THE APPLICATION OF GC-C-IRMS TO THE AUTHENTICATION AND CHARACTERISATION OF FOODS AND BEVERAGES

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CERTIFICATE OF AUTHORSHIP

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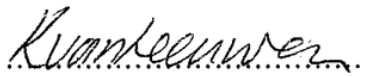

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DEDICATION

This thesis is dedicated to my husband and daughters for their love, support and belief in me during my PhD candidature.

And, to my father who passed away during my PhD candidature.

(14^{th} Aug 1944 – 17^{th} Aug 2015)

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OTHER PUBLICATIONS

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AUTHOR CONTRIBUTION STATEMENTS

Paper 1. KAvL (candidate) reviewed the literature and wrote the manuscript. FC, PDP and DR helped to evaluate and edit the manuscript.

Paper 2. KAvL (candidate) developed the research question, the method and experiments, analysed the data, interpreted the data and wrote the manuscript. MP developed the short vanillin method. FC, DR and PDP edited and evaluated the manuscript.

Paper 3. KAvL (candidate) developed the method and analysed the samples, interpreted the data and wrote the draft manuscript. FC and RB evaluated the data and edited the manuscript. DR and PDP gave a final edit of the manuscript.

Paper 4. KAvL (candidate) analysed and interpreted the results, completed all of the statistical analysis and wrote the manuscript. MP prepared the samples, developed the method and ran the samples. The experimental design with respect to fruit cultivation and growing regime was constructed by CM and KHL. FC, PDP and DR evaluated and edited the manuscript.

I declare that the author contribution statements above are accurate and I give permission for the candidate to include the papers and manuscripts in this thesis.

Signed by the supervisory team on behalf of the co-authors.

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Paul D. Prenzler	.....	.....
Danielle Ryan	.....	.....

LIST OF ABBREVIATIONS

‰ – “per mil”

15:0 – pentadecanoic acid

16:0 – palmitic acid

17:0 – heptadecanoic acid

18:0 – stearic acid

18:1 *cis*-11 – *cis*-11-octadecenoic acid

18:1 *cis*-9 – oleic acid

18:2*n*-6 – linoleic acids

18:3*n*-3 – α-linolenic acid

1σ – standard deviation

2σ – measurement of uncertainty

5-HMF – 5-hydroxymethylfurfural

AA – atomic absorption

Ala – alanine

ANOVA – one-way analysis of variance

Asn – asparagine

Asp – aspartate

BAS – Basilicata

C – carbon

C. ladanifer – Cistus ladanifer

C0 – control diet

C3 – Calvin photosynthetic pathway

C4 – Hatch-Slack photosynthetic pathway

C6 – diet supplemented with oil

CAM – Crassulacean acid metabolism photosynthetic pathway

CAP – Common Agricultural Policy

CBS – Caribou Hoof Standard

CD – spectrophotometry

CL0 – diet with *Cistus ladanifer*

CL6 – diet with both oil and *Cistus ladanifer*

CLA – conjugated linoleic acid

CO_2 – carbon dioxide

Conv – conventional

corr – corrected

CSIA – compound specific isotopic analysis

DM – dry matter

EA-IRMS – elemental analyser-isotope ratio mass spectrometry

EC – European Commission

ECF – ethyl chloroformate

EEC – European Economic Community

EI – electron impact ionization

ER – Emilia Romagna

Es-GC – enantioselective-gas chromatography

EtOH:pyr – ethanol:pyridine

EU – European Union

FA – fatty acid(s)

FAME – fatty acid methyl ester(s)

FID – flame ionisation detector

GABA – γ-aminobutyric acid

GC – gas chromatography

GC-C-IRMS – gas chromatography-combustion-isotope ratio mass spectrometry

GC-MS or GC/MS – gas chromatography-mass spectrometry

GC-P-IRMS – gas chromatography-pyrolysis-isotope ratio mass spectrometry

Gln – glutamine

Glu – glutamic acid

Glx – glutamate and glutamine combined

Gly – glycine

GMO – genetically modified organisms

H – hydrogen

H_2 – hydrogen

He – helium

HPLC – high-performace liquid chromatography

HR-NMR – high-resolution-nuclear magnetic resonance spectroscopy

HSD – Honest Significant Difference

HS-SPME – headspace-solid phase microextraction

IAEA – International Atomic Energy Agency

ICP-AES – inductively coupled plasma atomic emission spectroscopy

i-IST – internal isotopic standard

Ileu – isoleucine

IR – infrared spectroscopy

IRMS – isotope ratio mass spectrometry

iSTD – internal standard

KHS – Kudu Horn Standard

LC-PUFA – long chain-polyunsaturated fatty acids

Leu – leucine

MDGC – multidimensional gas chromatography

me-15:0 – methyl pentadecanoate

me-16:0 – methyl palmitate

me-17:0 – methyl heptadecanoate

me-18:0 – methyl stearate

me-18:1 *cis*-11 – methyl cis-11-octadecenoate

me-18:1 *cis*-9 – methyl oleate

me-18:2*n*-6 – methyl linoleate

me-18:3*n*-3 – methyl linolenate

MNM – methyl *N*-methylanthranilate

MS – mass spectrometry

N – nitrogen

N_2 – nitrogen

nd – not detected

NH_4^+ – ammonium

NL – neutral lipids

Nleu – norleucine

NO_3^- – nitrate

NOP – National Organic Program

Nval – norvaline

O – oxygen

O_2 – oxygen

Org – organic

PC – principal component

PCA – principal component analysis

PDO – protected denomination of origin

PGI – protected geographical origin

Phe – phenylalanine

PL – polar lipids

Pro – proline

Proc MIXED – MIXED procedure

PUFA – polyunsaturated fatty acids

RuBisCO – ribulose-1,5-biphosphate carboxylase/oxygenase

S – sulphur

SD – standard deviation

SEM – standard error of means

Ser – serine

SFA – saturated fatty acids

SNIF-NMR – site-specific isotopic fractionation nuclear magnetic resonance spectroscopy

