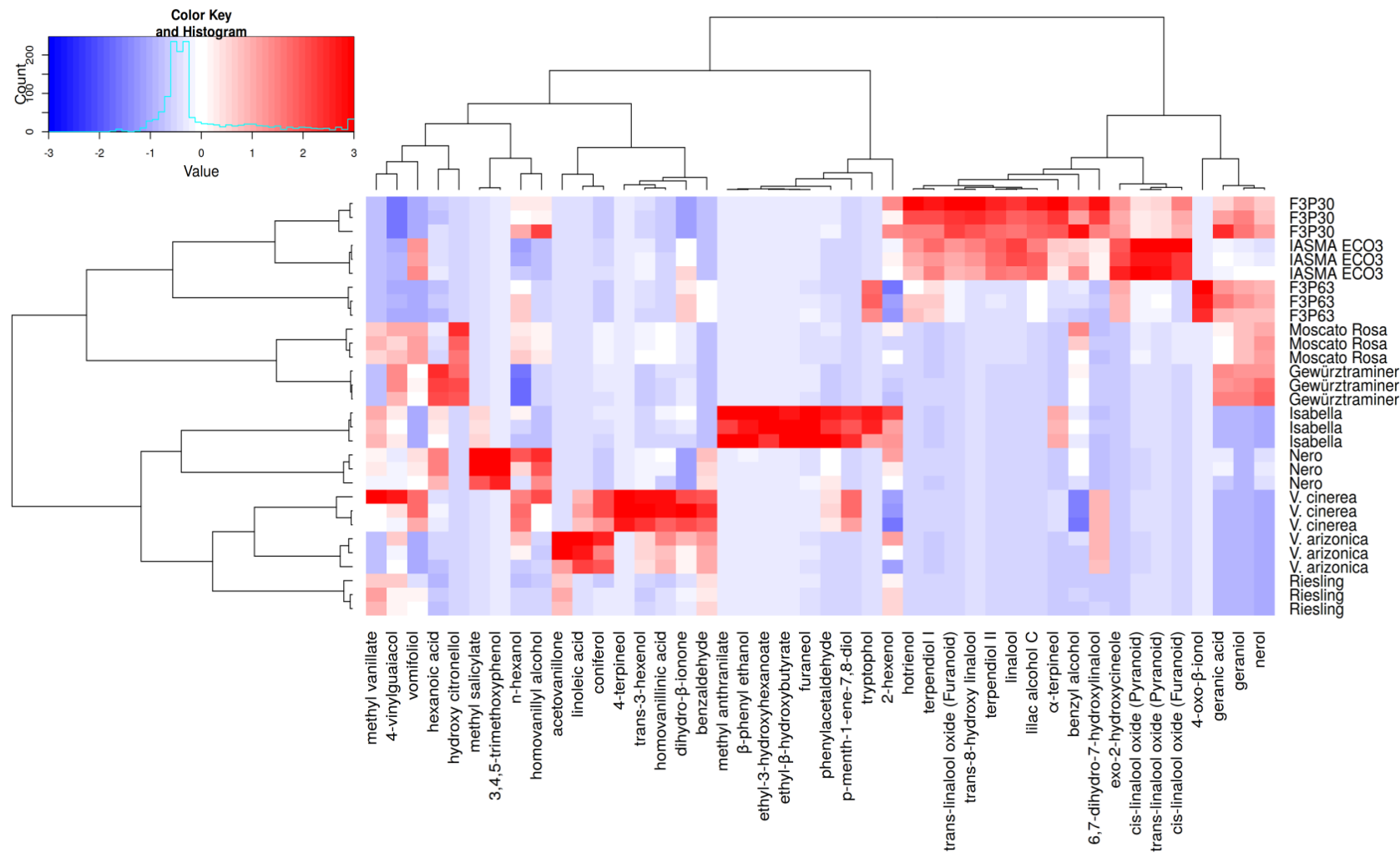
Figure 20 Hierarchical clustering and heat map visualization of free volatile aroma compounds identified in the grape samples
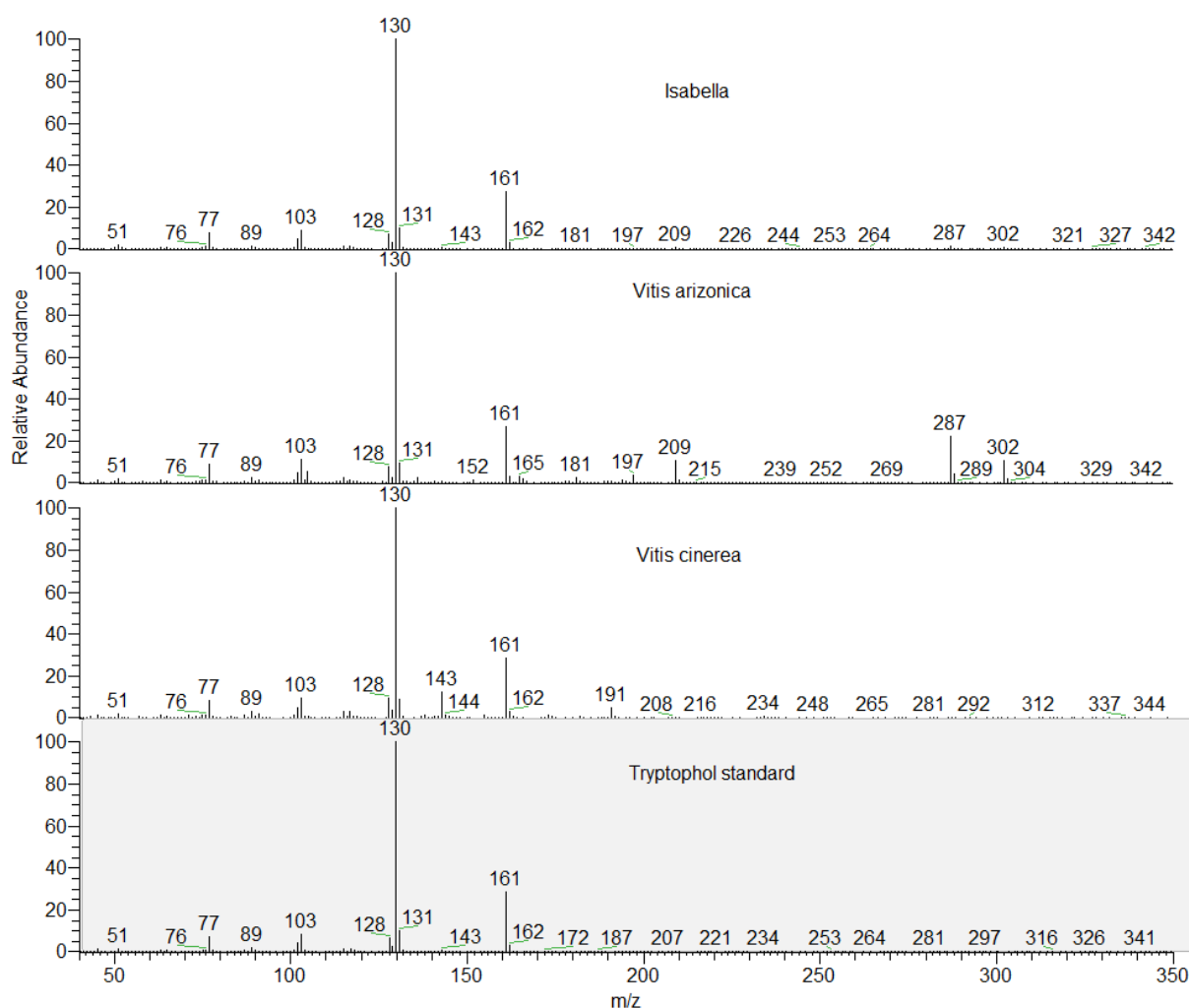
Figure 21 MS spectra of Tryptophol in standard and in samples

Monoterpenol linalool, terpendiol (I & II), *trans*-linalool oxide (furanoid & pyranoid) and *trans*-8-Hydroxy linalool were found to be higher in the free than the conjugated form in the F3P30 and IASMA ECO3 varieties. This is contrary to the general acceptance of higher concentrations of glycosidically conjugated monoterpenol in grapes. It also indicates that, no particular pattern of glycosidic accumulation of monoterpenols is common to all varieties and can be species-dependent. F3P30, F3P63, IASMA ECO3, Nero, Arizonica Texas and *Vitis cinerea* were studied here for the first time in this context. The database build in this study is readily applicable for the identification of these compounds in further studies for systematically profiling the thousands of genotypes available in the ampelographic collections. Moreover, among the other interesting results obtained in this study, it is worth mentioning the identification of tryptophol in higher concentrations (figure 21) in the

66

Isabella and Arizonica Texas cultivars. This compound was linked to the tryptophan metabolism, investigated for the production of 2-aminoacetophenone and other indole off-flavor in wine (F. Mattivi et al., 1999).

Parts of this study support the findings of (M Ugliano & Bartowsky, 2006; Maurizio Ugliano & Moio, 2008) that the volatile fraction obtained after enzymatic hydrolysis can increase the complexity of wine volatiles. Compounds like benzyl alcohol, beta-phenylethanol, geraniol and linalool showed very high concentrations after enzymatic hydrolysis. Specifically, monoterpene alcohols, such as geraniol, linalool and its oxides and citronellol, have low sensory thresholds suggesting that their release from odorless precursors can play an important role in the development of wine varietal flavor during winemaking. For example, geraniol was released in concentrations that were up to 10 times higher than its free concentration in the F3P30, IASMA ECO3, F3P63, Riesling and Moscato Rosa varieties. This suggests that the application of this type of enzymatic hydrolysis in winemaking can be considered a risky oenological practice, with the potential not simply to enhance, but also to drastically modify the aroma profile of wine. Since glycosylation is one of the predominant modifications in plants and many secondary metabolites undergo the process, the current approach can be applied to improve our understanding of the chemistry of secondary metabolites and their glycosidic counterparts.

4.5. Contributions

4.6. Images source

Images of grape Isabella, Gewürztraminer, Moscato Rosa, Nero and Riesling were adopted from, Origin of the picture: Julius Kühn-Institut Bundesforschungsinstitut für Kulturpflanzen (JKI) Institut für Rebenzüchtung Geilweilerhof-76833 Siebeldingen-Germany. Reproduced from http://www.vivc.de

5. Atmospheric pressure gas chromatography mass spectrometry (APGC-MS) based metabolomics profiling platform: development and application

5.1. Introduction

Gas chromatography coupled with mass spectrometry (GC-MS) is a powerful tool for simultaneously profiling a large number of plant metabolites. It is well-established and comprehensively used approach in metabolomics. Most widely used ionization technique in GC-MS is electron ionization (EI) which is a hard ionization technique and creates intense fragmentation of the parent molecule. The molecular ion in an EI spectrum is often present with very low abundance or sometimes absent. This in practice means that the molecular ion can be observed only for a limited set of major metabolites and this piece of information is frequently not available for low-abundance and trace metabolites, making their annotation more challenging.

In EI, the high energy electrons (70 eV) are used to collide with analyte molecules makes the production of further fragments, the similar way of fragmentation can be observed with different EI instrument. Since these spectral similarities, there are different databases of EI spectra, which are currently available for the identification of the compounds. Different databases like drugs, metabolites, poisons, pesticides, fungicides and common sample contaminants (Hurtado-Fernández et al., 2013). The NIST Mass Spectral Search Program[©] is one of the widely used commercial software for the compound identification in EI GC-MS experiments. Many GC-MS can perform chemical ionization (CI) and a gas chromatograph equipped with a high-resolution time of flight mass spectrometer (TOFMS) (Waters GCT) was available within the labs of Fondazione Mach to perform such experiments. Unfortunately, a preliminary attempt to use it (using methane, data not reported) failed since the measurements lacked both the sensitivity and the reproducibility to be used effectively within metabolomics experiments. The sensitivity is needed to obtain a reasonable coverage including the low abundance metabolites. The stability of the system is a pre-requisite to allow the comparison of several injections within the same sequence, which has been observed with the GCT as a general problem, given that this technique rarely has been used so far within metabolomics.

The gas chromatography hyphenated with atmospheric pressure ionization time of flight mass spectrometry is relatively new technique that is commercially launched by Waters

Corporation under the trade name APGC-MS. It offers several benefits over traditional EI GC-MS systems. Being a soft ionization technique it allows reduced levels of fragmentation and generates the spectrum conserving the molecular ion species (figure 22). Additionally the system offers high mass accuracy, which is extremely useful in structure elucidation of unknown compounds. The lack of spectral database (unlike EI) for compound identification is the main reason for the limited use of soft ionization techniques in GC-MS applications and vice versa. Recent studies from (Hurtado-Fernández et al., 2013; Pacchiarotta et al., 2013) Pacchiarotta et al. (2013) reported web based online database of the compounds using APCI technology. Pacchiarotta et al (2013) generated MS and MS2 spectra for 150 compounds and Hurtado-Fernández et al. (2013) reported spectra for 100 compounds in their publically available web based resource. Both databases jointly contains wide range of compounds such as amino acids, fatty acids, sugars and derivatives, phenolic acids and related compounds, flavonoids, organic acids, vitamins, nucleosides and nucleobases.
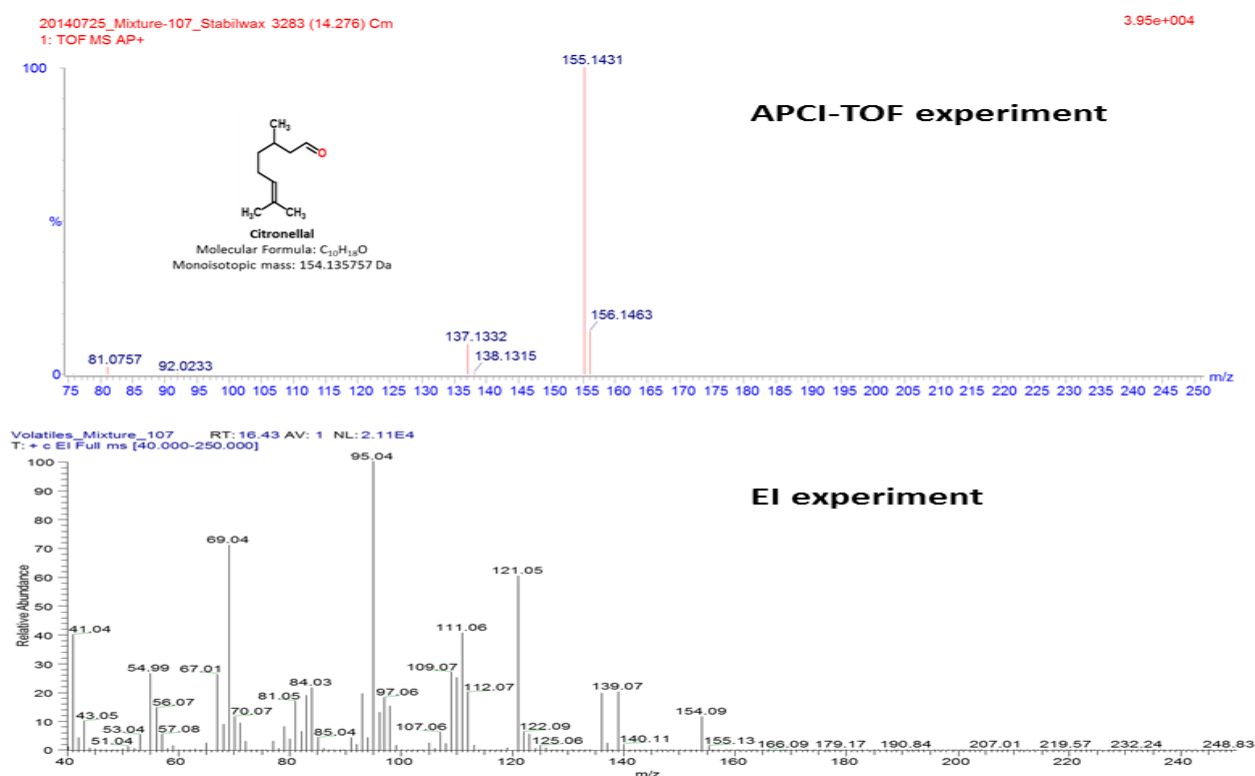
Figure 22 EI and APGC spectra of citronellal

We propose here some of the novel work done with development of APGC-MS technology based metabolomics platform. Two different case studies were conducted using grapes and Arabidopsis as an application to check the practicality of the platform.

5.2. Case study 1 – Grape

5.2.1. Materials and Methods

5.2.1.1. Grape samples

Six grape varieties (table 7) were collected from the two different locations in Texas, i.e. Blue Ostrich Vineyards 5611 FM Road 2382, Saint Jo, TX 76265, and Arche Vineyards 228 Wagner Rd, St Jo, TX 76265. Healthy and mature grape berries ($20°$-$22°$ Brix) were sampled and stored immediately at -80 $°$C prior to analysis.

Picture taken during sampling at Blue Ostrich Vineyards

Table 7 List of the grape varieties included in the study (Chapter 5)

LAB Code	Name	Berry Color	Location	°Brix
SYR	Syrah	Black	Arche wines	21.6
CHA	Chardonnay	White	Arche wines	24.4
CAB-1	Cabernet Sauvignon	Black	Arche wines	23
CAB-2	Cabernet Sauvignon	Black	Blue-Ostrich wines	24.4
TEM	Tempranillo	Noir	Blue-Ostrich wines	19.2
CHB	Chenin blanc	White	Blue-Ostrich wines	17

5.2.1.2. Chemicals and Reagents

Methanol, dichloromethane, ethanol, Pentane, sodium sulphate and ascorbic acid were purchased from Sigma Fisher Scientific USA. Citric acid, methyl benzoate and sodium azide were purchased from sigma Aldrich USA. The water used in the experiments was purified with a Mille-Q water purification system from Millipore (Bedford, MA, USA); SPE cartridges Isolate ENV+ (1 g, 6 mL) were obtained from Biotag (USA)

5.2.1.3. Sample Preparation

Sample preparation method was adapted from (Vrhovsek et al., 2014) with some modifications. 500 g grape berries were squeezed using household juicer (Model 54224 B23, Hamilton Beach) for 10 minutes, the freshly extracted grape juice was further centrifuged for 5min at 8000 rpm (Allegra 6R Centrifuge, Beckman Coulter). The upper clear part of the juice is decanted. 20 mL of this clear extract was taken for further extraction procedure and to it sodium azide (50 μL, of 1000 mg/L in water solution), citric acid (15 mg), ascorbic acid (15 mg) were added for to avoid any microbiological or enzymatic reaction (Fedrizzi et al., 2012). Methyl benzoate (10 μL of 1000 mg/L in Ethanol) was added as internal standard. The solution was then vortexed for 3 min in order to get all the contents mixed homogeneously and the extract was further used for the solid phase extraction procedure. All samples prepared in three technical replicates.

Isolute ENV+ cartridges were conditioned with 2 mL of methanol and 6 mL milliQ water (Milli Q Advantage A10 model, MilliQ, USA), and then the clear grape extract was eluted through cartridges. Later cartridges were washed with 20 mL of water to remove any leftover water soluble impurities and then volatiles were eluted with 20 mL of dichloromethane, elute was collected in a glass tube and 40 mL of pentane was added to it. To remove traces of moisture, the solution was dried with anhydrous Na_2SO_4 and then concentrated carefully using TurboVap® LV (Biotage, USA) to 200 μL, and subsequently analyzed by the APGC-TOFMS system.