SPME – solid phase microextraction

Thr – threonine

TSG – traditional specialty guaranteed

UN – unstructured covariance

Val – valine

VCDT – Vienna Canyon Diablo Troilite

V-PDB – Vienna Pee Dee Belemnite

VSMOW – Vienna Standard Mean Ocean Water

δ – delta

$\delta^{13}C$ – stable isotope ratio of carbon expression, $^{13}C/^{12}C$

$\delta^{15}N$ – stable isotope ratio of nitrogen expression, $^{15}N/^{14}N$

$\delta^{18}O$ – stable isotope ratio of oxygen expression, $^{18}O/^{16}O$

$\delta^{2}H$ – stable isotope ratio of hydrogen expression, $^{2}H/^{1}H$

$\delta^{34}S$ – stable isotope ratio of oxygen expression, $^{34}S/^{32}S$

ABSTRACT

Traceability and authenticity perform an important role in keeping the food and beverage industries honest for the protection of the general consumer and for the decent producer. The process to investigate the authenticity and traceability of food and beverages is through analytical techniques and methods. One such technique is the gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and compound specific isotopic analysis (CSIA) to analyse different compounds in a variety of matrices, as the name suggests, for isotope ratios of C and N.

The purpose of this thesis was the implementation GC-C-IRMS to develop methods to detect adulterations in distillates using $\delta^{13}C$ for vanillin; to trace dietary effects on intramuscular fat in sheep using $\delta^{13}C$ of fatty acid methyl esters; and to differentiate between different farming systems (organic vs. conventional) with $\delta^{13}C$ and $\delta^{15}N$ of amino acids from tomatoes.

The first study was the development of a method for a single compound, vanillin, for $\delta^{13}C$. The determination of whether a distillate has been adulterated, by the addition of synthetic vanillin, to enhance the overall quality of the beverage could be detected. Vanillin ranges were determined and compared with those in literature for synthetic, natural and ex-lignin vanillin. Distillate samples, rum, whisky etc, were analysed for their $\delta^{13}C$ vanillin value, which were compared against the determined vanillin ranges. The vanillin in 32 distillates were in the $\delta^{13}C$ range for ex-lignin, there was one spirit, however, which was found to have synthetic vanillin.

The second study was of greater analytical complexity than the first study as 4 fatty acids (FA) $\delta^{13}C$ values were analysed in polar (PL) and neutral (NL) lipids of the intramuscular fat of lambs. The lambs (24) were fed 4 different diets supplemented with oil and *Cistus ladanifer* L. (a tanniferous shrub). The research question was to understand whether the increase in intramuscular fat for lambs fed a diet supplemented with both oil and *C. ladanifer*, could be explained mostly by the incorporation of diet preformed FA or by increased *de novo* FA synthesis. It was shown that, with respect to 16:0, the increase of intramuscular FA was due to continual *de novo* FA synthesis for lambs fed a diet supplemented with oil and *C. ladanifer*. The results also showed that diets supplemented with oil prevented *de novo* FA synthesis from occurring; therefore, the inclusion of *C. ladanifer* to the diet repressed the oil effect.

The last study, of even greater analytical complexity, focussed on the analysis of 9 amino acids $\delta^{15}N$ and $\delta^{13}C$ values for the differentiation between tomatoes grown organically and conventionally. Amino acids are involved in many metabolic pathways in the plant; therefore, tracing the N uptake from the fertilizer to the plant and subsequent fruit could be possible. Glx functioned as an internal standard to remove the effects of external factors. The analysis of the amino acids Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and Phe for $\delta^{15}N$ and $\delta^{13}C$ with particular focus on Glx for $\delta^{13}C$, provided separation between tomatoes grown organically and tomatoes grown conventionally.

CHAPTER ONE

AIMS & OBJECTIVES

INTRODUCTION

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AIMS & OBJECTIVES

The overall aim of this study was to develop analytical methods for the authentication and characterisation of food and beverage matrices by measuring isotopic ratios of C and N using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Having extensively reviewed the literature, it was apparent that there were several more areas of the application of GC-C-IRMS that could be explored with regards to food and beverage authentication. In order to advance knowledge in this field of study, these applications needed to include analytical challenges due to matrix effects, chromatographic effects (particularly baseline separation of analytes); and sample preparation and derivatisation, which in GC-C-IRMS can introduce fractionation. Thus, three objectives were chosen covering these different aspects of methodological development and application:

1. To develop a $\delta^{13}C$ GC-C-IRMS method to enable differentiation of natural and synthetic vanillin in a variety of distillates for detection of beverage adulteration.

2. To determine, using $\delta^{13}C$, if fatty acids (FA) in the intramuscular fat of lambs fed with four different dietary regimes were produced due to a higher *de novo* synthesis or due to a higher accumulation of dietary fat.

3. To differentiate tomato fruits grown under conventional and organic farming regimes by analysis of the $\delta^{15}N$ composition of amino acids within the fruits.

INTRODUCTION

The authenticity, traceability and nutritional quality of food and beverages is becoming increasingly important around the world. There are many reasons for this for example food related illnesses and diseases have become more widespread and the use of genetically modified organisms (GMO) in foods are more prevalent. Also, consumers are increasingly aware of the food chain process and desire food grown organically rather than conventionally. Likewise the interest in high quality and authentic products as defined as PDO, PGI and TSG (protected denomination of origin, protected geographical origin and traditional specialty guaranteed, respectively) is strengthening. Finally as our understanding of the links between diet and health improves, consumers are increasingly interested in foods and beverages with the potential to lead to better health outcomes. An example of this is the research that shows that different lipids in the diet can have profound effects on susceptibility to certain diseases, such as heart disease, leading to dietary advice and consumer demands for certain types of lipids.

Food and beverages of high quality are more costly to produce, and these costs are then passed on to the consumer. Producers of foods and beverages of inferior quality, produced at a lower cost, could therefore be tempted to adulterate by the addition on flavour/aroma compounds to their produce to simulate high quality produce that demand higher market prices. This type of activity is fraudulent and is termed as adulteration. There are numerous other ways in which food and beverages can be adulterated, such as misrepresentation and/or mislabelling, substitution with an inferior product and extending a product via the supplementation of non-permissible additives with subsequent non-disclosure of such additives. Adulterations of products could pose serious health risks to consumers and loss of trust in the product for the consumer. To protect the consumer and

the producer, the European Union has set strict controls (EU Directive 79/112/EEC & Directive 2000/13/EC) to address the problem of adulteration of foods and beverages.

Traceability of food and beverages facilitates the production process of the foodstuffs to be followed from beginning (farm) to end (retailer). This process ensures confidence in the product for the consumer. Moreover, if a foodstuff was contaminated, being able to trace the production process for that foodstuff would allow the determination of the source of the contamination. Indeed the provision of the European Regulation EC, 178/2002 aims to eliminate all inequitable and counterfeit products for the benefit and the protection of both the consumer and producer.

However, while EU directives and regulations provide a legislative framework, implementation requires the availability of analytical methodologies capable of detecting fraudulent activity. Moreover, with increased consumer demand for healthy products (as mentioned above), analytical methods that are also capable of providing more information on the nutritional quality of food, are needed. This thesis will examine the possibilities that gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) offers in terms of determining food authenticity and nutritional quality. This will be achieved via three well-defined studies as outlined below. It should be noted that the versatility of GC-C-IRMS is demonstrated through studies of increasing analytical complexity as well as covering both authenticity and nutritional quality aspects, of importance to consumers. The studies, in increasing order of complexity, are:

- the determination of isotope ratios for a single element (carbon), for a single compound (vanillin), to determine if adulteration of spirits can be detected;

- the determination of isotope ratios for a single element (carbon), for a class of related compounds (fatty acid methyl esters), to determine if diet can influence the nutritional quality of lamb;
- the determination of isotope ratios for multiple elements (esp. carbon and nitrogen), for chemically varied compounds (amino acids), to determine if organically grown tomatoes can be distinguished from those grown by conventional methods.

Analysis of spirits

Of major concern for the beverage industry is the addition of flavour/aroma compounds to the beverage as it presents a loss of income for authentic producers. Additions of characteristic flavours and aromas to specific beverages can be especially difficult to detect and requires state of the art analytical instrumentation such as mass spectrometry based isotopic ratio instrumentation. Of late, GC-C-IRMS for $\delta^{13}C$ has been used to analyse for compounds characteristic to certain beverages, such as citrus liqueurs for monoterpene adulteration (Schipilliti, Bonaccorsi, Cotroneo, Dugo, & Mondello, 2013). GC-C-IRMS enables the analysis of single compounds (compound specific isotope analysis; CSIA) in a matrix whereas IRMS analyses the whole sample. The benefit of GC-C-IRMS is that compounds can be analysed separately for their isotopic ratios within a matrix and therefore if a compound was added to a matrix it would have a different isotope ratio to that of the matrix due to their different mechanisms of formation. Usually, with adulteration of a product to influence aroma/flavour, synthetic versions of the characteristic compound would be added as they are cheaper than the compound when produced naturally. This analysis becomes more difficult with the complexity of the compound. For example vanillin can be produced naturally, synthetically and can also be produced biosynthetically from naturally produced compounds like ferulic acid and

curcumin (from turmeric), or derived from lignin from wood (Greule et al., 2010; Ruff, Hör, Weckerle, König, & Schreier, 2002). The various production pathways impacts the isotopic ratios which can then be exploited for the detection of adulteration.

As distillates are generally stored and aged in oak barrels, compounds such as vanillin are leached into the beverage. One objective of this study was to develop an analytical method implementing GC-C-IRMS to establish if it is possible to determine the source of vanillin in a distillate (wooden barrel it is stored in, or by synthetic vanillin addition) by measuring its $\delta^{13}C$ value.

Analysis of intramuscular fat in lambs

From a nutritional quality perspective, saturated fatty acids (SFA), conjugated linoleic acid (CLA) and long chain-polyunsaturated fatty acids (LC-PUFA) and their subsequent impact on human health, are gaining increased attention. The development of cardiovascular diseases in humans is related to the intake of SFA, however intake of CLA isomers and LC-PUFA can be beneficial for human health resulting in reduced risk of cancer, diabetes and atherosclerosis (Jerónimo et al., 2010; Jerónimo, Alves, Prates, Santos-Silva, & Bessa, 2009; Vasta et al., 2009).