5.2.1.4. APGC-TOFMS Analysis

Analysis of was performed with a 7890A gas chromatograph (GC, Agilent Technologies, Wilmington, DE, USA) hyphenated to a time-of-flight mass spectrometer (SYNAPT™ G2, Waters Corp., Milford, MA, USA) through an atmospheric pressure ion source (APGC, Waters Corp.). Separation of fruit volatiles was carried out using an Restek Stabilwax column (0.25 mm i.d., 30m length and 0.25 μm thick film). 1 μL volume of liquid sample was injected into the GC using a 7693 autosampler (Agilent Technologies, Wilmington, DE, USA); injections were done in splitless mode. GC injector temperature was set at 230°C, oven temperature program was set as; initial temperature of 40°C with hold of 4 minutes, followed by a 6°C/min ramp to 250°C with a final hold of 5 min. Helium was used as carrier gas at 1 mL/min (constant flow). The sample was introduced into the MS via a heated transfer line held at 275 °C and with a 350 mL/min sheath gas (nitrogen) flow. Mass spectrometer was operated in TOF-MS[E] (20-40 eV) mode with corona current 2 μA, source temperature 150 °C, sample cone 20 V, cone gas flow 40 L/h and auxiliary gas flow of 80 liter per hour. Spectral recording was performed in centroid data format, resolution analyzer mode and positive polarity with a 0.2 sec scan time. The MS data was acquired within the mass range of m/z 50-1200 Daltons. The system was operated with the Waters Corporation MassLynx© software (Version 4.1 SCN 870).

5.2.1.5. Data processing and Annotation

Raw data was converted to mzXML format and then processed using XCMS (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006) for feature extraction, the features were further

annotated using the CAMERA annotation package (Kuhl, Tautenhahn, Böttcher, Larson, & Neumann, 2012). The peak area table obtained from XCMS and CAMERA annotation was further manually filtered by removing peak areas with very low intensity values (<100), also features with m/z values between 50 to 350 Daltons were only considered since this is the range of molecular weights of the fruit volatile compounds. The final table of features and peak area was used for performing principle component analysis (for both high energy and low-energy experiments). The principle component analysis was performed by using SIMCA P+ (version 12.0, Umetrics) software.

5.2.2. Results

5.2.2.1. APGC ionization of the fruit volatiles

In order to understand the ionization pattern of the fruit volatiles under APGC source conditions, several reference standards of fruit aroma compounds (acid, aldehyde, C_{13}-norisoprenoid, ester, furan, ketone, monoterpene, phenol, sesquiterpene and pyrazine) were analyzed in the system using standardized protocols. Protonated $[M+H]^{\bullet}$ species were observed mainly for most of the compounds as there was slight moisture present in the ion source. Loss of OH (-17.0028) group was observed in the case of compounds containing hydroxy group. Detailed information about ionization and adducts formations; neutral losses of compounds are shown in the table 8. This table was further used for feature extraction from samples. Furthermore, clean spectra for each compound were also preserved in the Masslynx$^{©}$ library format, which is also convertible to NIST MS library format for the future references. Figure 23 and 24 explains the ionization of the VOC methyl benzoate under the high and low-energy levels of the APGC instrument as well as under EI conditions and NIST MS library spectra respectively.
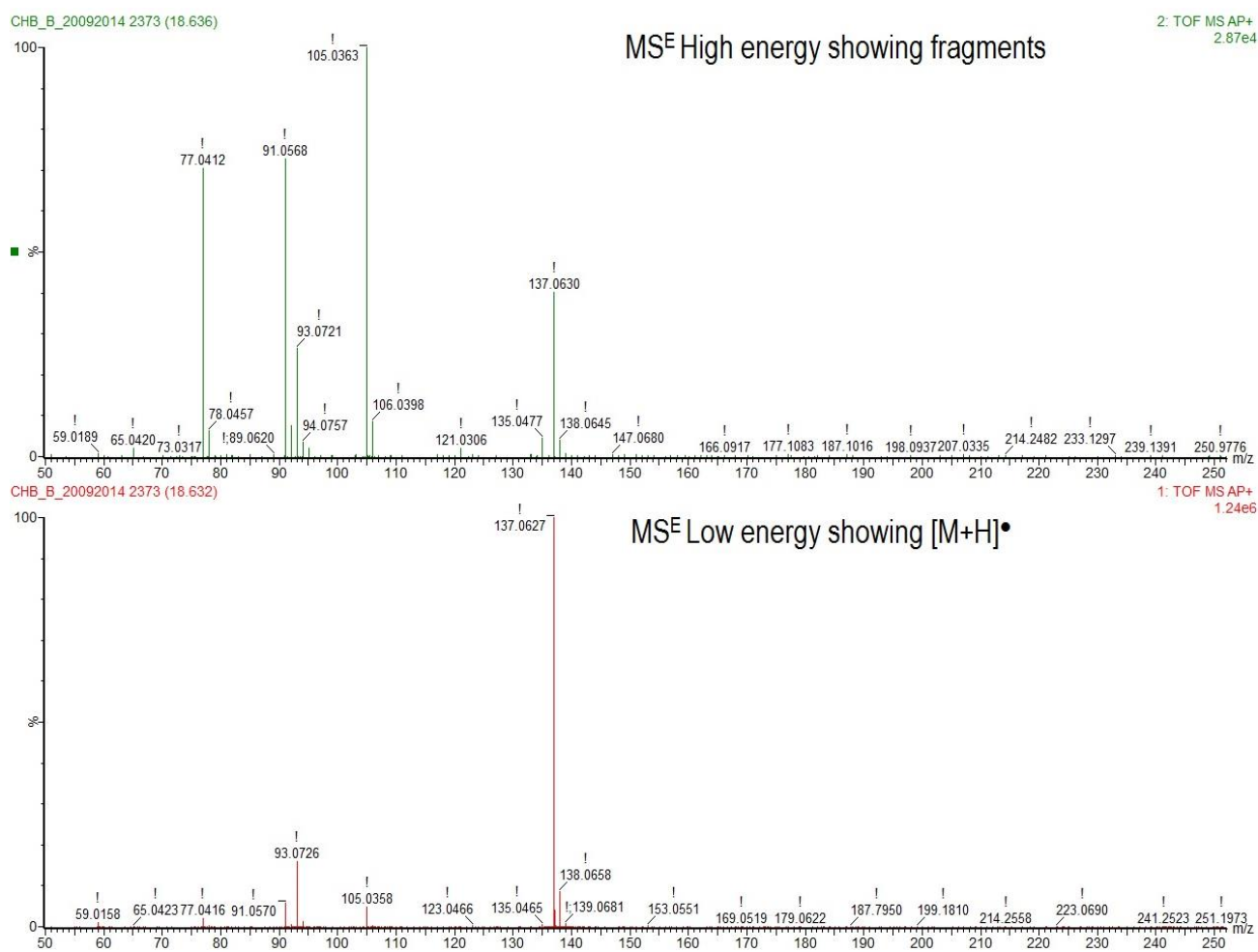
Figure 23 APGC spectra of Methyl Benzoate acquired in MSE mode
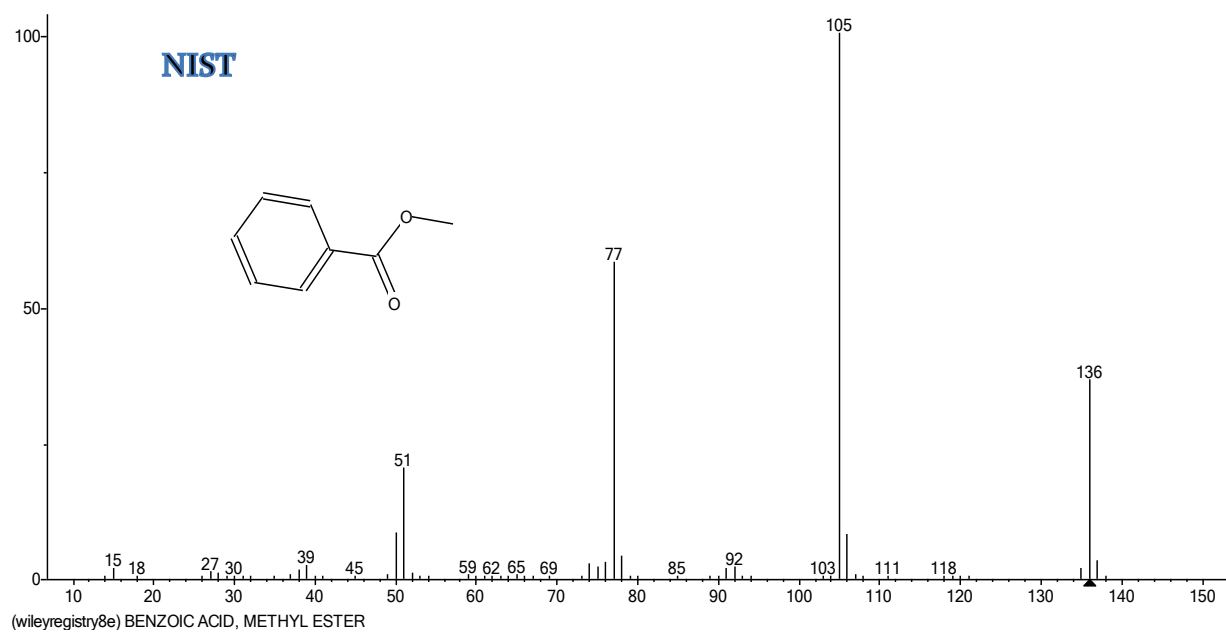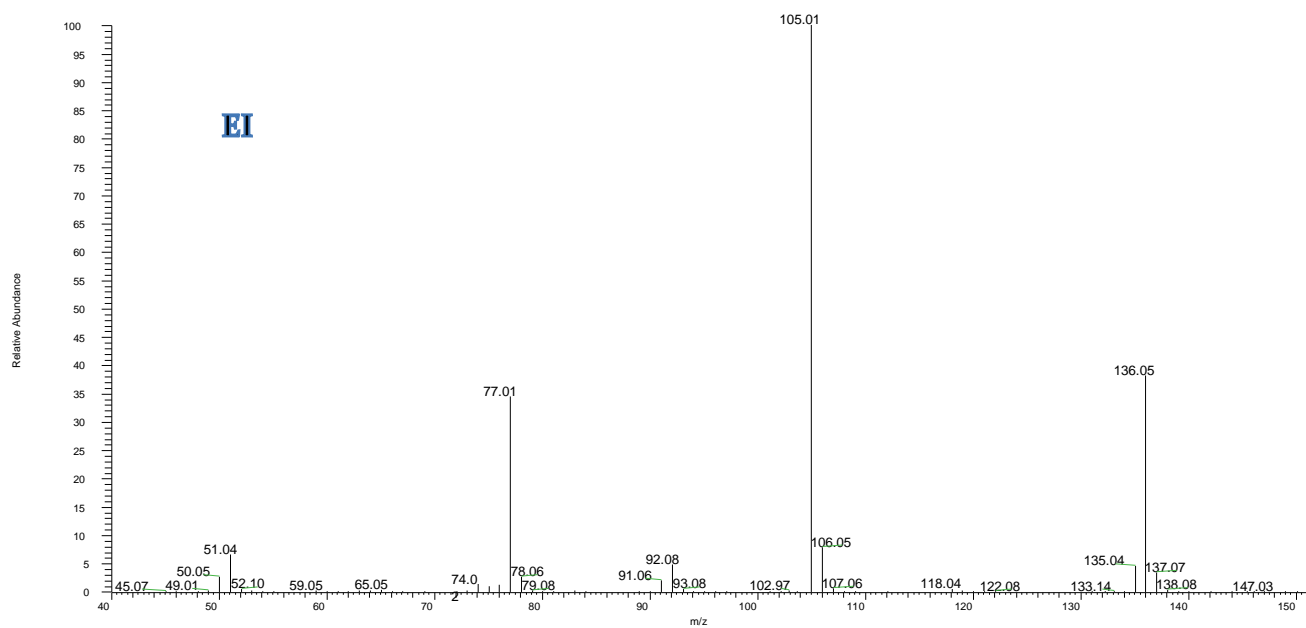
Figure 24 EI and NIST MS library spectra of Methyl Benzoate

Table 8. Peak table of the fruit volatile compounds generated from the analysis of reference standards

No	Metabolite	Class	Retention time (min)	Molecular formula	Monoisotopic mass	Adduct observed	Formula of adduct	Experimental monoisotopic mass of adduct	Theoretical monoisotopic mass of adduct	PPM error between theoretical mass and experimental mass
1	2-Ethylbutyric acid	Acid	21.16	C6H12O2	116.0837	M+H	C6H13O3	117.0911	117.0916	-4.5
2	3,4-Dihydroxybenzaldehyde	Aldehyde	16.86	C7H6O3	138.0317	M+H	C7H7O3	139.0761	139.0759	1.4
3	Cuminaldehyde	Aldehyde	21.79	C10H12O	148.0888	M+H	C10H13O	149.0981	149.0966	10.1
4	Dodecanal	Aldehyde	20.5	C12H24O	184.1827	M+H	C12H25O	185.191	185.1905	2.7
5	Phenyl acetaldehyde	Aldehyde	19.18	C8H8O	120.0575	M+H	C8H9O9	121.0657	121.0653	3.3
6	Undecanal	Aldehyde	18.43	C11H22O	170.1671	M+H	C11H23O	171.1757	171.1749	4.7
7	Anethole	Aromatic hydrocarbon	22.7	C10H12O	148.0888	M+H	C10H13O	149.0973	149.0966	4.7
8	Vanillylacetone (zingerone)	Benzenoid	37.22	C11H14O3	194.0943	M-(C2HO2)	C9H13O	137.0863	137.0966	3.09
	vanillylacetone (zingerone)	Benzenoid	37.22	C11H14O3	194.0943	M+	C11H14O3	194.0949	194.0943	NA
9	(±)-Theaspirane-I	C13-norisoprenoid	16.16	C13H22O	194.1671	M-OH	C13H21	177.1646	177.1643	1.7
	(±)-Theaspirane-II	C13-norisoprenoid	18.99	C13H22O	194.167	M+H	C13H23O	195.1753	195.1749	2
10	alpha-Ionol	C13-norisoprenoid	23.95	C13H22O	194.1671	M-OH	C13H21	177.166	177.1643	9.6
11	Theaspirane-I	C13-norisoprenoid	16.11	C13H22O	194.1671	M+H	C13H23O	195.1755	195.1749	3.1

	Theaspirane-II	C13-norisoprenoid	16.98	C13H22O	194.1671	M-OH	C13H21	177.165	177.1643	4
12	beta-Ionol	C13-norisoprenoid	24.83	C13H22O	194.1671	M-OH	C13H21	177.1657	177.1643	7.9
13	2-Phenoxyethyl isobutyrate	Ester	27.96	C12H16O3	208.1099	M-(C6H5O)	C6H11O2	115.0761	115.0759	0
	2-Phenoxyethyl isobutyrate	Ester	27.96	C12H16O3	208.1099	M+H	C12H17O3	209.1178	209.1178	1.7
14	Benzyl cinnamate	Ester	42.14	C16H14O2	238.0994	M-(CHO2)	C15H13	193.1171	193.1229	NA
	Benzyl cinnamate	Ester	42.14	C16H14O2	238.0994	M+	C16H14O2	238.1039	238.0994	NA
15	beta-Humulene	Ester	16.65	C15H24	204.1878	M+H	C15H25	205.1945	205.2056	-5.4
16	Butyl benzoate	Ester	23.29	C11H14O2	178.0994	M-(C4H7)	C7H7O2	123.0449	123.0446	2.4
17	Butyl butyrate	Ester	14.37	C8H16O2	144.115	M-(C2H4)	C6H13O2	117.0916	117.0916	0
18	Butyl heptanoate	Ester	16.57	C11H22O2	186.162	M-(C4H7)	C7H15O2	131.1075	131.1072	2.3
19	Butyl isobutyrate	Ester	10.07	C8H16O2	144.1150	M-(C2H4)	C6H13O2	117.0914	117.0916	-1.7
	Butyl isobutyrate	Ester	10.07	C8H16O2	144.1150	M+H	C8H17O2	145.1228	145.1229	-0.7
20	Cinnamyl acetate	Ester	28.13	C11H12O2	176.0837	M-(C2H3O2)	C9H9	117.071	117.0704	4.5
	Cinnamyl acetate	Ester	28.13	C11H12O2	176.0837	M-(C2H3O)	C9H9O	133.0653	133.0653	5.1
21	*cis*-3-hexenyl 3-methylbutanoate	Ester	16.03	C11H20O2	184.1463	M+H	C11H21O2	185.1544	185.1542	1.1
23	Ethyl-2-methylbutyrate	Ester	5.28	C7H14O2	130.0994	M-(C2H4)	[C5H10O2+H]	103.0759	131.1072	-1.5
	Ethyl-2-methylbutyrate	Ester	5.28	C7H14O2	130.0994	M+H	C7H15O2	131.107	103.0759	0
24	Ethyl anthranilate	Ester	30	C9H11NO2	165.079	M+H	C9H12NO2	166.0879	166.0868	6.6
	Ethyl anthranilate	Ester	30	C9H11NO2	165.079	M-(CHO2)	C8H10N	120.0651	120.0813	NA
	Ethyl anthranilate	Ester	30	C9H11NO2	165.079	M+	C9H11NO2	165.079	165.079	NA
25	Ethyl butyrate	Ester	6.2	C6H12O2	116.0837	M-(C2H4)	C4H9O2	89.0608	117.0916	5.1
	Ethyl butyrate	Ester	6.2	C6H12O2	116.0837	M+H	C6H13O2	117.0922	89.0603	5.6
26	Ethyl caprylate	Ester	14.81	C12H24O2	200.1776	M-(C2H4)	C10H21O2	173.1547	173.1542	6.2

	Ethyl caprylate	Ester	14.81	C10H20O2	172.1463	M+H	C10H21O2	173.1557	173.1542	8.7
27	Ethyl decanoate	Ester	19.1	C9H18O2	158.1307	M-(C2H4)	C7H15O2	131.107	131.1072	2.9
	Ethyl decanoate	Ester	19.1	C12H24O2	200.1776	M+H	C12H25O2	201.1861	201.1855	3
28	Ethyl heptanoate	Ester	12.44	C8H16O2	144.115	M-(C2H4)	C6H13O2	117.0916	117.0916	-1.5
	Ethyl heptanoate	Ester	12.44	C9H18O2	158.1307	M+H	C9H9O2	159.1385	159.1385	0
29	Ethyl hexanoate	Ester	10.03	C8H16O3	144.115	M-(C2H4)	C6H13O2	117.0916	145.1229	-1.7
	Ethyl hexanoate	Ester	10.03	C8H16O2	144.115	M+H	C8H17O2	145.1227	117.0914	-1.4
30	Ethyl isovalerate	Ester	5.3	C7H14O2	130.0994	M-(C2H4)	[C5H10O2+H]	103.0756	131.1072	-3.1
	Ethyl isovalerate	Ester	5.3	C7H14O2	130.0994	M+H	C7H15O2	131.1068	103.0759	-2.9
31	Ethyl nonanoate	Ester	17	C11H22O2	186.162	M-(C2H4)	C9H19O2	159.1388	158.1385	1.6
	Ethyl nonanoate	Ester	17	C11H22O2	186.162	M+H	C11H23O3	187.1701	187.1698	1.9
32	Ethyl phenylacetate	Ester	21.92	C10H12O2	164.0837	M-(C3H5O2)	C7H7	91.0551	91.0548	0
	Ethyl phenylacetate	Ester	21.92	C10H12O2	164.0837	M+H	C10H13O2	165.0916	165.0916	3.3
33	Ethyl salicylate	Ester	22.35	C9H10O3	166.063	M+H	C9H11O3	167.072	167.0708	7.2
34	Ethyl sorbate	Ester	16.38	C8H12O2	140.0837	M-(C2H5O)	C6H7O	95.0497	95.0497	-0.9
	Ethyl sorbate	Ester	16.38	C8H12O2	140.0837	M-(C2H4)	C6H9O2	113.0602	113.0603	0
	Ethyl sorbate	Ester	16.38	C8H12O2	140.0837	M+H	C8H13O2	141.0916	141.0916	0
35	Eugenyl acetate	Ester	29.82	C12H14O3	206.0943	M+H	C12H15O3	207.1033	207.1021	5.8
36	Geranyl benzoate	Ester	34.56	C17H22O2	258.162	M-(C7H5O2)	C10H17	137.1331	137.133	0.7
	Geranyl benzoate	Ester	34.56	C17H22O2	258.162	M+H	C17H23O2	259.17	259.1689	0.8
37	Geranyl butyrate	Ester	23.87	C14H24O2	224.1776	M-(C4H7O2)	C10H17	137.134	137.133	7.3
38	Geranyl phenylacetate	Ester	21.33	C18H24O2	272.1776	M+H	C18H25O2	273.2582	273.2582	0.7
39	Geranyl propionate	Ester	22.52	C13H22O2	210.162	M-(C3H5O2)	C10H17	137.1336	137.133	4.4