Both SFA, CLA and LC-PUFA are found in ruminant meats (Alves & Bessa, 2009; Nagao & Yanagita, 2005; Vasta et al., 2009). Manipulation of meat composition such that it contains increased concentrations of CLA and LC-PUFA, such as eicosapentaenoic and docosahexaenoic acids which are known to have anti-inflammatory, antithrombotic, and anti-atherogenic effects, and reduced concentrations of SFA, would be beneficial for the meat eating society (Givens, 2005; Jerónimo et al., 2009).

The concentration of CLA in meat is dependent on the diet of the animal and due to the ruminal biohydrogenation of the essential fatty acids linoleic acid and linolenic acid, which are solely derived from the diet, to form stearic acid from which other fatty acids are derived (Bessa et al., 2007; Sackmann et al., 2003; Vasta et al., 2009). Stearic, oleic and palmitic acid can also be formed by *de novo* synthesis. Increasing the amount of these essential fatty acids in the diet could increase the formation of these fatty acids in the muscle and fat of the ruminant (Jerónimo et al., 2010; Raes K, De Smet S, Balcaen A, Claeys E, & D., 2003). *De novo* synthesis by the animal is depressed when there is an abundance of fatty acids derived from the diet (Chilliard, 1993). Traceability of the ruminant meat to its food source should be possible, as the composition of fatty acids in ruminant meat can be derived from the diet. Implementing GC-C-IRMS for analysis $\delta^{13}C$ values of fatty acids in the intramuscular fat of lambs compared to dietary fatty acids for has increasing complexity compared to single compound analysis as for distillates especially since there are metabolic processes occurring in the rumen, such as unsaturated fatty acid hydrogenation and isomerisation and *de novo* synthesis of fatty acids (Alves & Bessa, 2009).

Analysis of tomatoes

As people become more aware of what they are eating through their own research, they have become more interested in how their food is produced and how healthy it is for them, for example, conventionally grown products versus organically grown products. Organic products are deemed healthier, friendlier for the environment and safer as they are not plied with insecticides or synthetic fertilizers like conventional products (Laursen, Schjoerring, Kelly, & Husted, 2014). Organic products, due to their production costs, command high prices from the consumer, whereas conventional products are much cheaper to produce. Adulterations such as mislabelling a conventionally grown product

as organic for increased profits are constantly occurring (Huck, Pezzei, & Huck-Pezzei, 2016) and are difficult to detect. How is it possible to differentiate between a tomato grown organically and a tomato grown conventionally, when physically the two look the same? It is known that synthetic fertilisers are different from organic fertilisers due to their mode of formation with respect to their $\delta^{15}N$ values. Synthetic fertilisers have $\delta^{15}N$ values between –6‰ and +6‰ and organic fertilisers (e.g. animal manures) have $\delta^{15}N$ values between +1‰ and +37‰ (Bateman & Kelly, 2007). As plants uptake nitrogen from the soil these values could be reflected in the plant and subsequently in the fruit of the plant (tomato). Indeed analysis of bulk (whole sample) tomatoes for $\delta^{15}N$ by can differentiate between plants grown organically and plants grown conventionally (Nakano, Uehara, & Yamauchi, 2003). However, the uptake of nitrogen by plants is more complicated than just a simple uptake of nitrogen from fertilizers, as there are other factors that affect nitrogen uptake, and the resultant $\delta^{15}N$ value. Factors such as temperature, soil type, moisture content, type of chemical fertilizer applied, differences in agricultural practices, variations in deposits of atmospheric nitrogen all play a role (Bateman, Kelly, & Woolfe, 2007; Choi, Ro, & Lee, 2003). It has also been shown that nitrogen can affect the $\delta^{13}C$ values of a plant due to high rates of fertiliser and can also be influenced by other environmental factors (Högberg, Johannisson, & Hällgren, 1993; Senbayram, Dixon, Goulding, & Bol, 2008).

Nitrogen uptake is facilitated by the conversion of nitrate and ammonia into amino acids. Amino acids in plants are involved in many metabolic pathways such as secondary metabolism, plant defense, and cell component and protein synthesis (Molero, Aranjuelo, Teixidor, Araus, & Nogués, 2011). Their measurement could be used to track the metabolic pathway of nitrogen in plants. Environmental parameters that could affect nitrogen uptake might also impact on amino acid composition and hence their isotopic ratio. Acknowledging all these factors, another objective of this study was to determine

if the analysis of amino acids in the tomatoes by GC-C-IRMS for $\delta^{15}N$ and $\delta^{13}C$ could differentiate between conventionally and organically grown tomatoes.

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CHAPTER TWO

INTRODUCTORY COMMENTS

LITERATURE REVIEW

Paper 1 van Leeuwen, K. A., Prenzler, P.D., Ryan, D., & Camin, F. (2014). Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry for Traceability and Authenticity in Foods and Beverages. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5): 814-837. DOI:10.1111/1541-4337.12096.

LITERATURE REVIEW UPDATE

REFERENCES

INTRODUCTORY COMMENTS

This chapter provides an overview of what isotopes are, the history of the analysis of isotopes by GC-C-IRMS, the measurement of isotopes in foodstuffs and beverages and why GC-C-IRMS is a powerful tool for determining authenticity and/or traceability of edible products. The literature review was undertaken to identify what was previously done with GC-C-IRMS for the analysis of food and beverages to help define the scope of my research. Following the manuscript is a brief summary of articles published since the review was submitted for publication (2 Jun 2014)

Comprehensive REVIEWS in Food Science and Food Safety

Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry for Traceability and Authenticity in Foods and Beverages

Katryna A. van Leeuwen, Paul D. Prenzler, Danielle Ryan, and Federica Camin

Abstract: As consumers demand more certainty over where their food and beverages originate from and the genuineness of ingredients, there is a need for analytical techniques that are able to provide data on issues such as traceability, authenticity, and origin of foods and beverages. One such technique that shows enormous promise in this area is gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). As will be demonstrated in this review, GC-C-IRMS is able to be applied to a wide array of foods and beverages generating data on key food components such as aroma compounds, sugars, amino acids, and carbon dioxide (in carbonated beverages). Such data can be used to determine synthetic and natural ingredients; substitution of 1 ingredient for another (such as apple for pear); the use of synthetic or organic fertilizers; and origin of foods and food ingredients, including carbon dioxide. Therefore, GC-C-IRMS is one of the most powerful techniques available to detect fraudulent, illegal, or unsafe practices in the food and beverages industries and its increasing use will ensure that consumers may have confidence in buying authentic products of known origin.

Keywords: adulteration, authenticity, food analysis, gas chromatography-combustion-isotope ratio mass spectrometry, traceability

Introduction

The global food industry is estimated to be worth U.S.$4 trillion in retail sales annually (Services 2012). At the same time, consumer awareness of food issues is increasing as evidenced by more stringent labeling requirements, growing awareness of genetically modified organisms (GMOs) and diseases linked to food ("mad cow disease" and "bird flu"), as well as the increase in the market for premium products, such as organic food, protected denomination of origin (PDO), protected geographical indication (PGI), and traditional speciality guaranteed (TSG). In view of the value of the global food industry and greater consumer demands on food, there is a necessity for regulations that protect consumers from dangerous or fraudulent practices and analytical methods to assist in enforcing regulations and detecting untoward activities.

In the food and beverage industries, 2 keywords associated with consumer protection are "authenticity" and "traceability." The word "authentic" is defined (Dictionary.com 2013) as "*the sense of actuality and lack of falsehood or misrepresentation. Authentic carries a connotation of authoritative certification that an object is what it is claimed to be.*" Nonauthentic food products arise through adulteration and fraud through practices such as mislabeling/misrepresentation, addition of flavors/aromas to enhance a product at a cheaper price, addition of nondisclosed additives to increase bulk, among others. For the food and beverage industry, the necessity for authentic products is in the best interest of the consumer, as nonauthentic products could pose health risks and/or consumer confidence may be dampened through an inferior product sold as the genuine item.

The word "trace" is defined (Dictionary.com 2013) as "*to follow, discover, or ascertain the course of development of (something),*" and "*to follow or be followed to source; date back.*" For the consumer, traceability of a food provides a level of confidence in the product that is being sold as there is a level of transparency, there is nothing to conceal in the production of the product. Traceability is also beneficial to trace back the provenance of the ingredients and the source of any contamination.

For example in Europe, with the establishment of the European Economic Community (EEC), rules such as the "Common Agricultural Policy" (CAP) in 1957 (Zobbe 2001) were introduced to control agricultural practices, supply, and expenses. Such Euro-wide rules eventually led, toward the end of 1978, to regulations to address authenticity and traceability practices such as labeling of foodstuffs (Council Directive, 79/112/EEC). One of the main outcomes of these rules is the elimination of all unfair competition and the trading of fake products. The milestone of the European Union food safety and traceability regulation is the

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Regulation (EC) Nr 178/2002, which defines "traceability" as "the ability to trace and follow a food, feed, food-producing animal, or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing, and distribution."

Methods for testing authenticity and providing data on traceability require robust analytical techniques that can be utilized by the various regulatory authorities around the world. The analytical methods currently used to determine the origin of a foodstuff or beverage and whether it has been adulterated are:

- separation techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC);
- structural analysis such as mass spectrometry (MS), high-resolution-nuclear magnetic resonance (HR-NMR) spectroscopy, and infrared spectroscopy (IR);
- stable isotope analysis such as site-specific isotopic-fractionation nuclear magnetic resonance (SNIF-NMR) spectroscopy and isotopic ratio mass spectrometry (IRMS);
- trace element analysis such as inductively coupled plasma atomic emission spectroscopy (ICP-AES) and atomic absorption spectroscopy (AA);
- bioanalytical techniques such as DNA profiling,

together with compositional analysis, data handling, and interpretation implementing multivariate statistics such as chemometrics (Lees 1998).

Of the many techniques available to aid in authenticity and traceability testing, IRMS has been widely used due to the high precision of the method, the requirement for small samples, and the fact that the same technique can be used for almost any type of food or beverage. Since the 1st report in 1977 of IRMS to detect adulteration of honey, by the addition of high-fructose corn syrup (Doner and White 1977), other applications have included authenticity testing and traceability studies of olive oil (Camin and others 2010), orange fruit (Rapisarda and others 2010), lamb (Piasentier and others 2003), and beef (Boner and Forstel 2004). Even though IRMS has been successfully applied to the detection of adulteration across a wide range of foods and beverages, a major drawback is that it is a bulk technique, that is, the data are for the whole sample, which may contain many hundreds or thousands of different chemical compounds. In order to provide more information about specific compounds in foods and beverages, IRMS may be coupled to a GC unit so that separation of compounds occurs prior to isotope ratio monitoring. Coupling of the GC to the IRMS may be via either a combustion or pyrolysis chamber giving information about C/N or H/O isotopes, respectively. Indeed, the development of GC-combustion (C)/pyrolysis (P)-IRMS is a powerful technique for adulteration and authenticity testing (Greule and others 2010), as it allows specific compounds to be targeted that are important to the overall make-up of the particular foodstuff or beverage.