40	Heptyl acetate	Ester	13.41	C9H18O2	158.1307	M+H	C9H19O2	159.0707	159.1358	NA
41	Hexyl acetate	Ester	22.45	C8H16O2	144.115	M+H	C8H17O2	145.1243	145.1229	9.6
42	Hexyl hexanoate	Ester	18.62	C12H24O2	200.1776	M-(C6H11)	C6H13O2	117.0917	117.0916	0.9
43	Isobutyl acetate	Ester	13	C6H12O2	116.0837	M+H	C6H13O2	117.0912	117.0916	0.9
44	Linalyl butyrate	Ester	20.05	C14H24O2	224.1776	M-(C4H7O2)	C10H17	137.1338	137.133	5.8
45	Linalyl propionate	Ester	18.56	C13H22O2	210.162	M-(C3H5O2)	C10H17	137.1333	137.133	2.2
46	Methyl 3-(methylthio)propionate	Ester	12.06	C5H10O2S	134.0401	M+H	C5H11O2S	135.0478	135.048	-1.5
47	Methyl anthranilate	Ester	29.49	C8H9NO2	151.0633	M+H	C8H10NO2	152.0692	152.0686	NA
48	Methyl caproate	Ester	8.78	C7H14O2	130.0994	M+H	C7H15O2	131.107	131.1072	-1.5
49	Methyl jasmonate	Ester	30.91	C13H20O3	224.1412	M+H	C13H21O3	225.1513	225.1491	9.8
50	Methyl n-methylanthranilate	Ester	20.72	C9H11NO3	165.079	M-(CH3O)	C8H8NO	134.0615	134.0606	4.2
	Methyl n-methylanthranilate	Ester	20.72	C9H11NO2	165.079	M+H	C9H12NO2	166.0875	166.0868	6.7
51	Methyl pelargonate	Ester	16.06	C10H20O2	172.1463	M+H	C10H21O2	173.1552	173.1543	5.8
52	Methyl salicylate	Ester	21.71	C8H8O3	152.0473	M+H	C8H9O3	153.056	153.0552	5.2
53	Methyl *trans*-cinnamate	Ester	26.93	C10H10O2	162.0681	M+H	C10H11O2	163.0766	163.0759	4.3
54	Neryl acetate	Ester	20.8	C12H20O2	196.1463	M-(C2H3O2)	C10H18	137.1331	137.133	0.7
55	n-Hexyl butanoate	Ester	14.47	C10H20O2	172.1463	M-(C6H11)	C4H9O2	89.0606	89.0603	1.2
	n-Hexyl butanoate	Ester	14.47	C10H20O2	172.1463	M+H	C10H21O2	173.1544	173.1543	3.4
56	n-Pentyl acetate	Ester	5.7	C7H14O2	130.0994	M-(C2H4)	[C5H10O2+H]	103.0757	131.1072	-2.3
	n-Pentyl acetate	Ester	5.7	C7H14O2	130.0994	M+H	C7H15O2	131.1069	103.0759	-1.9
57	Octyl acetate	Ester	15.68	C10H20O2	172.1463	M+H	C10H21O2	173.1541	173.1542	-0.6
58	Propyl propionate	Ester	12.12	C6H12O2	116.0837	M+H	C6H13O2	117.0914	117.0914	-1.7
59	Sabinene hydrate	Ester	15.4	C10H18O	154.1358	M-OH	C10H17	137.1329	137.133	-0.1

60	*trans*-2-Hexenyl acetate	Ester	18.55	C8H14O2	142.0994	M+H	C8H15O	143.1081	143.1072	4.9
61	alpha-Methylbenzyl acetate	Ester	20.33	C10H12O2	164.0837	M-(C2H3O2)	C8H9	105.0706	105.0704	1.9
62	alpha-Asarone	Ether	32.75	C12H16O3	208.1099	M+H	C12H17O3	209.1174	209.1178	-1.9
63	5-Hydroxymethyl furfural	Furan	33.31	C6H6O4	126.0317	M-OH	C6H5O2	109.0294	109.029	3.7
	5-Hydroxymethyl furfural	Furan	33.31	C6H6O3	126.0317	M+H	C6H7O3	127.0402	127.0395	5.5
64	3-Hexanone	Ketone	5.58	C6H12O	100.0888	M+H	C6H13O	101.0587	101.0966	NA
65	1R,2R,5R-(+)-Hydroxy-3-pinanone	Ketone	24.89	C10H16O2	168.115	M-(C2H3O2)	C8H13	109.1018	109.1017	0
	1R,2R,5R-(+)-Hydroxy-3-pinanone	Ketone	24.89	C10H16O2	168.115	M-OH	C10H15O	151.1123	151.1123	0
	1R,2R,5R-(+)-Hydroxy-3-pinanone	Ketone	24.89	C10H16O2	168.115	M+H	C10H17O2	169.1229	169.1229	0.9
66	2,6,6-Trimethylcyclohexanone	Ketone	11.95	C9H16O	140.1201	M+H	C9H17O	123.1174	123.1174	0
	2,6,6-Trimethylcyclohexanone	Ketone	11.95	C9H16O	140.1201	M-OH	C9H15	141.1279	141.1279	0
67	2-Nonanone	Ketone	13.79	C9H18O	142.1358	M+H	C9H19O	143.1441	143.1436	3.5
68	3-Octanone	Ketone	10.46	C8H16O	128.1201	M+H	C8H17O	129.1278	129.1279	-0.8
69	4-Hexen-3-one	Ketone	8.88	C6H10O	98.07317	M+H	C6H11O	99.0804	99.081	-6.1
70	6-Methyl-5-hepten-2-one	Ketone	10.19	C8H14O	126.1045	M-OH	C8H13	109.1018	109.1017	0.9
71	Damascenone	Ketone	22.51	C13H18O	190.1357	M+H	C13H19O	191.1444	191.1436	4.2
72	gamma-Octalactone	Lactone	24.24	C8H14O2	142.0994	M-OH	C8H13O	125.0972	125.0966	4.8
	gamma-Octalactone	Lactone	24.24	C8H14O2	142.0994	M+H	C8H15O2	143.1079	143.1072	4.9
73	(-)-Menthone	Monoterpene	15.28	C10H18O	154.1358	M+H	C10H19O	155.1434	155.1436	-1.3
74	(-)-Myrtenol	Monoterpene	22	C10H16O	152.1201	M-OH	C10H15	135.1177	135.1174	2.2
75	(-)-Rose oxide-I	Monoterpene	13.64	C10H18O	154.1358	M-OH	C10H17	137.133	137.133	0
	(-)-Rose oxide-II	Monoterpene	14.04	C10H18O	154.1357	M+H	C10H19O	155.1439	155.1436	1.9
76	(+)-alpha-Terpineol	Monoterpene	20.24	C10H18O	154.1358	M-OH	C10H17	137.1332	137.133	1.5

77	(+)-Camphene	Monoterpene	5.73	C10H16	136.1252	M+H	C10H17	137.1245	137.133	NA
78	(+)-Menthofuran	Monoterpene	15.79	C10H14O	150.1045	M+H	C10H15O	151.1125	151.1123	1.3
79	(+)-Menthone-I	Monoterpene	15.34	C10H18O	154.1358	M+H	C10H19O	155.143	155.1436	-3.9
	(+)-Menthone-II	Monoterpene	15.92	C10H18O	154.1358	M-OH	C10H17	137.1329	137.133	-0.7
80	(2E,6E)-Farnesol	Monoterpene	30.79	C15H26O	222.1984	M-OH	C15H25	205.1956	205.1956	0
81	(R)-(+)-Limonene	Monoterpene	8.96	C10H16	136.1251	M+H	C10H17	137.1329	137.133	-0.7
	(R)-(+)-Pulegone	Monoterpene	19.14	C10H16O	152.1201	M+H	C10H17O	153.1286	153.1279	4.6
82	(S)-(−)-Limonene	Monoterpene	11.37	C10H16	136.1252	M+H	C10H17	137.1329	137.133	-0.7
83	2-Phenoxyethanol (rose ether)	Monoterpene	27.98	C8H10O2	138.0681	M-OH	C8H9O	121.0651	121.0651	-1.7
84	3-Carene	Monoterpene	7.36	C10H16	136.1252	M+H	C10H17	137.0759	137.133	NA
85	5-Methylfurfural	Monoterpene	11.78	C6H6O2	110.0368	M+H	C6H7O2	111.0451	111.0446	4.5
86	Acetanisole	Monoterpene	27.94	C9H10O2	150.0681	M+H	C9H11O2	151.0773	151.0759	9.3
87	Acetovanillone	Monoterpene	35.2	C9H10O3	166.063	M+H	C9H11O3	167.0741	167.0708	NA
88	alfa-Pinene	Monoterpene	5.13	C10H16	136.1252	M+H	C10H17	137.133	137.133	0
89	Camphor	Monoterpene	14.44	C10H16O	152.1201	M+H	C10H17O	153.0647	153.1279	NA
90	Carvacrol	Monoterpene	29.15	C10H14O	150.1045	M+H	C10H15O	151.1128	151.1123	3.3
91	*cis*-Jasmone	Monoterpene	24.66	C11H16O	164.1201	M+H	C11H17O	165.1299	165.1279	12.1
92	Citral	Monoterpene	9.22	C10H16O	152.1201	M+H	C10H17O	153.1276	153.1279	-2
93	Citronellal	Monoterpene	14.48	C10H18O	154.1357	M-OH	C10H17	137.1334	137.133	2.9
	Citronellal	Monoterpene	14.48	C10H18O	154.1357	M+H	C10H19O	155.1441	155.1436	3.2
94	delta-Neoclovene-I	Monoterpene	17.47	C15H24	204.1878	M+H	C15H25	205.1958	205.1956	1
	delta-Neoclovene-II	Monoterpene	18.3	C15H24	204.1878	M+H	C15H25	205.1958	205.1956	1
95	dihydro-alpha-ionone	Monoterpene	22.43	C13H22O	194.1671	M+H	C13H23O	195.1768	195.1749	9.7

96	Eugenol	Monoterpene	28.4	C10H12O2	164.0837	M+H	C10H13O2	165.0916	165.0916	0
97	Geranic acid-I	Monoterpene	30.6	C10H16O2	168.115	M+H	C10H17O2	169.1239	169.1229	5.9
	Geranic acid-II	Monoterpene	31.26	C10H16O2	168.115	M-(CHO2)	C9H15	123.182	123.1174	6.5
98	Geraniol	Monoterpene	23.05	C10H18O	154.1357	M-OH	C10H17	137.1336	137.133	4.4
99	Geranyl acetone -I	Monoterpene	22.69	C13H22O	194.1671	M+H	C13H23O	195.1752	195.1749	2.8
	Geranyl acetone -II	Monoterpene	23.15	C13H22O	194.1671	M+H	C13H23O	195.1752	195.1749	NA
100	Linalool	Monoterpene	15.79	C10H18O	154.1357	M-OH	C10H17	137.1329	137.133	-1.3
	Linalool	Monoterpene	15.79	C10H18O	154.1357	M+H	C10H19O	155.1434	155.1436	-0.7
101	Linalool oxide-I	Monoterpene	13.06	C10H18O2	170.1307	M-OH	C10H17O	153.1285	153.1279	3.9
102	Linalool oxide-II	Monoterpene	12.6	C10H18O2	170.1307	M-OH	C10H17O	153.1287	153.1279	5.2
103	p-Cymene	Monoterpene	10.75	C10H14	134.1095	M-(CH3)	C9H11	119.0922	119.0861	NA
104	p-Menth-1-ene	Monoterpene	7.16	C10H18	138.1409	M-H	C10H17	137.0746	137.133	NA
105	Safranal	Monoterpene	15.87	C10H14O	150.1045	M+H	C9H15O	151.1126	151.1123	2
106	Terpinen-4-ol	Monoterpene	18.31	C10H18O	154.1358	M-OH	C10H17	137.133	137.133	0
107	*trans*-Beta farnesene	Monoterpene	19.68	C15H24	204.1878	M+H	C15H25	205.1963	205.1956	3.4
108	*trans*-Terpin	Monoterpene	27.23	C10H20O2	172.1463	M-(H3O2)	C10H17	137.134	137.133	5.2
	trans-Terpin	Monoterpene	27.23	C10H20O2	172.1463	M-(H3O)	C10H17O	153.1287	153.1279	7.3
109	*trans*-2-Octenal	Monoterpene	11.43	C8H14O	126.1045	M+H	C8H15O	127.1126	127.1123	2.4
110	2-Methoxy-4-vinylphenol	phenol	28.25	C9H10O2	150.0681	M+H	C9H11O2	151.0762	151.0759	2
111	4-Ethylphenol	Phenol	28.58	C8H10O	122.0732	M+H	C8H11O	123.0811	123.0817	0.8
112	Benzophenone	Phenol	32.96	C13H10O	182.0732	M+H	C13H11O	183.0824	183.081	7.6
113	2-Isobutyl-3-methoxypyrazine	Pyrazine	16.72	C9H14N2O	166.1106	M+H	C9H15N2O	167.1189	167.1189	-2.9
114	2-Methoxy-3-isopropyl pyrazine	Pyrazine	14.71	C8H12N2O	152.095	M+H	C8H13N2O	153.1039	153.1028	7.2

115	2-Methoxy-3-secbutylpyrazine	Pyrazine	16.25	C9H14N2O	166.1106	M+H	C9H15N2O	167.1208	167.1208	-14.3
116	Isopropyl methoxy pyrazine	Pyrazine	14.73	C8H12N2O	152.095	M+H	C8H15N2O	153.1046	153.1045	0.6
117	2-Methylthio-benzothiazole	S-compound (thiol)	32.18	C8H7NS2	181.002	M+H	C8H8NS2	182.0106	182.0098	4.4
118	2-Methylthiolan-3-one	S-compound (thiol)	16.78	C5H8OS	116.0296	M+H	C5H9OS	117.0378	117.0374	3.4
119	4-Methylthio-1-butanol	S-compound (thiol)	22.9	C5H12OS	120.0609	M-OH	C5H11S	103.0582	103.0581	1
120	6-Mercapto-1-hexanol	S-compound (thiol)	26.87	C6H14OS	134.0765	M+	C6H14OS	134.0601	134.0606	-3.7
121	(+)-Cedrol-I	Sesquiterpene	25.86	C15H26O	222.1984	M-OH	C15H25	205.1968	205.1956	5.8
	(+)-Cedrol-II	Sesquiterpene	27.54	C15H26O	222.1984	M-OH	C15H25	205.1968	205.1956	NA
122	(+)-Ledene	Sesquiterpene	20.1	C15H24	204.1878	M+H	C15H25	205.2151	205.1856	NA
123	alpha-Humulene	Sesquiterpene	19.66	C15H24	204.1878	M+H	C15H25	205.1951	205.1956	-2.4
124	Caryophyllene oxide	Sesquiterpene	25.34	C15H24O	220.1827	M-OH	C15H25O	203.1824	203.18	5.9
	Caryophyllene oxide	Sesquiterpene	25.34	C15H24O	220.1827	M+H	C15H25O	221.1918	221.1905	11.8
125	gamma-Humulene	Sesquiterpene	19.53	C15H24	204.1878	M+H	C15H25	205.1964	205.1956	3.9
125	Guaiene	Sesquiterpene	20.02	C15H24	204.1878	M+H	C15H25	205.1961	205.1956	2.4
126	Isolongifolene	Sesquiterpene	16.55	C15H24	204.1878	M+H	C15H25	205.1964	205.1956	3.9
127	beta-Caryophyllen	Sesquiterpene	25.31	C15H24	204.188	M-H	C15H23	203.1809	203.18	4.4
128	p-Propylanisole	--	18.59	C10H14O	150.1044	M+H	C10H15O	151.1122	151.1123	-0.7

16 compounds, (+) alpha-terpineol, (R)-(+)-pulegone, 3-carene, anethole, benzophenone, citronellal, delta-neoclovene, dihydro-alpha-ionone, geraniol, linalool oxide, methyl pelargonate, methyl salicylate, phenyl acetaldehyde, p-propyl anisole, sabinene hydrate, *trans*-terpin were putatively annotated by using table 8 parameters and CAMERA (Kuhl et al., 2012) annotation package and XCMS (Benton et al., 2008; Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006).

As the data was acquired in low and high-energy modes (figure 23), we have extracted features from two different functions; principle component analysis was performed on the feature extracted. Figure 25 shows the differentiation in the samples based on the features extracted from the low-energy data while figure 26 shows the differentiation in the samples based on the features extracted from the high-energy data. The preliminary analysis shows good separation of the all varieties in both energy based data acquisition.
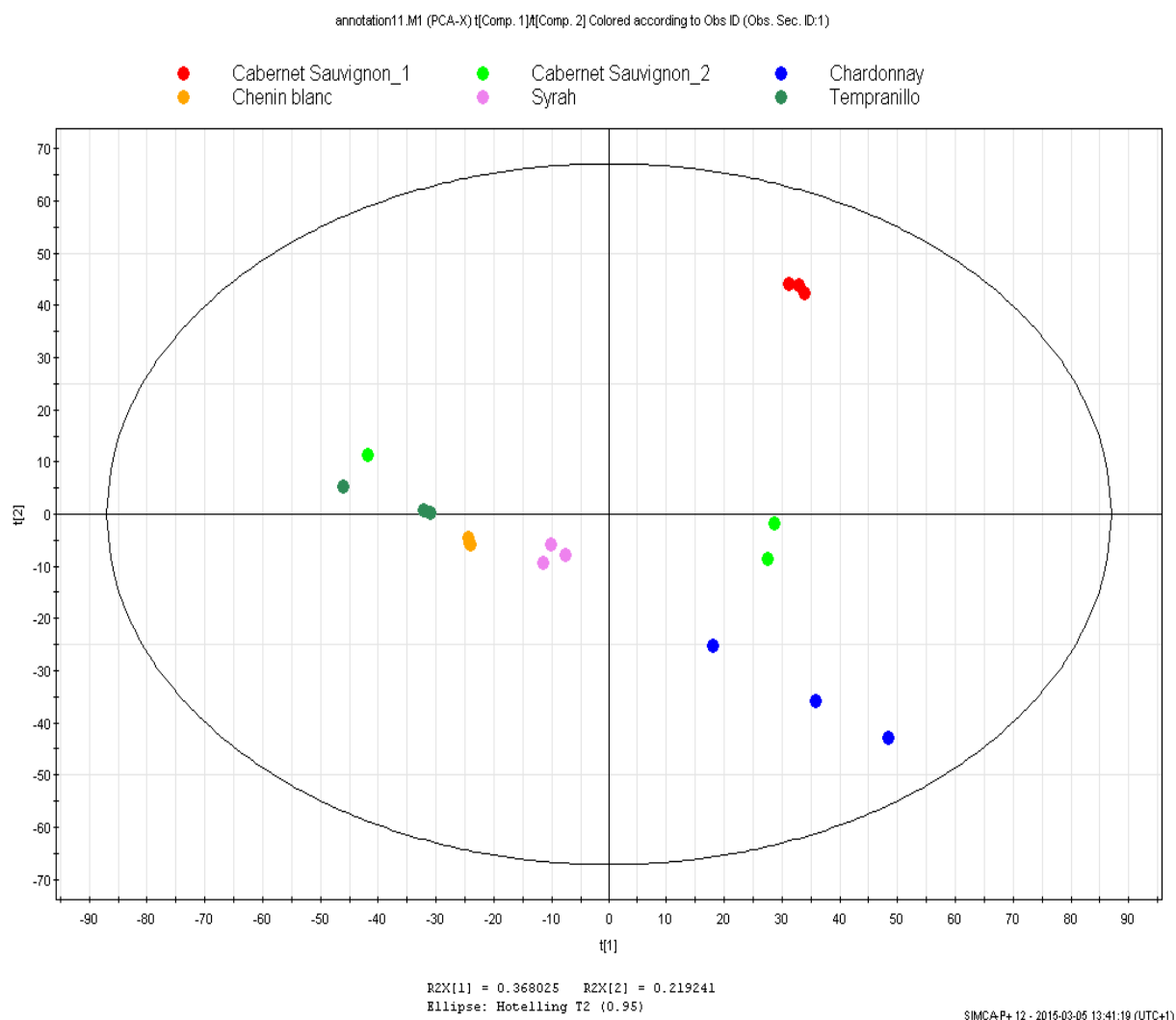
Figure 25 PCA score plot of features extracted in low-energy data acquisition

annotation12.M1 (PCA-X) t[Comp. 1]/t[Comp. 2] Colored according to Obs ID (Obs. Sec. ID:1)