The aim of this review is to provide a broad overview of the GC-C-IRMS technique covering the historical development of the method, a discussion on the natural variability of carbon (C) and nitrogen (N) stable isotope ratios, and an overview of the applications of the technique to a variety of foods and beverages for authentication and traceability purposes. The emphasis of the review will be on C-IRMS for several reasons: historically, GC-C-IRMS was developed before GC-P-IRMS; the principles of operation of the instruments are similar in most respects (Sessions 2006); and most importantly, in applications to food and beverages, results from combustion studies have been reported as stand-alone articles, whereas pyrolysis studies have almost always been reported in combination with combustion studies. In presenting this review, the power of the GC-C-IRMS technique is highlighted as a valuable tool in ensuring consumer confidence and safety in the global food industry.

Historical Development of GC-C-IRMS

The development of GC-C-IRMS instrumentation is underpinned by some of the most fundamental discoveries in science. As such, we briefly review these developments, which highlight the way in which this very applied technique has evolved due to fundamental developments in physics and chemistry.

In the middle to late 1800s (Svec 1985), chemists and physicists were exploring the nature of cathode rays, which led in 1895 to the discovery of particles with a negative charge (Svec 1985; Perrin 1986; Griffiths 1997; Thomson 2010). Joseph John Thomson replicated this experiment, improving the experimental design, and also utilized a Faraday cup to determine that cathode rays are definitely negatively charged particles (termed "electrons") and their velocity and mass-to-charge ratio (Thomson 2010). These initial stages of measuring particles in cathode rays mark the beginning of IRMS in the early 1900s with the design of the mass spectrograph by Thomson and others (Classen 1908; Dempster 1918). The various parties were experimenting with different methods of detection of elements and their isotopes.

During the decade 1910 to 1920, significant developments in MS yielded the mass spectrum of CO_2 (Thomson 1912; Budzikiewicz and Grigsby 2006) and the discovery of 2 isotopes of neon (Thomson 1913; Budzikiewicz and Grigsby 2006). Initially, it was thought that neon was composed of 2 gases giving a main ray at m/z 20 and a weaker ray at m/z 22. The assignment of the 2 rays to neon arose from the development of a new "velocity focusing" mass spectrograph.

In 1918, another type of mass spectrograph was reported, termed as "directional focusing" (Dempster 1918; Dempster 1948; Svec 1985; Squires 1998; Budzikiewicz and Grigsby 2006) and by 1948, the isotopic composition of 83 elements had been determined (Dempster 1948). A "double focusing" instrument was reported in 1934, which combined a radial electric field and a uniform magnetic field, creating velocity and direction focusing, thereby enhancing the measurement accuracy of the mass spectrograph due to sharpened images and intensity (Mattauch and Herzog 1934). Various versions of the "double focusing" instrument appeared between 1936 and 1941 (Bainbridge and Jordan 1936; Nier 1940; Straus 1941; Svec 1985). During this time, natural variation in isotopic abundances was discovered and the variance present in specific isotope ratios may carry significant information (Nier 1937; Nier and Gulbransen 1939).

The ability to measure variations in the abundances of isotopes in gases such as CO_2 was enhanced by the design of a mass spectrometer with dual inlet system in 1947. The incorporation of a "changeover valve" allowed 2 gas mixtures (sample and standard) to be alternatively interchanged, in seconds, and subsequently introduced to the mass spectrometer to be detected by 3 Faraday cups (Murphey 1947). Murphey employed this instrument to determine thermal diffusion factors of gas mixtures such as H_2 and D_2, H_2 and He, and D_2 and He (Murphey 1947). Improvements to this system and the incorporation of a reference standard allowed variations in the isotope abundance ratios of carbon and oxygen in CO_2 and O_2 to be measured with great accuracy (McKinney and others 1950; Brand 2004).

Over the next quarter of a century, there were continual improvements to mass spectrometer instruments and the first commercial (quadrupole) mass spectrometer coupled to a gas chromatograph was released in 1968 (Brock 2011). But it was not until 1976 (Sano and others 1976; Brand 1996) that a combustion system was incorporated between the GC outlet and the mass detector to convert the eluent to simple gases whose isotope ratios can be measured. In 1976, Sano developed this instrument to implement an isotope tracer technique for complex matrices such as metabolites in urine samples as an aid in the research of drug metabolites. The system included a CuO combustion reactor positioned after the GC and a multiple ion detection scheme (detecting ions of m/z 44 and 45) (Sano and others 1976; Brand 1996). This design was the prototype for the GC-C-IRMS instrument defined as "isotope-ratio-monitoring gas chromatography-mass spectrometry (IRM-GCMS)." The first commercial GC-C-IRMS was released in 1988, building on Sano's design with various features such as: a water trap (magnesium perchlorate); a trap for CO_2 (when analyzing for N_2); and the removal of nitrogen oxides through a reduction oven over copper metal. The instrument measured isotope ratios at natural abundance levels (Matthews and Hayes 1978) for C and N in any organic molecule that could be analyzed by GC.

A further enhancement to the overall instrumental design of the GC-C-IRMS was the incorporation of a flame ionization detector (FID). This allowed the "normal" gas chromatogram to be monitored from the main eluent, while the remainder passed through the combustion and isotope monitoring system. Interestingly, the application chosen to highlight the advantages of this advancement was the determination of the natural ^{13}C abundance of flavorings found in different foods to develop an authenticity test for foodstuffs. Hence, the ability for isotope ratio monitoring to be utilized in food authenticity/traceability has been recognized from the earliest stages of the development of the technique.

One of the requirements for precise measurement of isotope ratios in GC-C-IRMS is the necessity for strict baseline separation of components of interest, which may be difficult in complex sample matrices. Hence, there are ongoing efforts to improve separation of the peaks in the gas chromatograph so that the eluent travelling to the mass spectrometer is composed of a single component. One strategy for achieving this is to "heart-cut" the retention time interval of interest and divert the flow to a 2nd column, where further separation can take place prior to introduction to the IRMS. This multidimensional (MD) GC approach does allow better resolution of overlapping peaks, but it suffers from the drawback that 2 runs are required—the first to determine which region is to be heart-cut, and the second where MDGC is performed. Alternatively, in comprehensive 2-dimensional GC (GC × GC), the whole sample is passed through a 2nd column and therefore only one run is required. While the advantages of comprehensive GC × GC-C-IRMS have been demonstrated in sports drug testing (Tobias and others 2008), so far no food or beverage applications have been published (to the best of the authors' knowledge). Thus, a potential area of fruitful research may be to utilize MD and comprehensive GC-C-IRMS in authenticity and traceability studies.

Principles of Operation of GC-C-IRMS Instrumentation

As the name suggests, the basic components of a GC-C-IRMS are a gas chromatograph, which feeds eluent to a combustion system, and thence to an isotope ratio mass spectrometer. Details of these 3 instrumental components are documented in a review by Sessions (2006); hence, only a brief description is included in this review. While the gas chromatograph needs modification to accommodate the combustion/reduction tubes and so on for GC-C-IRMS, the principles of GC are no different from any other GC instrument, such as GC-FID or GC-MS. Sample introduction to the GC can occur in a variety of ways depending on the sample, including split/splitless injection, on column injection, solid-phase microextraction (SPME), and so on. The most important aspect is to guarantee the injection of analytes without fractionation (see below). After separation on the column, the eluent is split with a small part diverted to a "standard" detector such as FID or MS, while the other portion of the flow continues to the combustion system.

Combustion of the eluent occurs in 3 stages: an oxidation step, a reduction step, and the removal of water. Oxidation is facilitated by high temperature (800 to 1100 °C) and the use of oxidation catalysts such as CuO/Pt or CuO/NiO/Pt (Meier-Augenstein 1999). The principle elements of organic compounds, C, H, and N, are oxidized to the gases CO_2, H_2O, and NO_x, which are then passed into a reduction tube, where in the presence of elemental Cu at 500 to 700 °C, NO_x is reduced to N_2 and any remaining O_2 is removed (note that oxygen originating from the sample cannot be analyzed by IRMS in the system described here, since atmospheric oxygen would change the isotope ratios of oxygen in the combustion gases).

After the reduction step, water is removed from the analyte gas stream. This is a necessary step as the water can react with the gases inside the ion source (in the IRMS) creating ions of differing compositions with conflicting masses to the ions to be measured (known as "isobaric interference"). For example, water could protonate the $^{12}CO_2$ to $H^{12}CO_2^+$ that has the same m/z as the $^{13}C^{16}O_2^+$ peak of 45. Removal of water is typically accomplished via a countercurrent drier based on a Nafion™ membrane (Leckrone and Hayes 1998). For nitrogen analyses only, CO_2 must be removed from the gas stream, generally by cryogenic trapping (Merrit and others 1994), to prevent possible isobaric interferences with N_2 in the ion source (from production of $^{12}C^{16}O$).

An important component of the combustion assembly is a system for solvent diversion. During a run, the solvent peak elutes first; this peak must be removed because the solvent reduces the performance of the oxidation column by overloading the column with organic material. The most common system, referred to as backflushing, acts to reverse the He flow through the oxidation reactor such that the solvent is vented out by an exhaust valve, before it can reach the oxidation column. Once the solvent is vented, eluent from the sample is allowed through the combustion system. The backflush system can also play a role in regeneration of the oxidation reactor by introducing O_2 into the reversing He stream. A new system has recently been proposed, which is based upon the use of a cooled injection system and auxiliary vacuum for solvent removal (Flenker and others 2007).

Dried gases from the combustion system travel to the IRMS detector and are subjected to electron-impact ionization and a succeeding magnetic-sector or electromagnetic analyzer, deflecting the generated ions by molecular weight to be subsequently detected by precisely positioned (in the MS focal plane) Faraday cups. For carbon, there are 3 detecting Faraday cups that monitor the various isotopologues of CO_2, namely, m/z 44 ($^{12}C^{16}O^{16}O$), m/z 45 ($^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$), and m/z 46 ($^{12}C^{18}O^{16}O$). For nitrogen, these 3 Faraday cups are arranged to capture isotopologues of N_2—m/z 28 ($^{14}N^{14}N$), m/z 29 ($^{14}N^{15}N$), and m/z 30 ($^{15}N^{15}N$). During the chromatographic run, the Faraday cups