R2X[1] = 0.324836   R2X[2] = 0.203449
Ellipse: Hotelling T2 (0.95)

SIMCA-P+ 12 - 2015-03-05 13:48:15 (UTC+1)
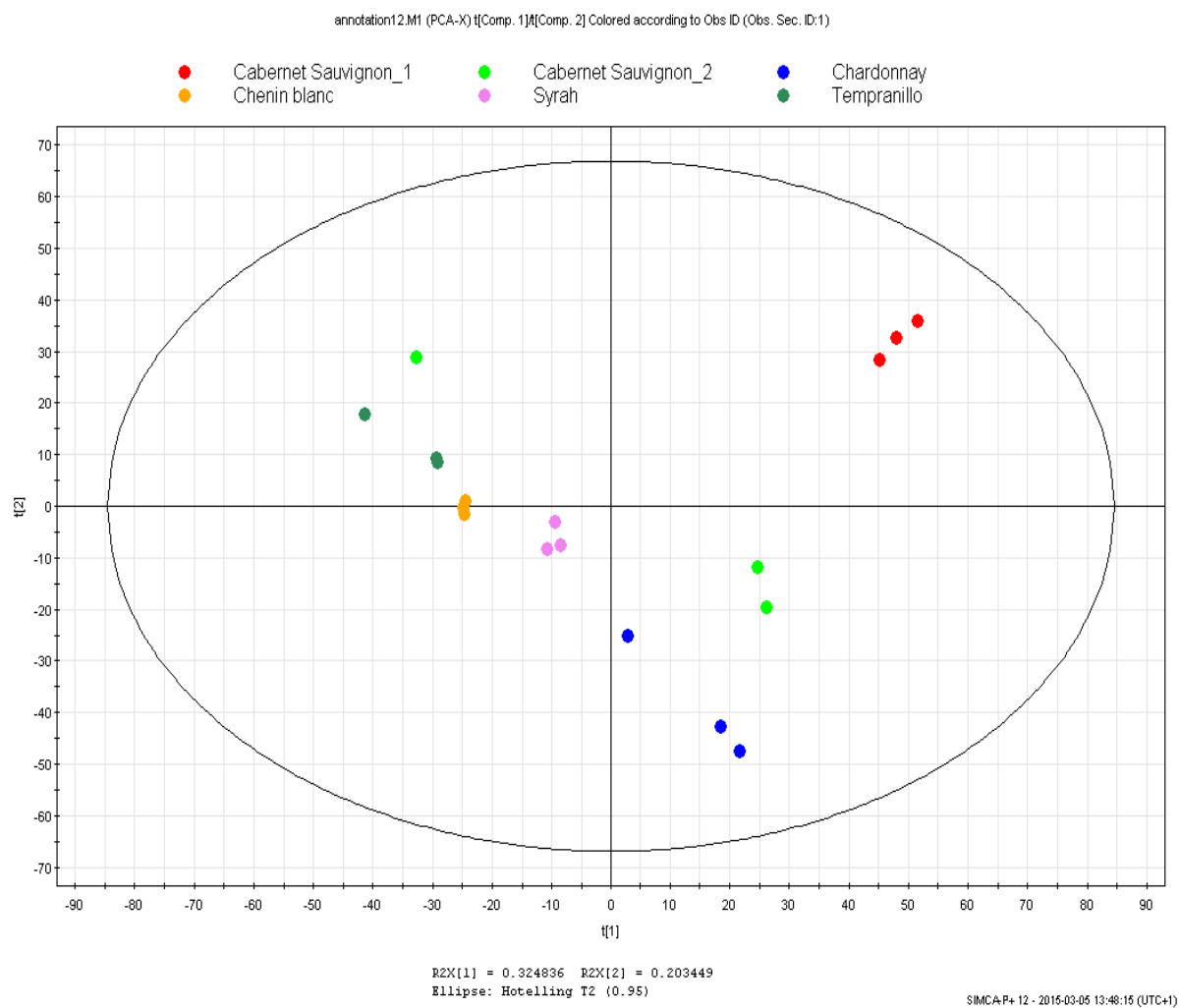
Figure 26 PCA score plot of features extracted in high-energy data acquisition

5.3. Case study 2- Arabodopsis

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Metabolic Phenotyping Using Atmospheric Pressure Gas Chromatography-MS

Vladimir Shulaev,[2] Ghaste Manoj,[2,3] Steven Lai,[1] Carolina Salazar,[2] Nobuhiro Suzuki,[2] Janna Crossley,[2] Feroza Kaneez Coudhury,[2] Khadiza Zaman,[2] Ron Mittler,[2] James Langridge,[1] Robert Plumb,[1] Fulvio Mattivi,[3] Giuseppe Astarita[1]
[1] Waters Corporation, Milford, MA, USA
[2] Department of Biological Sciences, College of Arts and Sciences, University of North Texas, Denton, TX, USA
[3] Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy

APPLICATION BENEFITS

Atmospheric pressure gas chromatography mass spectrometry (APGC-MS) provides molecular ion information, which is typically absent when traditional vacuum source (i.e., electron ionization) gas chromatography mass spectrometry (GC-MS) is employed. This application note highlights the use of APGC-MS[E] for analysis in metabolomics.

INTRODUCTION

Gas chromatography coupled with mass spectrometry is a well established analytical approach to metabolomics. The most widely used ionization technique is electron ionization (EI), which produces highly fragmented spectra that are library searchable. The molecular ion in an EI spectrum is often absent or of very low abundance. The lack of molecular ion information, especially for derivatized compounds in complex mixtures, can lead to incorrect identification when using spectral matching alone.

Atmospheric pressure GC (APGC) is a soft chemical ionization technique that generates a mass spectrum in which there is minimal fragmentation and conservation of the molecular ion (Figure 1). Additionally, when APGC is combined with a time of flight mass spectrometer (TOF-MS) for exact mass MS[E] analysis, comprehensive precursor and fragment ion spectral data is obtained for every detectable component in a complex sample. GC-MS[E] offers a simple route to providing high selectivity and confidence for simultaneous identification and quantitation in a single analysis.

Here we report an APGC-TOF MS[E] profiling approach and its application to metabolic fingerprinting of Arabidopsis.

WATERS SOLUTIONS

SYNAPT® G2-S HDMS®
Mass Spectrometer

APGC System

Progenesis® QI Software

MassLynx® Software v4.1 SCN870

KEY WORDS

Metabolomics, GC, atmospheric pressure gas chromatography

1

EXPERIMENTAL

GC conditions

GC system:	7890A GC
Column:	HP-5MS column, 30 m length, 0.25 mm I.D., and 0.25 µm film thickness (Agilent Technologies)
Carrier gas:	Helium 1 mL/min
Temp. gradient:	Initial 70 °C, 5 °C/min to 310 °C, hold 1 min
Injection type:	split mode (split 4:1)
Injector temp.:	230 °C
Injection vol.:	1 µL
Make-up gas:	Nitrogen 400 mL/min
Transfer line temp.:	310 °C

MS conditions

MS system:	SYNAPT G2-S HDMS
Mode of operation:	TOF-MS^E
Ionization:	APGC
Corona current:	3 µA
Cone voltage:	20 V
Source temp.:	150 °C
Cone gas:	10 L/h
Auxiliary gas flow:	500 L/h
MS gas:	Nitrogen
Acquisition range:	50 to 1200
Transfer CE:	Ramp 20 to 40 V

Data management

Progenesis QI Software v1.0

MassLynx Software v4.1 SCN870

Sample preparation

Arabidopsis thaliana seeds were grown under controlled conditions. Seedlings were harvested and polar metabolites were extracted and derivatized. The dried polar phase was methoximated for 90 minutes at 45 °C and trimethylsilylated for 30 minutes at 37 °C.
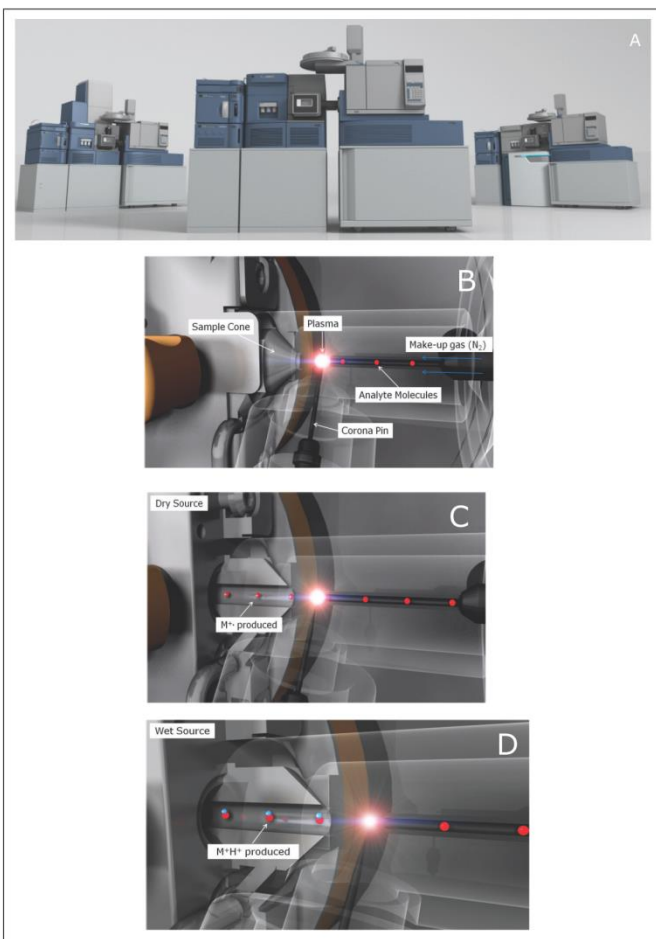
Figure 1. A) An APGC System can be coupled to various Waters MS instruments, including the Xevo® TQ-S and the SYNAPT G2-S. The changeover from UPLC® to APGC takes less than five minutes. B) The APGC source consists of an ion chamber with a corona pin inside. The GC column enters the source via a heated transfer line. Corona discharge creates nitrogen plasma within this region. Radical cations generated in this plasma interact and ionize the analyte molecules. The ions created are then transferred to the mass analyzer. C) Under dry source conditions the predominant method of ionization is charge transfer, generating molecular radical cations [M+•]. This method favours relatively non-polar molecules. D) When a protic solvent, such as water or methanol, is added to the source, the predominant ionisation is proton transfer, generating [M+H]+ ions. This method favors relatively polar molecules.

RESULTS AND DISCUSSION

APGC-MS analysis of commercially available pure reference compounds of known plant metabolites was performed. Following the analysis, an in-house APGC reference database was created containing retention times, and accurate mass-to-charge ratio (m/z) for precursor and fragment ions (Figure 2). APGC provided abundant molecular ions with minimal fragmentation at low collision energy (Figure 2A). To add confidence to compound identification, collision energy was ramped from 20 to 40 eV in the high energy function to generate maximum information from fragment ions (Figure 2A). Due to the use of charge exchange chemical ionization, elevated collision energy data resulted in a spectrum similar to the traditional EI data (Figure 2B).
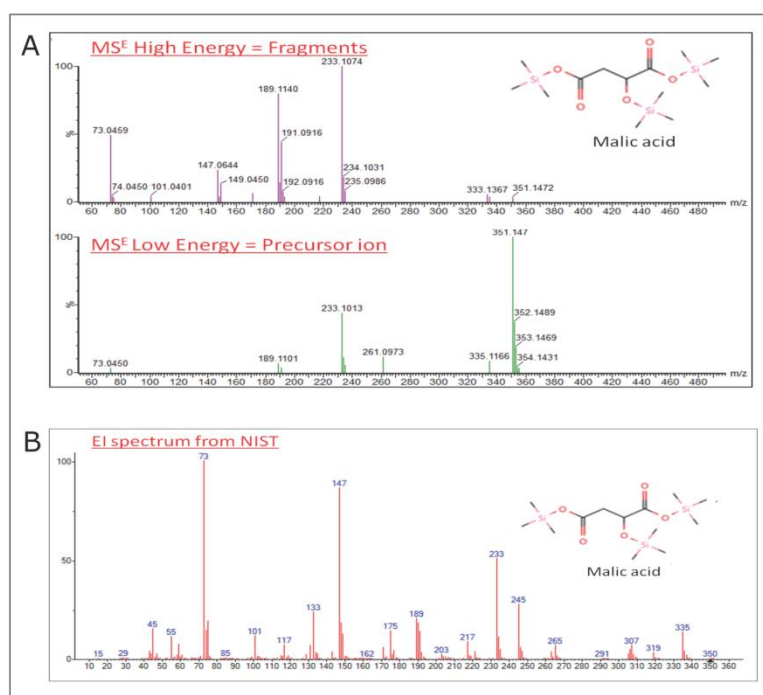
Figure 2. A) Synthetic reference standards were derivatized and analyzed by APGC-SYNAPT HDMS using MS^E acquisition mode. This mode alternates between low and elevated collision energy to acquire both precursor and product ion information in a single analytical run. Advanced software algorithms align precursor and fragment spectra according to retention time and links them together. An in-house database of derivatized precursors and fragments, and retention times was created. B) EI spectrum of derivatized malic acid, obtained from the NIST library, was comparable with the MS^E spectrum obtained by ramping up the collision energy from 20 to 40 eV in the high energy function.

Plant extracts were analyzed using APGC-TOF MS^E and raw data were imported to Progenesis QI Software for processing and analysis (Figure 3A and B). Multivariate statistical analysis highlighted the molecular differences between groups of samples (Figure 4A and B). The Progenesis QI search engine Metascope allowed us to query experimentally-derived or in-house databases. We were able to customize the search parameters for the metabolite identification according to multiple orthogonal measures such as mass accuracy, retention time and fragmentation matching (Figure 4C). Additionally, if ion mobility is activated, collision cross-section (CCS) data, which reflect the ionic shape of the metabolites in the gas phase, can also be mined for identification.
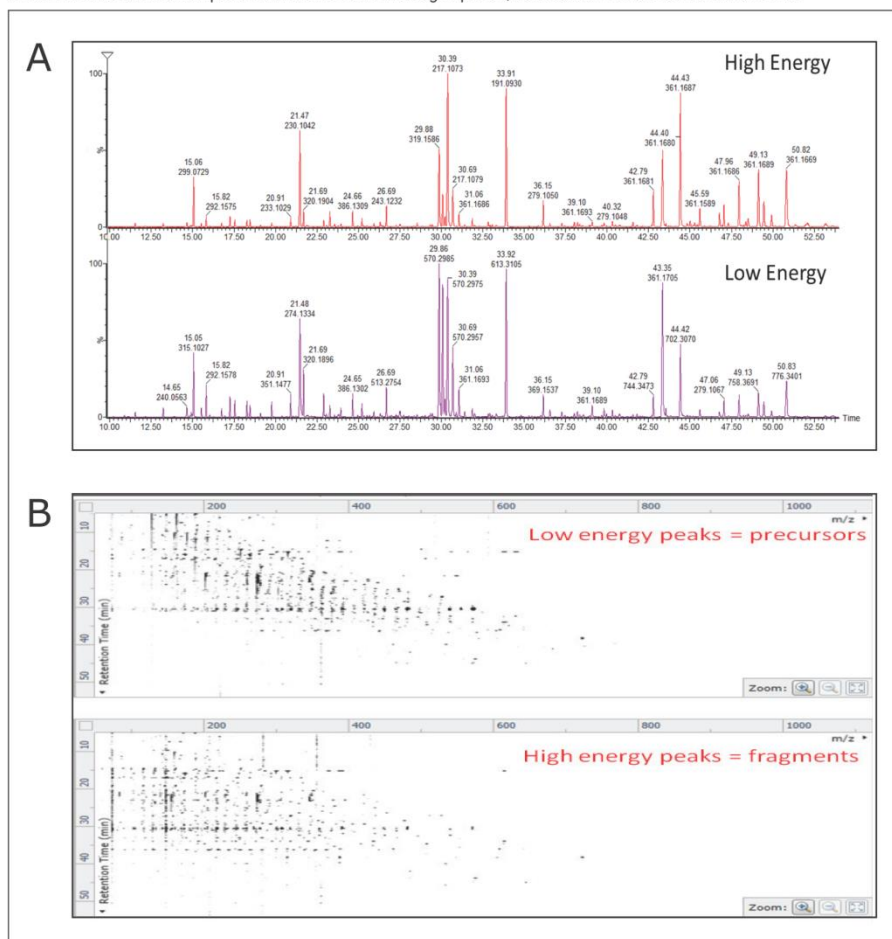
Figure 3. Biological samples were analyzed using APGC-MS$_E$ which provided information for both the intact precursor ions (at low collision energy) and the fragment ions (high collision energy). A) APGC-MS^E chromatogram of the Col-O Arabidopsis leaf extract. B) Data were imported as bidimentional maps (retention times versus m/z) and processed using Progenesis QI.
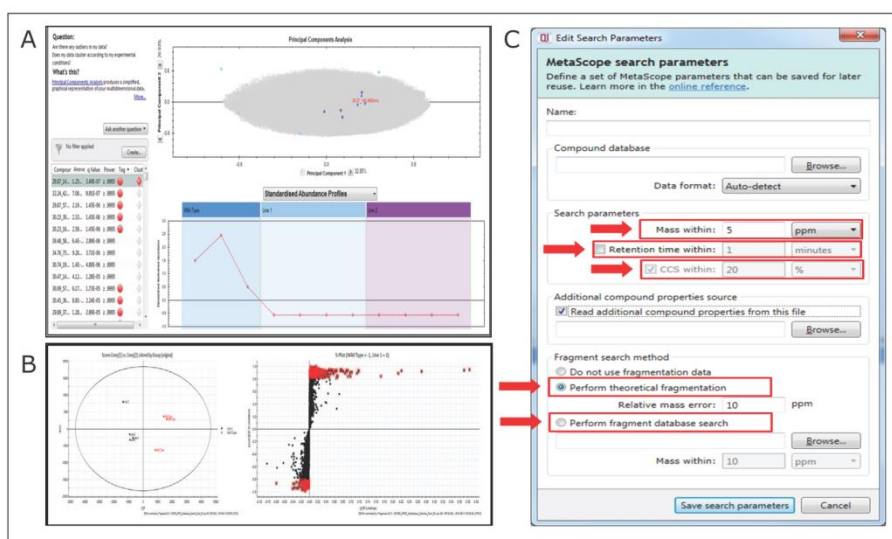
Figure 4. A) PCA model for wild type and mutant lines. B) OPLS-DA model for Arabidopsis Col-O wild type plants and mutant line. C) The Progenesis QI search engine allowed us to query experimentally-derived or in-house databases.

Sample data, generated with the low energy (precursor ion) spectrum and high energy (fragment ion) spectrum of each component, was searched against both the in-house and commercial mass spectrum libraries (Figure 5A and B). The identification score described the level of confidence obtained for each library hit based on accurate mass for precursor and fragment matching, retention time, and isotope ratios (Figure 5A and B).
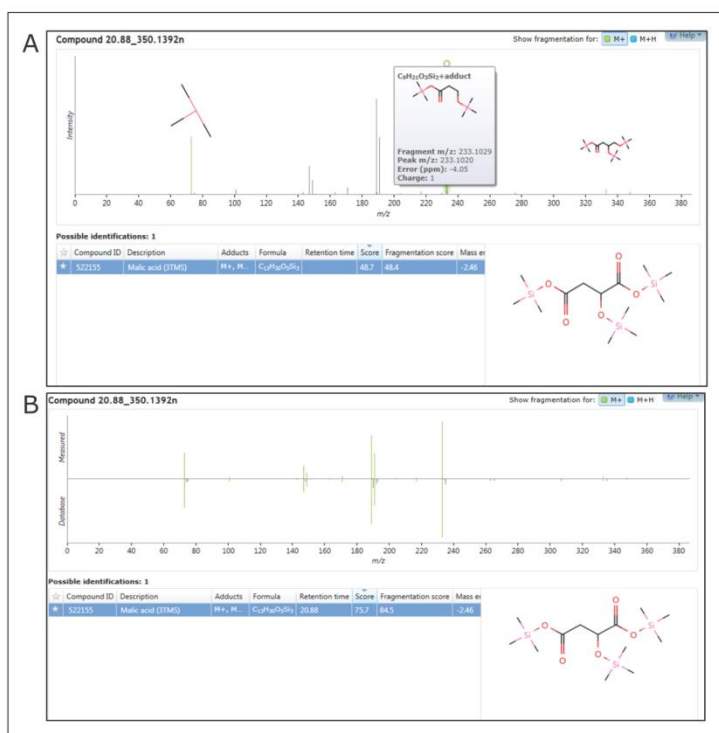
Figure 5. A) APGC-MS^E allowed the identification of malic acid extracted from a biological matrix, using theoretical fragmentation within Progenesis QI. The spectra of fragment ions were aligned with co-eluting precursor ions and matched against theoretically generated spectra (green signals). B) The identification score improved when identification of malic acid was conducted using an in-house database within Progenesis QI. The spectra of fragment ions were aligned with co-eluting precursor ions and matched against experimentally generated spectra (green signals).

As structurally similar metabolites often co-elute, the concurrent acquisition of an intact molecular ion, along with fragmentation data for sub-structural determination, was particularly useful. In combination with accurate mass measurement, the molecular ion helps determine the limits of chemical composition, which can subsequently be used along with the fragmentation data for more detailed and specific structural elucidation of both known and unknown metabolites.

A summary workflow for the APGC-TOF-MS^E approach:

1. An in-house database of derivatized metabolites was generated using APGC-MS^E

2. APGC provided abundant molecular ions with minimal fragmentation at low collision energy, which are typically missing when traditional vacuum source GC-MS is employed.

3. Due to the use of charge exchange chemical ionization, the elevated collision energy data resulted in a spectrum similar to the traditional EI data.

4. APGC-MS approach was used for metabolic fingerprinting of a set of Arabidopsis mutants.

5. Sample data generated with the low and high energy function (MS^E) were searched against the in-house database using the search engine within Progenesis QI.

CONCLUSIONS

APGC-TOF MS^E with Progenesis QI is a valuable solution for metabolomics applications. The use of orthogonal information for the metabolite identification, including accurate mass, retention time, and theoretical or measured fragmentation, increased the confidence of metabolite identification while decreasing the number of false positive identifications.

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5.4. Discussion

Ever increasing applications of GC-MS technology needs to address new requirements of the user some of them are soft ionization and preservation of molecular ion in the spectrum. The current technique of APGC-MS has shown its possible potentials in the future of GC-MS analysis. With the analysis of commercially available pure reference compounds of known fruit aroma compounds, we observed that APGC-MS provided abundant molecular ions with minimal fragmentation at low collision energy. For further confidence level in compound identification, the collision energy was ramped from 20 to 40 eV in the high-energy function to generate maximum information from fragment ions (figure 23). The use of charge exchange chemical ionization and elevated collision energy data resulted in a spectrum similar to the traditional EI data (figure 24) which is fascinating result and opens the use of EI spectral database for the compound identification.

To my belief, the main reason that limits the use of APGC-MS technology is the lack of spectral databases that compliments the compound identification. There are many studies recently coming up with small spectral databases based on APGC ionization (Hurtado-Fernández et al., 2013; Pacchiarotta et al., 2013). We also report our database containing spectra, retention times, and accurate mass-to-charge ratio (m/z) for precursor and fragment ions of 128 Fruit VOCs, the current database is also available in Masslynx$^©$ library format.

The experimental results from the analysis of selected grape samples showed that by using the features and further statistical tools we were able to discriminate the different grape varieties. The use of orthogonal information for the metabolite identification (accurate mass, retention time, and theoretical or measured fragmentation) increased the confidence of metabolite identification. Sixteen aroma compounds were putatively identified using metabolomics annotation tools.

Overall experience with the APGC-MS suggested its capacity to play a potential alternative approach to the common EI technique, if supported by spectral databases for compound identifications. The results from the analysis of grape and Arabidopsis samples also proposed that it is a valuable solution for GC-MS based metabolomics experiments. This is the first step

towards the exploration of this technology, which in future needs focused studies utilizing all possibilities of the technique.

5.5. Contributions

6. Thesis conclusions

The sensory quality of the fruits is widely determined by the qualitative and quantitative composition of volatile organic compound present in it. This thesis comprises research on the mapping of volatile compounds in grape. The work mainly describes the development and application of different gas chromatography mass spectrometry techniques, advanced data analysis strategies, statistical tools and some of novel multidimensional datasets of the grape VOCs. In the experiment of comprehensive mapping of VOCs, 124 grape cultivars were profiled for their VOCs content by the use of headspace solid phase microextraction and gas chromatography mass spectrometry. Additionally, automated pipeline and in-house database of grape VOCs were generated for the identification of the compounds, this work was done in collaboration with data management team at FEM. The annotation resulted into the "level 1" identification of 118 VOCs of different chemical classes and reports the dataset information which will allow to classify the most cultivated and distributed grape cultivars on the basis of their aromatic profile.

The glycosylated precursors are considered as a storehouse of aroma of grapes, many aroma active compounds are preserved in the grape berries in the form of these precursors in very high concentrations. We performed analysis of free and bound aroma compounds in 10 selected grape genotypes and successfully annotated 66 compounds. Many compounds showed qualitative and quantitate differences in free and glycosylated conjugated form. Cultivars Nero and species viz. *Vitis arizonica* and *Vitis cinerea* were studied for first time with this approach. The data produced will be beneficial for wine producers, in order to obtain information about not only the directly available free fraction but also their precursors that can significantly change its aroma during winemaking. The methodology used was simple, practical and reproducible and could be of general interest for the study of aroma precursors in other matrices.

The last part of the thesis was dedicated to study the development and applications of atmospheric pressure ionization gas chromatography mass spectrometry (APGC-MS), the relatively new technique in the field of GC-MS analysis. It offers several benefits over traditional EI GC-MS systems, as soft ionization based reduced levels of fragmentation and conservation of the molecular ion species. An APGC-MS method was developed and several for the analysis of several fruit volatile reference compounds was performed using

standardized protocols for the understanding of ionization patterns. To add confidence to compound identification the data was acquired into MS^E mode where collision energy was ramped from 20 to 40 eV. The low-energy function produced spectra with minimum fragmentation containing molecular ion species and high energy function generated spectra similar to EI. The final database contains spectra of 135 compounds and information like retention times, and accurate mass-to-charge ratio (m/z) for precursor and fragment ions. In a case study of grapes the clean spectra of 135 reference standards were saved in a Masslynx$^©$ library format which can be useful for any further studies. Six different grape varieties were analyzed by the same protocol and 16 aroma compounds were putatively identified using metabolomics annotation tools. This suggests that the APGC-MS is a valuable solution for GC-MS based metabolomics experiments and good possible alternative to traditional EI based systems.

The wide range of grape varieties studied in the thesis and three different datasets were created out of which the dataset from chapter 3 and chapter 5 jointly reports 177 VOCs. Some of new grape cultivars were studied in this thesis like Girelli hybrids and some non-vinifera cultivars. All the datasets generated in the study can be used as comprehensive repository of VOCs in selected grape cultivars. Most importantly, this database represents the significant portion of the grape secondary metabolism and a necessary part of grape metabolome. Moreover, the protocols reported in the study were tested with the grape as sample fruit and further can be extended to more fruit commodities.

7. References

Aprea, E., Gika, H., Carlin, S., Theodoridis, G., Vrhovsek, U., & Mattivi, F. (2011). Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry. *Journal of Chromatography A, 1218*(28), 4517–4524. doi:10.1016/j.chroma.2011.05.019

Arapitsas, P., Speri, G., Angeli, A., Perenzoni, D., & Mattivi, F. (2014). The influence of storage on the "chemical age" of red wines. *Metabolomics, 10*, 816–832. doi:10.1007/s11306-014-0638-x

Azzolini, M., Fedrizzi, B., Tosi, E., Finato, F., Vagnoli, P., Scrinzi, C., & Zapparoli, G. (2012). Effects of Torulaspora delbrueckii and Saccharomyces cerevisiae mixed cultures on fermentation and aroma of Amarone wine. *European Food Research and Technology, 235*(2), 303–313. doi:10.1007/s00217-012-1762-3

Baek, H. H., & Cadwallader, K. R. (1999). Contribution of Free and Glycosidically Bound Volatile Compounds to the Aroma of Muscadine Grape Juice. *Journal of Food Science, 64*(3), 441–444. doi:10.1111/j.1365-2621.1999.tb15059.x

Balogh, M. P. (2009). *The Mass Spectrometry Primer*. Waters Corporation.

Benton, H. P., Wong, D. M., Trauger, S. a., & Siuzdak, G. (2008). XCMS2: Processing tandem mass spectrometry data for metabolite identification and structural characterization. *Analytical Chemistry, 80*(16), 6382–6389. doi:10.1021/ac800795f

Boido, E., Fariña, L., & Carrau, F. (2013). Characterization of glycosylated aroma compounds in tannat grapes and feasibility of the near infrared spectroscopy application for their prediction. *Food Analytical Methods, 6*, 100–111. doi:10.1007/s12161-012-9423-5

Boido, E., Lloret, A., Medina, K., Fariña, L., Carrau, F., Versini, G., & Dellacassa, E. (2003). Aroma composition of Vitis vinifera Cv. tannat: the typical red wine from Uruguay. *Journal of Agricultural and Food Chemistry, 51*(18), 5408–13. doi:10.1021/jf030087i

Boss, P., Pearce, A., Zhao, Y., Nicholson, E., Dennis, E., & Jeffery, D. (2015). Potential Grape-Derived Contributions to Volatile Ester Concentrations in Wine. *Molecules, 20*(5), 7845–7873. doi:10.3390/molecules20057845

Boutou, S., & Chatonnet, P. (2007). Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some of the main odorants causing off-flavours in wine. *Journal of Chromatography A, 1141*, 1–9. doi:10.1016/j.chroma.2006.11.106

Buck, L., & Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell, 65*(1), 175–187. doi:10.1016/0092-8674(91)90418-X

Buttery, R. G., Ling, L. C., & Wellso, S. G. (1982). Oat leaf volatiles: possible insect attractants. *Journal of Agricultural and Food Chemistry, 30*, 791–792. doi:10.1021/jf00112a045

Caputi, L., Carlin, S., Ghiglieno, I., Stefanini, M., Valenti, L., Vrhovsek, U., & Mattivi, F. (2011). Relationship of changes in rotundone content during grape ripening and winemaking to manipulation of the "peppery" character of wine. *Journal of Agricultural and Food Chemistry, 59*, 5565–5571. doi:10.1021/jf200786u

Carnesecchi, S., Schneider, Y., Ceraline, J., Duranton, B., Gosse, F., Seiler, N., & Raul, F. (2001). Geraniol, a component of plant essential oils, inhibits growth and polyamine biosynthesis in human colon cancer cells. *The Journal of Pharmacology and Experimental Therapeutics, 298*(1), 197–200.

Chen, F., Tholl, D., Bohlmann, J., & Pichersky, E. (2011). The family of terpene synthases in plants: A mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. *Plant Journal, 66*, 212–229. doi:10.1111/j.1365-313X.2011.04520.x

Coelho, E., Rocha, S. M., Delgadillo, I., & Coimbra, M. a. (2006). Headspace-SPME applied to varietal volatile components evolution during Vitis vinifera L. cv. "Baga" ripening. *Analytica Chimica Acta, 563*, 204–214. doi:10.1016/j.aca.2005.11.018

Cramer, G. R., Ergül, A., Grimplet, J., Tillett, R. L., Tattersall, E. a R., Bohlman, M. C., … Cushman, J. C. (2007). Water and salinity stress in grapevines: Early and late changes in transcript and metabolite profiles. *Functional and Integrative Genomics, 7*, 111–134. doi:10.1007/s10142-006-0039-y

Degenhardt, J., Köllner, T. G., & Gershenzon, J. (2009). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry, 70*(15-16), 1621–1637. doi:10.1016/j.phytochem.2009.07.030

Des Gachons, C. P., Van Leeuwen, C., Tominaga, T., Soyer, J. P., Gaudillère, J. P., & Dubourdieu, D. (2005). Influence of water and nitrogen deficit on fruit ripening and aroma potential of Vitis vinifera L cv Sauvignon blanc in field conditions. *Journal of the Science of Food and Agriculture, 85*(May 2004), 73–85. doi:10.1002/jsfa.1919

Díaz de León-Sánchez, F., Pelayo-Zaldívar, C., Rivera-Cabrera, F., Ponce-Valadez, M., Ávila-Alejandre, X., Fernández, F. J., … Pérez-Flores, L. J. (2009). Effect of refrigerated storage on aroma and alcohol dehydrogenase activity in tomato fruit. *Postharvest Biology and Technology, 54*, 93–100. doi:10.1016/j.postharvbio.2009.07.003

Doneva-Sapceska, D., Dimitrovski, A., Milanov, G., & Vojnovski, B. (2006). Free and Potentially Volatile Monoterpenes in Grape Varieties. *Bulletin of the Chemists and Technologists of Macedonia, 25*(1), 51–56.

Dourtoglou, V., Antonopoulos, A., Dourtoglou, T., & Lalas, S. (2014). Discrimination of varietal wines according to their volatiles. *Food Chemistry, 159*, 181–7. doi:10.1016/j.foodchem.2014.03.032

Dunkel, A., Steinhaus, M., Kotthoff, M., Nowak, B., Krautwurst, D., Schieberle, P., & Hofmann, T. (2014). Nature's chemical signatures in human olfaction: A foodborne

perspective for future biotechnology. *Angewandte Chemie - International Edition, 53*, 7124–7143. doi:10.1002/anie.201309508

Ebeler, S. E., & Thorngate, J. H. (2009). Wine chemistry and flavor: looking into the crystal glass. *Journal of Agricultural and Food Chemistry, 57*(18), 8098–108. doi:10.1021/jf9000555

El Hadi, M. A. M., Zhang, F. J., Wu, F. F., Zhou, C. H., & Tao, J. (2013). Advances in fruit aroma volatile research. *Molecules, 18*, 8200–8229. doi:10.3390/molecules18078200

Emanuelli, F., Battilana, J., Costantini, L., Le Cunff, L., Boursiquot, J.-M., This, P., & Grando, M. S. (2010). A candidate gene association study on muscat flavor in grapevine (Vitis vinifera L.). *BMC Plant Biology, 10*(1), 241. doi:10.1186/1471-2229-10-241

Emanuelli, F., Lorenzi, S., Grzeskowiak, L., Catalano, V., Stefanini, M., Troggio, M., … Grando, M. S. (2013). Genetic diversity and population structure assessed by SSR and SNP markers in a large germplasm collection of grape. *BMC Plant Biology, 13*, 39. doi:10.1186/1471-2229-13-39

Farneti, B., Masuero, D., Costa, F., Magnago, P., Malnoy, M., Costa, G., … Mattivi, F. (2015). Is there room for improving the nutraceutical composition of apple? *Journal of Agricultural and Food Chemistry*, 150227090900001. doi:10.1021/acs.jafc.5b00291

Fedrizzi, B., Carlin, S., Franceschi, P., Vrhovsek, U., Wehrens, R., Viola, R., & Mattivi, F. (2012). D-optimal design of an untargeted HS-SPME-GC-TOF metabolite profiling method. *The Analyst, 137*(16), 3725–31. doi:10.1039/c2an16309h

Fenoll, J., Manso, A., Hellín, P., Ruiz, L., & Flores, P. (2009). Changes in the aromatic composition of the Vitis vinifera grape Muscat Hamburg during ripening. *Food Chemistry, 114*(2), 420–428. doi:10.1016/j.foodchem.2008.09.060

Fernández-González, M., & Di Stefano, R. (2004). Fractionation of glycoside aroma precursors in neutral grapes. Hydrolysis and conversion by Saccharomyces cerevisiae. *LWT - Food Science and Technology, 37*(4), 467–473. doi:10.1016/j.lwt.2003.11.003

Figueiredo, A., Fortes, A. M., Ferreira, S., Sebastiana, M., Choi, Y. H., Sousa, L., … Pais, M. S. (2008). Transcriptional and metabolic profiling of grape (Vitis vinifera L.) leaves unravel possible innate resistance against pathogenic fungi. *Journal of Experimental Botany, 59*(12), 3371–3381. doi:10.1093/jxb/ern187

Fitzjohn, R. G. (2012). Diversitree: Comparative phylogenetic analyses of diversification in R. *Methods in Ecology and Evolution, 3*(Pagel 1994), 1084–1092. doi:10.1111/j.2041-210X.2012.00234.x

Flamini, R., De Rosso, M., De Marchi, F., Dalla Vedova, A., Panighel, A., Gardiman, M., … Bavaresco, L. (2013). An innovative approach to grape metabolomics: Stilbene profiling by suspect screening analysis. *Metabolomics, 9*, 1243–1253. doi:10.1007/s11306-013-0530-0

Flamini, R., De Rosso, M., Panighel, A., Dalla Vedova, A., De Marchi, F., & Bavaresco, L. (2014). Profiling of grape monoterpene glycosides (aroma precursors) by ultra-high performance-liquid chromatography-high resolution mass spectrometry (UHPLC/QTOF). *Journal of Mass Spectrometry : JMS*, *49*(12), 1214–22. doi:10.1002/jms.3441

Gershenzon, J. (2007). Plant volatiles carry both public and private messages. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(13), 5257–5258. doi:10.1073/pnas.0700906104

Ghaste, M., Narduzzi, L., Carlin, S., Vrhovsek, U., Shulaev, V., & Mattivi, F. (2015). Chemical composition of volatile aroma metabolites and their glycosylated precursors that can uniquely differentiate individual grape cultivars. *Food Chemistry*, *188*, 309–319. doi:10.1016/j.foodchem.2015.04.056

Gil, M., Bottini, R., Berli, F., Pontin, M., Silva, M. F., & Piccoli, P. (2013). Volatile organic compounds characterized from grapevine (Vitis vinifera L. cv. Malbec) berries increase at pre-harvest and in response to UV-B radiation. *Phytochemistry*, *96*, 148–157. doi:10.1016/j.phytochem.2013.08.011

Girard, B., & Fukumoto, L. (2002). Volatile terpene constituents in maturing Gewürztraminer grapes from British Columbia. *American Journal of Enology and Viticulture*, *2*(March), 99–109. Retrieved from http://www.ajevonline.org/content/53/2/99.short

Goff, S. a, & Klee, H. J. (2006). Plant volatile compounds: sensory cues for health and nutritional value? *Science (New York, N.Y.)*, *311*(2006), 815–819. doi:10.1126/science.1112614

Gould, M. N. (1997). Cancer chemoprevention and therapy by monoterpenes.pdf, *105*(June), 977–979.

Grando, M. S., Versini, G., Nicolini, G., & Mattivi, F. (1993). Selective use of wine yeast strains having different volatile phenols production. *Vitis*, *32*, 43–50.

Grimplet, J., Van Hemert, J., Carbonell-Bejerano, P., Díaz-Riquelme, J., Dickerson, J., Fennell, A., … Martínez-Zapater, J. M. (2012). Comparative analysis of grapevine whole-genome gene predictions, functional annotation, categorization and integration of the predicted gene sequences. *BMC Research Notes*, *5*, 213. doi:10.1186/1756-0500-5-213

Gunata, Y. Z., Bayonove, C. L., Baumes, R. L., & Cordonnier, R. E. (1985). The aroma of grapes I. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *Journal of Chromatography A*, *331*, 83–90. doi:10.1016/0021-9673(85)80009-1

Günata, Z. (2013). Biosynthesis of C13-norisoprenoids in vitis vinifera: Evidence of carotenoid cleavage dioxygenase (ccd) and secondary transformation of norisoprenoid compounds. *ACS Symposium Series*, *1134*(i), 97–107. doi:10.1021/bk-2013-1134.ch009

Guth, H. (1997). Identification of character impact odorants of different white wine varieties. *Journal of Agricultural and Food Chemistry*, *2*(3), 3022–3026. Retrieved from http://pubs.acs.org/doi/abs/10.1021/jf9608433

Hendriks, M. M. W. B., Eeuwijk, F. a Van, Jellema, R. H., Westerhuis, J. a., Reijmers, T. H., Hoefsloot, H. C. J., & Smilde, A. K. (2011). Data-processing strategies for metabolomics studies. *TrAC - Trends in Analytical Chemistry*, *30*(10), 1685–1698. doi:10.1016/j.trac.2011.04.019

Hu, C., & Xu, G. (2013). Trends in Analytical Chemistry Mass-spectrometry-based metabolomics analysis for foodomics. *Trends in Analytical Chemistry*, *52*, 36–46. doi:10.1016/j.trac.2013.09.005

Huang, A.-C., Burrett, S., Sefton, M. A., & Taylor, D. K. (2014). Production of the Pepper Aroma Compound, (–)-Rotundone, by Aerial Oxidation of α-Guaiene.

Huang, A.-C., Sefton, M. a., Sumby, C. J., Tiekink, E. R. T., & Taylor, D. K. (2015). Mechanistic Studies on the Autoxidation of α-Guaiene: Structural Diversity of the Sesquiterpenoid Downstream Products. *Journal of Natural Products*, *78*(1), 131–145. doi:10.1021/np500819f

Hurtado-Fernández, E., Pacchiarotta, T., Longueira-Suárez, E., Mayboroda, O. a, Fernández-Gutiérrez, A., & Carrasco-Pancorbo, A. (2013). Evaluation of gas chromatography-atmospheric pressure chemical ionization-mass spectrometry as an alternative to gas chromatography-electron ionization-mass spectrometry: avocado fruit as example. *Journal of Chromatography. A*, *1313*, 228–44. doi:10.1016/j.chroma.2013.08.084

J. Marais. (1983). Terpenes in the aroma of grapes and wines. *South African Journal of Enology and Vticulture*, *4*(2), 49–58.

Jaillon, O., Aury, J.-M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., … Wincker, P. (2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature*, *449*(September), 463–467. doi:10.1038/nature06148

Jiang, B., & Zhang, Z. (2010). Volatile compounds of young wines from cabernet sauvignon, cabernet gernischet and chardonnay varieties grown in the loess plateau region of China. *Molecules*, *15*, 9184–9196. doi:10.3390/molecules15129184

Kuhl, C., Tautenhahn, R., Böttcher, C., Larson, T. R., & Neumann, S. (2012). CAMERA: an integrated strategy for compound spectra extraction and annotation of liquid chromatography/mass spectrometry data sets. *Analytical Chemistry*, *84*(1), 283–9. doi:10.1021/ac202450g

Kulakiotu, E. K., Thanassoulopoulos, C. C., & Sfakiotakis, E. M. (2004). Biological Control of Botrytis cinerea by Volatiles of "Isabella" Grapes. *Phytopathology*, *94*(9), 924–31. doi:10.1094/PHYTO.2004.94.9.924

Lund, S. T., & Bohlmann, J. (2006). The molecular basis for wine grape quality--a volatile subject. *Science (New York, N.Y.), 311*(2006), 804–805. doi:10.1126/science.1118962

Maicas, S., & Mateo, J. J. (2005). Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. *Applied Microbiology and Biotechnology, 67*(3), 322–35. doi:10.1007/s00253-004-1806-0

Martin, D. M., Chiang, A., Lund, S. T., & Bohlmann, J. (2012). Biosynthesis of wine aroma: transcript profiles of hydroxymethylbutenyl diphosphate reductase, geranyl diphosphate synthase, and linalool/nerolidol synthase parallel monoterpenol glycoside accumulation in Gewürztraminer grapes. *Planta, 236*(3), 919–29. doi:10.1007/s00425-012-1704-0

Mateo, J. J., & Jiménez, M. (2000). Monoterpenes in grape juice and wines. *Journal of Chromatography A, 881*, 557–567. doi:10.1016/S0021-9673(99)01342-4

Mathieu, S., Terrier, N., Procureur, J., Bigey, F., & Günata, Z. (2005). A Carotenoid Cleavage Dioxygenase from Vitis vinifera L.: Functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. *Journal of Experimental Botany, 56*(420), 2721–2731. doi:10.1093/jxb/eri265

Mattivi, F., Vrhovsek, U., Malacarne, G., Masuero, D., Zulini, L., Stefanini, M., … Guella, G. (2011). Profiling of resveratrol oligomers, important stress metabolites, accumulating in the leaves of hybrid Vitis vinifera (Merzling × Teroldego) genotypes infected with Plasmopara viticola. *Journal of Agricultural and Food Chemistry, 59*, 5364–5375. doi:10.1021/jf200771y

Mattivi, F., Vrhovšek, U., & Versini, G. (1999). Determination of indole-3-acetic acid, tryptophan and other indoles in must and wine by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A, 855*, 227–235. doi:10.1016/S0021-9673(99)00696-2

May, B., Lange, B. M., & Wüst, M. (2013). Biosynthesis of sesquiterpenes in grape berry exocarp of Vitis vinifera L.: Evidence for a transport of farnesyl diphosphate precursors from plastids to the cytosol. *Phytochemistry, 95*, 135–144. doi:10.1016/j.phytochem.2013.07.021

Mendes-pinto, M. M. (2009). Carotenoid breakdown products the — norisoprenoids — in wine aroma. *Archives of Biochemistry and Biophysics, 483*(2), 236–245. doi:10.1016/j.abb.2009.01.008

Metafa, M., & Economou, A. (2012). Comparison of solid-phase extraction sorbents for the fractionation and determination of important free and glycosidically-bound varietal aroma compounds in wines by gas chromatography-mass spectrometry. *Central European Journal of Chemistry, 11*(2), 228–247. doi:10.2478/s11532-012-0154-7

Murtagh, F., & Legendre, P. (2014). Ward's Hierarchical Agglomerative Clustering Method: Which Algorithms ImplementWard's Criterion? *Journal of Classification, 31*, 274–295. doi:10.1007/s00357-

Myles, S., Boyko, A. R., Owens, C. L., Brown, P. J., Grassi, F., Aradhya, M. K., ... Buckler, E. S. (2011). Genetic structure and domestication history of the grape. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 3530–3535. doi:10.1073/pnas.1009363108

Nasi, A., Ferranti, P., Amato, S., & Chianese, L. (2008). Identification of free and bound volatile compounds as typicalness and authenticity markers of non-aromatic grapes and wines through a combined use of mass spectrometric techniques. *Food Chemistry, 110*(3), 762–768. doi:10.1016/j.foodchem.2008.03.001

Official Journal of the European Union. (2004). Directive 2004/42/CE of the european parliament and of the council of 21 April 2004.

Oksman-Caldentey, K. M., & Saito, K. (2005). Integrating genomics and metabolomics for engineering plant metabolic pathways. *Current Opinion in Biotechnology, 16*, 174–179. doi:10.1016/j.copbio.2005.02.007

Oliveira, J. M., Faria, M., Sá, F., Barros, F., & Araújo, I. M. (2006). C6-alcohols as varietal markers for assessment of wine origin. *Analytica Chimica Acta, 563*(1-2 SPEC. ISS.), 300–309. doi:10.1016/j.aca.2005.12.029

Ong, P. K., & Acree, T. E. (1999). Similarities in the aroma chemistry of Gewürztraminer variety wines and lychee (Litchi chinesis sonn.) fruit. *Journal of Agricultural and Food Chemistry, 47*(2), 665–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10563950

Pacchiarotta, T., Derks, R. J., Hurtado-Fernandez, E., Van, B. P., Henneman, A., Schiewek, R., ... A, O. M. (2013). Online spectral library for GC-atmospheric pressure chemical ionization-ToF MS. *Bioanalysis, 5*(12), 1515–1525. doi:10.4155/bio.13.116

Pacifico, S., D'Abrosca, B., Scognamiglio, M., Gallicchio, M., Potenza, N., Piccolella, S., ... Fiorentino, A. (2011). Metabolic profiling of strawberry grape (Vitis × labruscana cv. "Isabella") components by nuclear magnetic resonance (NMR) and evaluation of their antioxidant and antiproliferative properties. *Journal of Agricultural and Food Chemistry, 59*(14), 7679–87. doi:10.1021/jf200926t

Patel, R., Prajapati, J. P., & Balakrishnan, S. (2014). Recent Trends in Packaging of Dairy and Food Products, (2003), 118–124.

Pawliszyn, J. (1999). Quantitative aspects of SPME. In *Applications of solid phase microextraction* (pp. 3–21).

Pawliszyn, J. (2012). Theory of Solid-Phase Microextraction. *Handbook of Solid Phase Microextraction, 38*(July), 13–59. doi:10.1016/B978-0-12-416017-0.00002-4

Pawliszyn, J., Pawliszyn, B., & Pawliszyn, M. (1997). Solid Phase Microextraction (SPME). *The Chemical Educator, 2*(4), 1–7. doi:10.1007/s00897970137a

Perkins, M. L., Zerdin, K., Rooney, M. L., D'Arcy, B. R., & Deeth, H. C. (2007). Active packaging of UHT milk to prevent the development of stale flavour during storage. *Packaging Technology and Science, 20*(July 2006), 137–146. doi:10.1002/pts.749

Pichersky, E., Noel, J. P., & Dudareva, N. (2006). Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science (New York, N.Y.), 311*(2006), 808–811. doi:10.1126/science.1118510

Pinho, P. G. de, & A.Bertrand. (1995). Analytical Determination of Furaneol (2,5-Dimethyl-4-Hydroxy-3(2H)-Furanone). Application to Differentiation of White Wines From Hybrid and Various Vitis vinifera Cultivars. *American Journal of Enology and Viticulture, 46*(2), 181–186.

Rapp, A., Volkman, C., & Niebergall, H. (1993). Analysis of volatile aroma compounds of grapevine: Characterization of Riesling and Riesling derived cultivars. *Vitis, 32*, 171–178.

Ribéreau-Gayon, P., Boidron, J. N., & Terrier, A. (1975). Aroma of Muscat grape varieties. *Journal of Agricultural and Food Chemistry, 23*(6), 1042–1047. Retrieved from http://pubs.acs.org/doi/abs/10.1021/jf60202a050

Risticevic, S., Deell, J. R., & Pawliszyn, J. (2012). Solid phase microextraction coupled with comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry for high-resolution metabolite profiling in apples : Implementation of structured separations for optimization of sample preparati. *Journal of Chromatography A, 1251*, 208–218. doi:10.1016/j.chroma.2012.06.052

Robinson, A. L., Boss, P. K., Solomon, P. S., Trengove, R. D., Heymann, H., & Ebeler, S. E. (2013). Origins of Grape and Wine Aroma. Part 1. Chemical Components and Viticultural Impacts. *American Journal of Enology and Viticulture, 65*(1), 1–24. doi:10.5344/ajev.2013.12070

Robinson, A. L., Boss, P. K., Solomon, P. S., Trengove, R. D., Heymann, H., & Ebeler, S. E. (2013). Origins of Grape and Wine Aroma. Part 1. Chemical Components and Viticultural Impacts. *American Journal of Enology and Viticulture, 65*(1), 1–24. doi:10.5344/ajev.2013.12070

Robinson, A. L., Boss, P. K., Solomon, P. S., Trengove, R. D., Heymann, H., & Ebeler, S. E. (2014). Origins of Grape and Wine Aroma. Part 2. Chemical and Sensory Analysis. *American Journal of Enology and Viticulture, 65*(1), 25–42. doi:10.5344/ajev.2013.13106

Roland, A., Vialaret, J., Razungles, A., Rigou, P., & Schneider, R. (2010). Evolution of S -cysteinylated and s -glutathionylated thiol precursors during oxidation of Melon B. and sauvignon blanc musts. *Journal of Agricultural and Food Chemistry, 58*, 4406–4413. doi:10.1021/jf904164t

Rowan, D. D. (2011). Volatile Metabolites. *Metabolites, 1*, 41–63. doi:10.3390/metabo1010041

Ruiz-García, L., Hellín, P., Flores, P., & Fenoll, J. (2014). Prediction of Muscat aroma in table grape by analysis of rose oxide. *Food Chemistry, 154*, 151–157. doi:10.1016/j.foodchem.2014.01.005

Ryona, I., & Sacks, G. L. (2013). Behavior of glycosylated monoterpenes, C13-norisoprenoids, and benzenoids in Vitis vinifera cv riesling during ripening and following hedging. *ACS Symposium Series, 1134*, 109–124. doi:10.1021/bk-2013-1134.ch010

Sánchez-Palomo, E., Díaz-Maroto, M. C., & Pérez-Coello, M. S. (2005). Rapid determination of volatile compounds in grapes by HS-SPME coupled with GC-MS. *Talanta, 66*, 1152–1157. doi:10.1016/j.talanta.2005.01.015

Sarry, J., & Gunata, Z. (2004). Plant and microbial glycoside hydrolases: Volatile release from glycosidic aroma precursors. *Food Chemistry, 87*(4), 509–521. doi:10.1016/j.foodchem.2004.01.003

Schauer, N., Zamir, D., & Fernie, A. R. (2005). Metabolic profiling of leaves and fruit of wild species tomato: A survey of the Solanum lycopersicum complex. *Journal of Experimental Botany, 56*(410), 297–307. doi:10.1093/jxb/eri057

Schievano, E., D'Ambrosio, M., Mazzaretto, I., Ferrarini, R., Magno, F., Mammi, S., & Favaro, G. (2013). Identification of wine aroma precursors in Moscato Giallo grape juice: a nuclear magnetic resonance and liquid chromatography-mass spectrometry tandem study. *Talanta, 116*, 841–51. doi:10.1016/j.talanta.2013.07.049

Schneider, R., Charrier, F., Moutounet, M., & Baumes, R. (2004). Rapid analysis of grape aroma glycoconjugates using Fourier-transform infrared spectrometry and chemometric techniques. *Analytica Chimica Acta, 513*(1), 91–96. doi:10.1016/j.aca.2003.11.082

Schreier, P., Drawert, F., & Junker, A. (1976). Identification of Volatile Constituents from Grapes. *Journal of Agricultural and Food Chemistry, 24*(2), 331–336.

Shulaev, V., Cortes, D., Miller, G., & Mittler, R. (2008). Metabolomics for plant stress response. *Physiologia Plantarum, 132*, 199–208. doi:10.1111/j.1399-3054.2007.01025.x

Shulaev, V., Sargent, D. J., Crowhurst, R. N., Mockler, T. C., Folkerts, O., Delcher, A. L., … Folta, K. M. (2011). The genome of woodland strawberry (Fragaria vesca). *Nature Genetics, 43*(2), 109–116. doi:10.1038/ng.740

Shulaev, V., Silverman, P., & Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature*. doi:10.1038/385718a0

Skouroumounist, G. K., & Winterhalter, P. (1994). Glycosidically Bound Norisoprenoids from Vitis vinifera Cv . Riesling Leaves, *64*, 1068–1072.

Smith, C. a, Want, E. J., O'Maille, G., Abagyan, R., & Siuzdak, G. (2006). XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry*, *78*(3), 779–87. doi:10.1021/ac051437y

Smith, C. a, Want, E. J., O'Maille, G., Abagyan, R., Siuzdak, G., & CA Smith, J Elizabeth, G O'Maille,Ruben Abagyan, and G. S. (2006). XCMS: processing mass spectrometry data for metabolite profiling using Nonlinear Peak Alignment,Matching,and Identification. *ACS Publications*, *78*(3), 779–87. doi:10.1021/ac051437y

Son, H. S., Hwang, G. S., Kim, K. M., Ahn, H. J., Park, W. M., Van Den Berg, F., ... Lee, C. H. (2009). Metabolomic studies on geographical grapes and their wines using 1H NMR analysis coupled with multivariate statistics. *Journal of Agricultural and Food Chemistry*, *57*, 1481–1490. doi:10.1021/jf803388w

Song, J., & Bangerth, F. (1996). The effect of harvest date on aroma compound production from " Golden Delicious " apple fruit and relationship to respiration and ethylene production, *8*, 259–269.

Song, J., Shellie, K. C., Wang, H., & Qian, M. C. (2012). Influence of deficit irrigation and kaolin particle film on grape composition and volatile compounds in Merlot grape (Vitis vinifera L.). *Food Chemistry*, *134*(2), 841–850. doi:10.1016/j.foodchem.2012.02.193

Strauss, C. R., Wilson, B., Anderson, R., & Williams, P. J. (1987). Flavorants in Riesling Grapes. *American Journal of Enology and Viticulture*, *38*(1), 23–27.

Sun, Q., Gates, M. J., Lavin, E. H., Acree, T. E., & Sacks, G. L. (2011). Comparison of odor-active compounds in grapes and wines from vitis vinifera and non-foxy American grape species. *Journal of Agricultural and Food Chemistry*, *59*, 10657–10664. doi:10.1021/jf2026204

Swiegers, J. H., Bartowsky, E. J., Henschke, P. a., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research*, *11*(2), 139–173. doi:10.1111/j.1755-0238.2005.tb00285.x

Tarr, P. T., Dreyer, M. L., Athanas, M., Shahgholi, M., Saarloos, K., & Second, T. P. (2013). A metabolomics based approach for understanding the influence of terroir in Vitis Vinifera L. *Metabolomics*, *9*, 170–177. doi:10.1007/s11306-013-0497-x

Tasin, M., Bäckman, A. C., Bengtsson, M., Ioriatti, C., & Witzgall, P. (2006). Essential host plant cues in the grapevine moth. *Naturwissenschaften*, *93*, 141–144. doi:10.1007/s00114-005-0077-7

Tautenhahn, R., Patti, G. J., Rinehart, D., & Siuzdak, G. (2012). XCMS online: A web-based platform to process untargeted metabolomic data. *Analytical Chemistry*, *84*, 5035–5039. doi:10.1021/ac300698c

Ugliano, M., & Bartowsky, E. (2006). Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three Saccharomyces yeast strains.

Journal of Agricultural and Food Chemistry, 6322–6331. Retrieved from http://pubs.acs.org/doi/abs/10.1021/jf0607718

Ugliano, M., & Moio, L. (2008). Free and hydrolytically released volatile compounds of Vitis vinifera L. cv. Fiano grapes as odour-active constituents of Fiano wine. *Analytica Chimica Acta*, *621*(1), 79–85. doi:10.1016/j.aca.2008.03.002

Usta, J., Kreydiyyeh, S., Knio, K., Barnabe, P., Bou-Moughlabay, Y., & Dagher, S. (2009). Linalool decreases HepG2 viability by inhibiting mitochondrial complexes I and II, increasing reactive oxygen species and decreasing ATP and GSH levels. *Chemico-Biological Interactions*, *180*, 39–46. doi:10.1016/j.cbi.2009.02.012

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., … Viola, R. (2010). The genome of the domesticated apple (Malus × domestica Borkh.). *Nature Genetics*, *42*(10), 833–839. doi:10.1038/ng.654

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. a., Cestaro, A., Pruss, D., … Viola, R. (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE*, *2*(12). doi:10.1371/journal.pone.0001326

Vianna, E., & Ebeler, S. E. (2001). Monitoring ester formation in grape juice fermentations using solid phase microextraction coupled with gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry*, *49*(2), 589–595. doi:10.1021/jf000907g

Voirin, S. G., Baumes, R. L., Gunata, Z. Y., Bitteur, S. M., Bayonove, C. L., & Tapiero, C. (1992). Analytical methods for monoterpene glycosides in grape and wine. I. XAD-2 extraction and gas chromatographic-mass spectrometric determination of synthetic glycosides. *Journal of Chromatography*, *590*(2), 313–28. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1560099

Voirin, S. G., Baumes, R. L., Sapis, J., & Bayonove, C. L. (1992). Analytical methods for monoterpene and wine glycosides in grape II . Qualitative and quantitative determination monoterpene glycosides in grape*. *Journal of Chromatography*, *595*, 269–281.

Vrhovsek, U., Lotti, C., Masuero, D., Carlin, S., Weingart, G., & Mattivi, F. (2014). Quantitative metabolic profiling of grape, apple and raspberry volatile compounds (VOCs) using a GC/MS/MS method. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *966*, 132–139. doi:10.1016/j.jchromb.2014.01.009

Wehrens, R., Weingart, G., & Mattivi, F. (2014). MetaMS: An open-source pipeline for GC-MS-based untargeted metabolomics. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *966*, 109–116. doi:10.1016/j.jchromb.2014.02.051

Wightman, J., & Price, S. (1997). Some effects of processing enzymes on anthocyanins and phenolics in Pinot noir and Cabernet Sauvignon wines. *American Journal of Enology and Viticulture*, (46), 39–48. Retrieved from http://www.ajevonline.org/content/48/1/39.short

Williams, P., Cynkar, W., & Francis, I. (1995). Quantification of glycosides in grapes, juices, and wines through a determination of glycosyl glucose. *Journal of Agricultural and Food Chemistry*, *12*, 121–128. Retrieved from http://pubs.acs.org/doi/abs/10.1021/jf00049a023

Winterhalter, P., Sefton, M. A., & Williams, P. J. (1990). Volatile C13-Norisoprenoid Compounds in Riesling Wine Are Generated From Multiple Precursors. *American Journal of Enology and Viticulture*, *41*(4), 277–283.

Winterhalter, P., & Skouroumounis, G. K. (1997). Glycoconjugated aroma compounds: occurrence, role and biotechnological transformation. *Advances in Biochemical Engineering/biotechnology*, *55*, 73–105. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9017925

Wood, C., Siebert, T. E., Parker, M., Capone, D. L., Elsey, G. M., Pollnitz, A. P., … Herderich, M. J. (2008). From wine to pepper: Rotundone, an obscure sesquiterpene, is a potent spicy aroma compound. *Journal of Agricultural and Food Chemistry*, *56*(10), 3738–3744. doi:10.1021/jf800183k

Yang, C., Wang, Y., Liang, Z., Fan, P., Wu, B., Yang, L., … Li, S. (2009). Volatiles of grape berries evaluated at the germplasm level by headspace-SPME with GC-MS. *Food Chemistry*, *114*(3), 1106–1114. doi:10.1016/j.foodchem.2008.10.061

Yu, S. G., Hildebrandt, L. a, & Elson, C. E. (1995). Geraniol, an inhibitor of mevalonate biosynthesis, suppresses the growth of hepatomas and melanomas transplanted to rats and mice. *The Journal of Nutrition*, *125*(March), 2763–2767.

Zhuang, S.-R., Chen, S.-L., Tsai, J.-H., Huang2, C.-C., Wu2, T.-C., Liu2, W.-S., … Wang1, and C.-K. (2009). Effect of Citronellol and the Chinese Medical Herb Complex on Cellular Immunity of Cancer Patients Receiving Chemotherapy/Radiotherapy. *Phytotherapy Research*, *23*, 785–790. doi:10.1002/ptr

8. Appendix

8.1. Supplementary data

Table 9 Average concentrations (μg/Kg) and standard deviation for the compounds identified in the free fraction of grapes

	F3P30		F3P51		F3P63		Riesling		Gewürztraminer		Moscato Rosa		Isabella		Nero		AT		VC	
Compounds	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD
Acid																				
Hexanoic acid	58.3	11.3	71.1	5.9	49.9	6.4	50.9	6.6	219.2	11.9	71.6	6.9	105.9	14.3	166.3	18.8	58.7	7.2	74.6	7.8
Linoleic acid	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	165.3	23.1	74.6	9.1
Alcohol																				
trans 3-hexenol	49.1	7.8	144.7	26.1	51.1	12.3	22.7	3.9	10.2	1.0	161.5	14.3	24.9	2.9	151.7	10.4	357.7	25.4	1013.1	98.5
n-hexanol	307.1	56.0	170.6	19.5	301.6	43.1	202.0	11.7	96.0	7.8	309.5	14.0	222.1	49.5	332.2	119.5	272.6	68.8	422.3	13.0
Tryptophol	----	----	----	----	18.8	2.6	----	----	----	----	----	----	22.8	7.1	----	----	----	----	----	----
Coniferol	----	----	10.7	3.5	----	----	15.4	11.0	20.4	3.4	27.1	1.5	15.9	5.5	----	----	163.0	31.8	151.2	30.0
beta Phenyl ethanol	24.3	2.2	11.2	3.4	13.4	1.5	23.3	4.4	22.9	3.1	23.2	2.5	3002.9	256.2	55.2	48.9	69.2	6.2	8.5	2.7
Benzyl alcohol	51.9	10.7	37.7	5.3	19.5	2.4	17.3	1.3	26.8	1.2	35.7	8.6	23.0	1.7	23.6	3.4	18.1	1.6	5.7	0.8
2-Hexenol	186.5	30.0	144.6	6.8	84.2	11.2	163.0	9.0	114.5	6.8	136.8	14.8	212.9	27.6	166.7	26.6	143.7	51.3	83.5	9.5
Aldehyde																				
Benzaldehyde	----	----	----	----	2.4	0.1	3.5	0.5	----	----	----	----	----	----	3.9	0.5	5.4	0.3	8.1	0.9
Phenylacetaldehyde	19.1	3.3	10.9	0.4	12.5	1.0	10.4	1.2	6.0	0.4	15.3	0.9	112.4	16.6	31.1	5.5	10.8	0.6	38.2	3.8
Benzenoid																				
Methyl vanillate	----	----	----	----	----	----	1.4	0.2	----	----	1.2	0.1	1.4	0.1	0.7	0.2	----	----	1.7	1.7
4-vinylguaiacol	----	----	1.3	0.1	1.3	0.1	4.0	0.6	5.8	0.5	4.9	0.5	3.7	0.2	2.7	0.2	3.7	0.8	5.4	3.1
Homovanillyl alcohol	33.0	21.5	----	----	4.9	0.7	4.4	0.7	5.1	0.7	19.1	1.2	----	----	53.2	10.5	3.7	0.4	28.9	23.3
Homovanillinic acid	4.5	1.9	9.3	2.6	9.0	0.2	11.9	8.2	13.8	2.8	34.8	2.1	20.2	6.8	24.9	18.3	90.9	16.9	165.4	27.3
Acetovanillone	----	----	----	----	----	----	3.7	0.7	----	----	----	----	----	----	----	----	9.1	3.7	----	----
C13-Norisoprenoid																				
Dihydro beta ionone	----	----	9.6	2.2	12.2	1.2	6.1	0.8	5.9	0.6	5.9	0.3	6.3	1.5	----	----	11.6	2.9	25.4	3.6
4-oxo beta ionol	----	----	----	----	2.7	0.4	----	----	----	----	----	----	----	----	----	----	----	----	----	----

3 hydroxy beta damascone	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
vomifoliol	----	----	33.1	1.8	----	----	13.3	4.4	15.8	1.4	30.7	1.1	----	----	17.8	3.6	----	----	36.5	6.1
Ester																				
Methyl salicylate	----	----	----	----	----	----	----	----	----	----	----	----	4.4	0.4	15.6	3.3	----	----	----	----
Ethyl 3-hydroxyhexanoate	----	----	----	----	----	----	----	----	----	----	----	----	8.0	1.3	----	----	----	----	----	----
Ethyl beta hydroxybutyrate	----	----	----	----	----	----	----	----	----	----	----	----	478.0	34.5	----	----	----	----	----	----
Methyl anthranilate	----	----	----	----	----	----	----	----	----	----	----	----	14.1	2.9	----	----	----	----	----	----
Furanone																				
Furaneol	8.5	1.7	8.5	2.0	11.1	9.1	3.5	4.6	2.2	1.7	0.5	0.1	380.7	6.5	5.8	9.6	4.9	6.9	2.3	0.3
Monoterpene																				
cis Linalool oxide (Pyranoid)	166.9	18.7	679.5	31.4	68.1	2.5	7.7	0.3	1.6	0.2	50.2	6.9	1.1	0.1	----	----	----	----	4.3	1.4
p-menth-1-ene-7,8-diol	----	----	----	----	----	----	----	----	----	----	----	----	8.6	0.9	----	----	----	----	6.3	1.0
Lilac alcohol C	7.2	1.3	5.9	0.4	1.4	0.1	----	----	----	----	----	----	----	----	----	----	----	----	----	----
6,7-Dihydro-7-hydroxylinalool	52.5	10.6	20.5	1.6	4.5	1.0	5.9	0.4	4.9	0.5	4.1	0.6	3.4	0.8	3.5	0.6	30.8	1.2	31.9	0.2
cis Linalool oxide (Furanoid)	112.6	19.9	206.8	23.4	----	----	----	----	----	----	10.6	1.9	----	----	----	----	----	----	----	----
Hotrienol	56.1	18.8	30.2	4.9	18.8	8.8	----	----	----	----	----	----	----	----	----	----	----	----	----	----
trans Linalool oxide (Pyranoid)	584.8	36.4	2093.5	64.8	222.5	8.9	23.5	0.5	2.7	0.3	44.4	5.4	1.9	0.7	----	----	----	----	2.1	0.2
Terpendiol II	2122.8	335.0	1751.9	107.2	100.7	22.3	9.4	2.1	4.3	1.7	33.8	5.3	----	----	6.7	5.0	7.4	5.3	7.2	7.8
Hydroxy Citronellol	----	----	----	----	----	----	----	----	5.0	0.4	5.0	0.8	----	----	----	----	----	----	----	----
trans Linalool oxide (Furanoid)	403.2	64.6	205.5	21.8	40.4	5.2	----	----	----	----	----	----	----	----	----	----	----	----	----	----
Terpendiol I	2017.2	446.4	1561.0	187.7	869.7	73.4	81.8	14.3	22.2	2.2	20.9	1.2	10.8	2.0	27.1	13.6	11.4	3.6	31.6	7.7
Linalool	3846.2	576.2	3813.2	649.4	139.8	55.6	25.2	1.4	----	----	124.1	23.2	----	----	----	----	----	----	9.4	2.3
alpha Terpineol	73.4	12.0	27.6	4.7	4.0	1.2	----	----	----	----	----	----	38.0	0.9	----	----	----	----	3.6	0.3
Nerol	15.9	2.2	6.1	1.8	15.8	1.2	----	----	22.0	2.5	18.2	0.7	----	----	4.8	0.5	----	----	----	----
trans 8 Hydroxy linalool	535.6	100.6	316.3	35.8	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
Geraniol	187.1	28.7	59.1	9.2	158.3	22.0	----	----	186.5	10.2	139.7	2.8	----	----	----	----	----	----	----	----
Geranic acid	92.5	40.3	31.4	2.2	87.7	15.1	----	----	92.0	4.1	40.4	1.7	----	----	21.6	9.4	----	----	----	----
Phenol																				
3,4,5-Trimethoxyphenol	----	----	----	----	----	----	----	----	----	----	----	----	----	----	4.2	0.4	----	----	----	----

[ATA]Arizonica Texas, [VC]Vitis cinerea

Table 10 Average concentrations (μg/Kg) and standard deviation for the compounds released after enzymatic hydrolysis of grapes

	F3P30		F3P51		F3P63		Riesling		Gewürztraminer		Moscato Rosa		Isabella		Nero		VAT		Cinerea	
Compounds	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD	Conc	± SD
Acid																				
Hexanoic acid	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	7.6	2.5
Linoleic acid	----	----	93.5	107.2	37.9	17.7	----	----	32.0	26.3	----	----	----	----	123.6	53.5	573.5	26.0	827.7	45.5
Alcohol																				
trans 3-hexenol	2.0	0.4	4.5	0.5	4.6	1.6	9.3	0.6	3.5	0.6	14.3	1.8	29.6	5.3	96.1	3.1	157.9	2.6	119.7	0.9
n-hexanol	13.5	3.1	18.6	1.3	30.3	7.8	52.3	0.5	15.7	3.6	25.8	3.4	224.7	58.7	153.5	1.1	525.6	6.4	289.7	3.7
Tryptophol	17.5	4.3	35.0	4.6	24.1	4.4	26.9	0.4	20.0	3.0	12.5	0.6	518.4	105.9	38.7	9.5	218.7	4.0	68.0	2.2
Coniferol	37.8	17.4	55.5	1.2	58.0	19.3	150.0	1.4	44.7	17.7	21.3	2.9	318.2	68.3	234.9	188.1	1318.6	61.5	519.9	20.1
beta Phenyl ethanol	321.8	52.1	161.0	13.1	182.0	43.8	385.4	75.5	158.1	30.1	317.5	16.3	9420.3	1843.0	525.0	12.6	7049.6	520.8	2449.2	51.4
Benzyl alcohol	426.4	86.5	204.8	5.6	268.5	63.9	619.5	12.5	300.3	65.8	520.4	22.9	1451.7	197.8	1397.2	65.4	3708.6	60.0	1120.4	13.2
2-Hexenol	----	----	----	----	----	----	8.5	0.3	----	----	----	----	17.1	4.5	----	----	33.8	0.1	14.8	0.3
4-Methoxyphenethyl alcohol	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	22.2	0.6	498.8	71.8
Anisyl alcohol	----	----	----	----	----	----	----	----	----	----	----	----	----	----	127.8	6.8	205.9	2.6	226.2	4.9
Cinnamyl alcohol	----	----	----	----	----	----	5.6	0.2	----	----	----	----	----	----	----	----	332.6	1.9	15.8	0.8
Aldehyde																				
Benzaldehyde	2.7	0.5	----	----	1.9	0.0	4.1	0.3	2.2	0.3	3.2	0.2	----	----	----	----	27.8	3.1	11.0	0.4
Phenylacetaldehyde	4.5	0.1	2.5	0.2	6.1	0.2	11.7	0.1	7.0	0.8	6.8	0.9	46.7	11.0	12.3	0.8	5.7	0.3	11.5	0.4
Benzenoid																				
Eugenol	3.7	0.8	2.6	0.3	1.7	0.4	4.4	0.1	14.3	2.7	4.4	0.1	81.3	16.4	4.7	0.2	190.9	1.6	214.0	5.3
Methyl vanillate	12.6	3.4	5.2	0.7	7.0	2.3	68.2	2.2	17.1	4.5	15.7	1.4	121.9	33.3	27.4	2.3	19.5	0.3	74.3	1.5
4-vinylguaiacol	13.5	4.2	12.1	4.2	33.4	7.3	61.8	2.7	46.8	7.8	3.8	0.9	195.8	37.1	36.3	30.7	158.0	7.8	93.8	9.6
Homovanillyl alcohol	20.3	7.6	6.6	0.8	10.1	3.1	94.3	3.3	55.7	12.2	70.9	4.9	11.0	1.9	1213.1	199.1	225.9	3.5	1448.0	24.3
Homovanillinic acid	26.5	14.7	50.3	0.6	48.2	14.2	143.9	1.5	30.1	14.0	13.8	3.7	141.9	28.7	276.1	274.8	779.6	18.7	513.5	17.8
Acetovanillone	80.4	22.1	26.7	2.3	31.7	10.2	203.1	6.2	60.7	13.4	46.4	4.1	210.5	42.9	----	----	162.3	3.7	----	----

C13-Norisoprenoid																				
C13-Norisoprenoid																				
3-4-dihydro-3-oxoactinidiol I	2.5	0.5	4.0	0.5	----	----	4.8	0.2	----	----	----	----	21.8	4.9	14.5	1.2	28.5	0.6	19.1	0.4
3-4-dihydro-3-oxoactinidiol II	2.8	0.6	3.5	0.5	----	----	7.8	0.2	----	----	----	----	15.4	2.5	13.8	0.5	55.4	2.1	31.6	0.4
3-4-dihydro-3-oxoactinidiol III	3.9	1.0	6.2	0.7	----	----	16.4	0.7	----	----	----	----	10.4	2.2	16.2	0.4	47.1	1.1	24.5	0.5
9-Hydroxy megastigma-4-6-dien-3-one	5.5	1.4	8.7	1.8	3.7	1.2	5.8	0.3	----	----	----	----	----	----	10.3	0.4	36.4	1.0	245.0	7.4
Dihydro beta ionone	6.2	1.2	4.5	1.6	5.6	2.2	3.7	0.2	7.5	1.6	4.3	0.9	10.7	2.1	37.8	2.3	150.7	4.5	133.5	1.2
4-oxo beta ionol	7.3	2.0	7.2	0.6	8.7	2.5	38.3	1.8	5.0	1.7	4.9	0.2	92.9	20.6	36.6	3.4	----	----	114.5	1.0
3 hydroxy beta damascone	36.9	14.6	34.9	3.2	28.4	10.8	68.9	2.0	25.1	6.5	22.4	1.2	66.6	23.0	155.8	7.4	164.7	3.2	194.8	2.4
vomifoliol	43.4	18.0	61.6	8.7	48.9	14.4	149.5	3.7	52.9	15.4	33.6	3.5	101.9	14.3	505.0	200.2	119.5	3.0	155.0	4.3
Dihydro-3-oxo beta ionol	84.8	20.0	28.1	4.2	32.3	9.1	137.4	2.9	33.4	7.6	27.1	1.7	336.0	67.9	335.7	37.3	89.4	3.8	665.4	10.2
3-keto alpha ionol	196.2	37.5	155.8	24.9	108.9	33.8	364.8	12.3	49.6	22.0	61.9	1.7	54.9	16.6	923.7	56.5	292.3	22.1	1810.8	35.0
3,4-Dihydroactinidol	----	----	----	----	----	----	----	----	----	----	----	----	3.3	0.9	4.2	0.2	----	----	10.7	0.5
Coumarin																				
Scopoletin	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	1067.8	6.0	----	----
Ester																				
Methyl salicylate	1.9	0.5	1.0	0.2	4.2	1.4	1.3	0.1	1.8	0.4	2.8	0.2	24.4	7.4	319.7	2.0	3.3	0.1	1.8	0.1
Ethyl 3-hydroxyhexanoate	----	----	----	----	----	----	----	----	----	----	----	----	87.6	10.6	----	----	----	----	----	----
Ethyl beta hydroxybutyrate	----	----	----	----	----	----	----	----	----	----	----	----	2072.4	151.2	----	----	----	----	----	----
Methyl anthranilate	----	----	----	----	----	----	----	----	----	----	----	----	16.5	3.4	----	----	----	----	----	----
Furanone																				
Furaneol	1.3	0.4	0.9	0.1	1.1	0.3	2.1	0.4	1.1	0.2	1.2	0.1	183.3	23.1	2.4	1.2	3.2	0.1	2.7	0.0
Monoterpene																				
1-p-menthen-9-al I	3.2	1.9	4.2	0.1	0.6	0.2	0.7	0.0	0.5	0.1	0.7	0.1	0.3	0.1	----	----	3.9	0.1	----	----
1-p-menthen-9-al II	3.6	2.0	4.5	0.0	0.7	0.3	0.8	0.0	0.6	0.1	0.8	0.1	0.3	0.1	----	----	4.1	0.1	----	----
Lilac alcohol A	10.1	2.5	9.0	0.8	11.5	3.2	----	----	----	----	----	----	----	----	----	----	49.2	1.0	----	----
cis Linalool oxide (Pyranoid)	14.0	2.6	18.3	2.3	22.8	6.0	13.2	0.2	6.5	0.5	4.1	0.5	3.4	0.9	35.5	1.4	14.9	0.2	19.6	0.3

p-menth-1-ene-7,8-diol	16.2	8.8	4.5	0.6	22.3	6.0	88.9	3.0	10.0	2.4	5.1	0.7	495.3	99.0	29.2	5.5	33.7	0.5	866.9	6.6
Lilac alcohol C	22.5	5.1	14.9	1.6	28.4	8.4	2.0	0.0	----	----	1.7	0.1	2.6	0.4	4.3	0.2	107.6	1.4	1.6	0.2
6,7-Dihydro-7-hydroxylinalool	23.3	9.4	25.9	1.8	8.4	1.8	4.4	0.1	4.9	1.0	4.1	0.2	1.1	0.3	4.6	1.4	6.9	0.4	4.3	0.3
4-terpineol	27.3	13.5	43.6	2.0	13.1	4.5	4.2	0.2	3.1	0.6	4.9	0.4	----	----	----	----	----	----	----	----
beta-Citronellol	27.6	3.9	6.9	0.7	30.0	8.9	1.8	0.1	24.3	5.8	10.8	1.2	28.0	4.6	12.4	0.9	----	----	----	----
Hydroxy nerol	30.7	15.6	29.7	3.1	32.4	4.8	----	----	27.1	6.2	19.0	8.7	12.9	3.2	17.0	1.9	----	----	----	----
Lilac alcohol B	33.3	7.9	14.3	1.0	43.2	12.6	2.2	0.1	----	----	1.9	0.1	----	----	5.4	0.7	73.6	1.4	----	----
cis Linalool oxide (Furanoid)	34.8	18.8	111.7	2.9	15.5	4.1	26.3	0.5	10.0	0.8	8.7	0.2	3.9	0.2	33.8	2.2	24.0	0.8	40.7	1.5
Hotrienol	52.9	32.6	95.7	39.4	35.5	15.9	4.0	0.2	----	----	3.2	0.4	----	----	----	----	----	----	----	----
trans Linalool oxide (Pyranoid)	66.3	9.0	41.8	5.2	36.5	9.6	21.4	0.3	8.4	1.6	9.1	0.8	15.6	1.8	17.6	1.1	90.6	1.4	41.7	0.3
Terpendiol II	72.2	19.8	86.3	18.2	19.3	5.7	3.9	0.3	2.6	0.8	3.4	0.2	----	----	----	----	----	----	----	----
Hydroxy Citronellol	99.6	30.9	31.6	2.9	52.6	15.8	21.2	0.7	59.6	12.2	31.7	2.2	22.6	4.8	71.7	7.7	7.5	0.4	5.3	0.3
trans Linalool oxide (Furanoid)	114.1	51.6	371.8	19.8	62.8	18.4	35.8	0.2	13.9	1.6	16.3	0.7	16.7	3.1	22.3	2.4	23.1	1.2	38.5	0.5
Terpendiol I	183.0	20.1	284.0	81.9	414.3	114.6	31.0	0.7	8.8	3.0	32.7	1.5	13.2	0.7	48.7	2.4	----	----	62.5	1.6
Linalool	224.8	30.6	200.5	41.9	221.9	65.2	41.5	0.6	12.8	2.8	84.2	7.6	8.9	0.9	38.8	1.4	----	----	3.5	0.3
alpha Terpineol	245.8	147.6	391.3	7.9	145.8	27.9	63.6	1.5	30.6	7.4	44.7	3.6	750.9	128.2	17.2	0.3	4.1	0.8	43.4	0.3
Nerol	312.0	39.3	188.5	16.2	451.2	120.3	7.8	0.5	281.8	77.7	234.7	31.3	134.9	29.2	247.5	3.5	5.1	0.4	15.1	0.1
cis 8 Hydroxy linalool	592.9	135.7	511.5	40.9	665.7	183.2	302.0	7.7	201.8	40.2	321.9	35.9	145.8	32.5	500.8	41.0	1805.1	29.2	----	----
trans 8 Hydroxy linalool	763.2	175.9	632.3	121.5	286.4	84.0	47.7	1.4	25.2	4.5	125.1	15.3	47.7	9.0	100.9	7.6	797.0	9.2	----	----
Geraniol	860.1	99.2	603.1	63.4	1177.5	290.8	62.2	0.8	1490.7	324.1	1945.8	204.2	230.6	54.9	512.3	9.5	40.9	0.4	30.1	0.9
Geranic acid	1771.9	20.2	1071.5	137.6	1536.1	313.7	35.4	0.7	1185.1	232.9	827.8	45.9	28.8	7.3	545.6	24.1	----	----	50.9	1.2
exo-2-Hydroxycineole	----	----	----	----	----	----	21.4	0.3	----	----	----	----	36.9	4.6	23.0	0.7	53.5	0.7	21.0	0.2
Phenol																				
Chavicol	1.9	0.2	----	----	----	----	----	----	----	----	----	----	----	----	----	----	29.2	0.6	2.8	0.2
3,4,5-Trimethoxyphenol	41.9	17.1	35.3	2.0	33.7	11.4	158.7	5.7	46.8	12.8	39.4	2.1	133.2	25.4	204.6	41.7	18.6	0.5	103.1	0.8
Quinone																				
2-Methoxyhydroquinone	----	----	----	----	----	----	6.9	0.2	----	----	32.5	3.6	44.7	6.4	191.2	51.1	238.8	12.4	529.3	6.9

[ATA]Arizonica Texas, [VC]Vitis cinerea

8.2. Retention Index Literaure for table 6

1. Andersen, J.F., Mikolajczak, K.L., and Reed, D.K. 1987a. Analysis of peach bark volatiles and their electroantennogram activity with lesser peachtree borer, Synanthedon pictipes (Grote & Robinson). J. Chem. Ecol. 13:2103-2114.

2. Aubert, C., Ambid, C., Baumes, R., and Günata, Z. (2003). Investigation of bound aroma constituents of yellow-fleshed nectarines (Prunus persica L. cv. Springbright). Changes in bound aroma profile during maturation. J. Agric. Food Chem. 51:6280-6286.

3. Baek, Cadwallader, Marroquin & Silva, (1997). Identification of predominant aroma compounds in muscadine grape juice; J. Food Science, 62, 2, 249-253.

4. Boido, E., Fariña, L., & Carrau, F. (2013). Characterization of glycosylated aroma compounds in tannat grapes and feasibility of the near infrared spectroscopy application for their prediction. Food Analytical Methods, Volume 6, Issue 1, 100-111.

5. Boulanger, R., Chassagne, D., and Crouzet, J. (1999). Free and bound flavour components of amazonian fruits. 1: Bacuri. Flavour and Fragrance Journal. 14:303-311.

6. Choi, H.-S. (2003). Character impact odorants of citrus hallooing [(C. unshiu Marcov x C. sinensis Osbeck) x C. reticulata Blanco] cold-pressed peel oil. Journal of Agricultural and Food Chemistry. 51: 2687-2692.

7. López-Vázquez Cristina, Bollaín María Herminia, Moser Sergio, Orriols Ignacio, (2010) Characterization and Differentiation of Monovarietal Grape Pomace Distillate from Native Varieties of Galicia, Journal of Agricultural and Food Chemistry., 58 (17), 9657–9665.

8. Cullere, L., Escudero, A., Cacho, J., and Ferreira, V. (2004). Gas chromatography-olfactometry and chemical quantitative study of the aroma of six premium quality Spanish aged red wines. Journal of Agricultural and Food Chemistry. 52 (6) 1653-1660.

9. Chassagne David, Boulanger Renaud, Crouzet Jean, Enzymatic hydrolysis of edible Passiflora fruit glycosides, (1999). Food Chemistry Volume 66, Issue 3, 281–288.

10. Davies, N.W. (1990). Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. Journal of Chromatography A. 503:1-24.

11. Engel, K. H., and Tressl, R. (1983). Formation of aroma components from nonvolatile precursors in passion fruit. Journal of Agricultural and Food Chemistry. 31:998-1002.

12. Fischer & Grosch (1987); Lebensm Wiss Technol, 20, 233-236.

13. Fukami, K., Ishiyama, S., Yaguramaki, H., Masuzawa, T., Nabeta, Y., Endo, K., and Shimoda, M. (2002). Identification of distinctive volatile compounds in fish sauce. Journal of Agricultural and Food Chemistry. 50:5412-5416.

14. Hognadottir, A., and Rouseff, R.L. (2003). Identification of aroma active compounds in orange essence oil using gas chromatography-olfactometry and gas chromatography- mass spectrometry. J. Chromatogr. A. 998:201-211.

15. Klesk, K., Quian, M., and Martin, R.R. (2004). Aroma extract dilution analysis of cv. Meeker (Rubus idaeus L.) Red raspberries from Oregon and Washington. Journal of Agricultural and Food Chemistry. 52:5155-5161.

16. Lee S. J., Noble A.C. (2003). Characterization of odor-active compounds in Californian Chardonnay wines using GC-olfactometry and GC-mass spectrometry. Journal of Agricultural and Food Chemistry. 51:8036-8044.

17. Lee, S.-J., Umano, K., Shibamoto, T., and Lee, K.-G. (2005). Identification of volatile components in basil (Ocimum basilicum L.) and thyme leaves (Thymus vulgaris L.) and their antioxidant properties. Journal of Agricultural and Food Chemistry. 91:131-137.

18. Nishimura, O. (1995) Identification of the characteristic odorants in fresh Rhizomes of ginger (Zingiber officinale Roscoe) using aroma extract dilution analysis and modified multidimensional gas chromatography-mass spectroscopy. Journal of Agricultural and Food Chemistry. 43:2941-2945.

19. Olivero, J., Gracia, T., Payares, P., Vivas, R., Diaz, D., Daza, E., and Geerlings, P.(1997). Molecular structure and gas chromatographic retention behavior of the components of ylang-ylang oil. J. Pharm. Sci. 86:625-630.

20. Ong, P.K.C., and Acree, T.E. (1999). Similarities in the aroma chemistry of Gewürztraminer variety wines and Lychee Fruit (Litchi chinesis Sonn.). Journal of Agricultural and Food Chemistry. 47:665-670.

21. Qian, M., and Reineccius, G. (2003). Potent aroma compounds in Parmigiano Reggiano cheese studied using a dynamic headspace (purge-trap) method. Flavour and Fragrance Journal. 18:252-259.

22. Ruther, J. (2000). Retention index database for identification of general green leaf volatiles in plants by coupled capillary gas chromatography-mass spectrometry. Journal of Chromatography A 890:313-319.

23. Selli, S., Cabaroglu, T., Canbas, A., Erten, H., Nurgel, C., Lepoutre, J.P., and Gunata, Z. (2004) Volatile composition of red wine from cv. Kalecik Karasi grown in central Anatolia. Food Chemistry. 85:207-213.

24. Ulrich, D., Hoberg, E., Rapp, A., and Kecke, S. (1997). Analysis of strawberry flavour-discrimination of aroma types by quantification of volatile compounds. Z. Lebensm. Unters. Forsch. A. 205:218-223.

25. Valim, M.F., Rouseff, R.L., and Lin, J. 2003. Gas chromatographic-olfactometric characterization of aroma compounds in two types of cashew apple nectar. Journal of Agricultural and Food Chemistry. 51:1010-1015.

26. G. Versini, A. Rapp, F. Reniero, H. M. (1991). Structural identification and presence of some p-menth-1-enediols in grape products. *Vitis, 30,* 143–149.

27. Internal database collection of grape and wine aroma compounds at, Department of Food Quality and Nutrition, Fondazione Edmund Mach

8.3. List of tables in the thesis

8.4. List of figures in the thesis

Curriculum Vitae

Manoj Shahaji Ghaste
Food Quality and Nutrition Department
Research and Innovation Centre
Fondazione Edmund Mach
Via E. Mach 1, 38010, S. Michele all' Adige, (TN) Italy
manoj.ghaste@fmach.it / manoj.ghaste@gmail.com
+39 3801540648

Education

- **Doctor of Philosophy (PhD)**

  Since Sept. 2011-ongoing
  GMPF PhD Fellow at Fondazione Edmund Mach and PhD candidate at International Doctoral School in Biomolecular Science, University of Trento, Italy.
  Thesis Title - Comprehensive Mapping of VOCs in Fruits
  Supervisor - Dr. Fulvio Mattivi, **Advisor** - Prof. Vladimir Shulaev

- **Master of Science (MSc)**

  2005-2007, Department of Agrochemicals and Pest Management, Shivaji University, Kolhapur, India

- **Bachelor of Science (BSc)**

  2002-2005, Shivaji University, Kolhapur, India

Relevant Experiences

- **Research Assistant:** April 2010-March 2011

  Institute of Environmental Research, Technical University of Dortmund, Dortmund, Germany

- **Senior Research Fellow:** December 2008-March 2010

  Agricultural Chemistry Division, National research Centre for Grapes, Pune, India

- **Project Assistant:** April 2007-December 2008

  Insecticide Testing Laboratory, Maharashtra state department of agriculture, Pune, India

Research Stays abroad

As a part of the PhD programme, spent time July 5- September 20 (2012) and June 28- September 30 (2014) at Department of Biological Sciences, University of North Texas (USA) in the metabolomics and plant signaling research laboratory under the supervision of Prof. Vladimir Shulaev.

Personal Details

Birth date	: 14th November 1983
Nationality	: Indian
Languages	: Marathi, Hindi, English and moderate Italian

Peer Reviewed Publications

1. Chemical Composition of Volatile Aroma Metabolites and their Glycosylated Precursors Uniquely Differentiates Individual Grape Cultivars, *Food Chemistry 188 (2015) 309–319,* **Manoj Ghaste**, Luca Narduzzi, Silvia Carlin, Urska Vrhovsek, Vladimir Shulaev and Fulvio Mattivi

2. Bioefficacy, dissipation kinetics and safety evaluation of selected insecticides in Allium cepa L., *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes, Volume 47, Issue 7, 2012,* Satya Srinivas P., Kaushik Banerjee, Manjusha R. Jadhav, **Manoj S. Ghaste** & K. E. Lawande

3. Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf cutting ants. *Proceedings of National Academy of Science of United States of America, PNAS 2011 108 (5) 1955-1960.* Schoenian I, Spiteller M, **Ghaste M**, Wirth R, Herz H, Spiteller D

4. Multiresidue Analysis of Multiclass Plant Growth Regulators in Grapes by Liquid Chromatography Tandem Mass Spectrometry. *Journal of AOAC International VOl.94 3 2011.* Oulkar D, Banerjee K, **Ghaste M**, Ramteke S, Naik D, Patil S, Jadhav M, Adsule P.

5. Optimization of two-dimensional gas chromatography time-of-flight mass spectrometry for separation and estimation of the residues of 160 pesticides and 25 persistent organic pollutants in grape and wine. *Journal of Chromatography A, 1217 (2010) 3881–3889.* Dasgupta S, Banerjee K, Patil S**, Ghaste M**, K Dhumal, Adsule P

6. Multiresidue Analysis of 50 Pesticides in Grape, Pomegranate and Mango by Gas Chromatography-Ion Trap Mass Spectrometry. *Journal of Agricultural and Food*

Chemistry, 2010, 58 (3), pp 1447–1454. Savant R, Banerjee K, Utture S, Patil S, Dasgupta S, **Ghaste M**, Adsule P

Application Note

1. Metabolic Phenotyping Using Atmospheric Pressure Gas Chromatography-MS, *Application Note APNT134832772 (2015)– Waters Corporation*, Vladimir Shulaev, **Ghaste Manoj**, Steven Lai, Carolina Salazar, Nobuhiro Suzuki, Janna Crossley, Feroza Kaneez Choudhury, Khadiza Zaman, Ron Mittler, James Langridge, Robert Plumb, Fulvio Mattivi, Giuseppe Astarita

Poster and Oral Presentations

1. Improving the annotation of unknown metabolites via integration of GCMS and LCMS metabolomics: A close look into the complexity of grapevine glycosides, *Gracious: the bilateral Israel-Italy grapevine symposium: physiology, genetics & genomics, October 29-31, 2014, Sede Boquer, Israel.* **Manoj Ghaste**, Luca Narduzzi, Silvia Carlin, Urska Vrhovsek, Vladimir Shulaev, Fulvio Mattivi

2. Enhanced metabolite profiling using Atmospheric Pressure Gas Chromatography (APGC) coupled with ion mobility MS63rd ASMS Conference on Mass Spectrometry and Allied May 31 - June 4, 2015 - St. Louis, Missouri. **Manoj Ghaste**, Giuseppe Astarita, Fulvio Mattivi, Vladimir Shulaev

3. Fusion of GC/MS and LC/HRMS Data to improve unknown volatile precursors Identification and confirmation in Grape, MET-GR III workshop: *Metabolic and Protein Network analysis in Systems Biology, September 18-20, 2014, Patras, Greece.* Luca Narduzzi, **Manoj Ghaste,** Fulvio Mattivi

4. Metabolomics Profiling using Atmospheric Pressure Gas Chromatography-Mass Spectrometry, 62nd ASMS, Conference on Mass Spectrometry and Allied Topics will take place in Baltimore, MD, USA, 15–19 June 2014. Vladimir Shulaev, **Manoj Ghaste**, Steven Lai, Carolina Salazar, Nobuhiro Suzuki, Janna Crossley, Ron Mittler, James Langridge, Giuseppe Astarita, Fulvio Mattivi

5. Atmospheric Pressure Gas Chromatography-Mass Spectrometric Profiling of VOCs in Fruits, 38th International Symposium on Capillary chromatography and 11th GCxGC Symposium, May 18 - 23, 2014 Riva del Garda, Italy. **Manoj Ghaste**, Vladimir Shulaev, Fulvio Mattivi

6. Metabolomics Profiling Using Atmospheric Pressure Gas Chromatography-MS, German Society for Mass Spectrometry (DGMS) 2015 from 1-4 March in Wuppertal, Germany.Weibchen Gunnar, Shulaev Vladimir, **Manoj Ghaste**, Lai Steven, Salazar

Carolina, Suzuki Nobuhiro, Crossley Janna, Mittler Ron, Langridge James, Astarita Giuseppe, Mattivi Fulvio

7. Targeted screening of free and glycosidically conjugated VOCs in Wine, *3rd MS Food Day Conference, Trento, Italy*. **Manoj Ghaste**, Silvia Carlin, Luca Narduzzi, Marco Stefanini, Urska Vhrovsek, Fulvio Mattivi

8. Establishing a GC-MS based metabolomics platform. *36th International Symposium on Capillary Chromatography, Riva del Garda, Italy.*Gerog J. Weingart, Bruno Fedrizzi, Silvia Carlin, **Manoj Ghaste**, Urska Vhrovsek, Pietro Franceschi, Matthias Scholz, Ron Wehrens, Fulvio Mattivi

9. Multiresidue Analysis of Plant Growth Regulators in Grape by LCESI-MSMS, Oulkar D, Banerjee K, Ramteke S, Patil S, **Ghaste M**, Naik D, Adsule P *International Conference on Emerging Trends in Chemistry, Department of Chemistry,Pune University, Pune, INDIA. January 5-7, 2010*

10. Application of comprehensive GCXGC-time-of-flight mass Spectrometry in resolving matrix influences in multiresidue analysis of 165 pesticides in grape and wine. Banerjee K, Dasgupta S,Patil S, R Savant, Oulkar D, M Jadhav, Patil S, **Ghaste M**, Adsule P. *2nd Latin American Pesticide Residue Workshop, Santa Fe, Argentina. June 8-11, 2009*

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ॐ सह नाववतु। सह नौ भुनक्तु। सह वीर्यं करवावहै।

तेजस्वि नावधीतमस्तु मा विद्विषावहै।

ॐ शान्तिः शान्तिः शान्तिः ॥

ॐ सर्वे भवन्तु सुखिनः सर्वे सन्तु निरामयाः।

सर्वे भद्राणि पश्यन्तु मा कश्चिद्दुःखभाग्भवेत्।

ॐ शान्तिः शान्तिः शान्तिः ॥

Oh Almighty! May he protect all of us!

May he cause us to enjoy!

May we acquire strength together!

May our knowledge become brilliant!

May we not hate each other!

Oh Almighty! May peace prevail! Everywhere!

Oh Almighty! May everybody be happy!

May all be free from ailments!

May all see what is auspicious!

May no one be subject to miseries!

Oh Almighty! May peace prevail! Everywhere!

kathopanishada 2:6:19 - India - 1400 b.c.e.