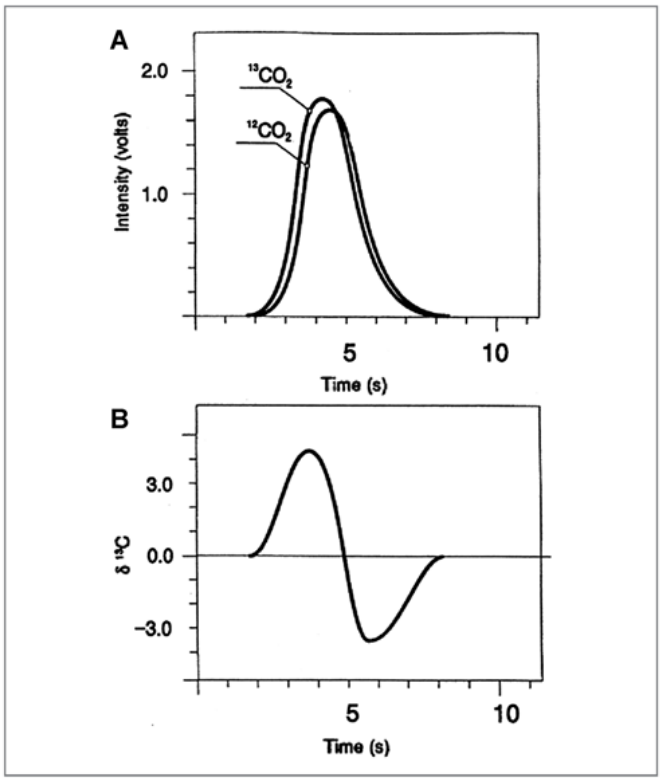

Figure 1–Chromatographic traces generated by GC-C-IRMS. (a) Time shift due to isotopic effect on retention time of CO_2. (b) The resulting S-shaped peak due to the 45/44 ratio (Meier-Augenstein 1999).

continuously record ion currents and these data are processed to give relative isotopic abundances as described below.

Processing of GC-C-IRMS Data

The output from a GC-C-IRMS consists of partially overlayed chromatograms that correspond to the ions detected by the Faraday cups. For carbon isotope monitoring, there will be 3 traces corresponding to m/z 44, 45, and 46 (Figure 1a shows 2 traces; m/z 44 ($^{12}C^{16}O_2$) and m/z 45 ($^{13}C^{16}O_2$), while for nitrogen, 2 traces are common m/z 28 and 29, with a 3rd trace optional (m/z 30). The peaks of isotopologues are shifted in time, because the heavier isotopologue elutes milliseconds before the lighter isotopologue as a result of chromatographic isotope effects (Matucha and others 1991) (see Figure 1).

The 1st step in data processing is that of peak detection whereby peak start and stop parameters are defined (Zhang and others 2012). From there, ion current ratios can be calculated for the detected peaks via summation, curve fitting, or linear regression. The summation approach is most commonly used (Zhang and others 2012) and is explained in detail in Ricci and others (1994). Briefly, once peak start and stop times have been defined, major and minor isotope traces are integrated from which isotope ratios can be calculated; background correction on the ion currents is mandatory and can be performed either before or after peak detection and integration (Zhang and others 2012). It should be noted that algorithms have been developed (Goodman and Brenna 1994; Bluck and Coward 2004) to accommodate some coelution of peaks in GC-C-IRMS; however, to the best of our knowledge, these algorithms have not been used in any real-life applications and we strongly emphasize the need for baseline separation of peaks.

Having calculated the isotope ratios (such as $^{13}C/^{12}C$) for each peak in the sample chromatogram, further data processing is required to report these ratios relative to ratios derived from standard reference substances (as shown in Figure 1b). This relative deviation is expressed using the isotope delta (δ) notation, which was first described for C by Urey (1948) and adapted as follows (Brand and Coplen 2012):

$$\delta^i E = \frac{({}^i R_{SA} - {}^i R_{REF})}{{}^i R_{REF}}$$

where ^{i} is the mass number of the heavier isotope of element E (for example, ^{13}C or ^{15}N);

R_{SA} is the respective isotope number ratio of a sample (such as for C: number of ^{13}C atoms/number of ^{12}C atoms or as approximation $^{13}C/^{12}C$).

R_{REF} is that of international recognized reference materials, namely, Vienna Pee Dee Belemnite (VPDB) for CO_2 and Air for N_2.

The delta values are multiplied by 1000 and are expressed in units of "per mil" (‰).

In GC-C-IRMS, correct calibration of the instrument is essential. This involves introducing the reference gas (either CO_2 or N_2) during the run via the reference gas inlet when there is no interference with other eluting peaks and/or using organic standards that can be added to the sample if they elute in analyte-free regions or analyzed in the run in the proximity of the samples (Sessions 2006). The isotope ratios of both the reference gases and the standards are calibrated against secondary isotopic reference materials with certified values (Brand and others 2014).

The precision of the GC-C-IRMS instrument has to be excellent, namely, 10^{-5} atom% deviation to determine the $^{13}C/^{12}C$ isotopic ratio (Brand 1996; Meier-Augenstein 1997). However, in order to achieve the necessary precision and accuracy, the analytical workflow must be carefully considered. It is essential for reproducibility that the following are considered: maintaining a clean working environment; extensive method development aimed at producing the optimal running conditions of the instrument (columns, injector systems/methods, splits, flow rates, isotopic calibration, and so on); appropriately selected internal standards (Caimi and others 1994; Mosandl 1995; Mosandl and others 1995); avoidance of analysis-induced fractionation, as a result of derivatization, improper injecting methods, incomplete sample combustion, and so on and suitable sampling and storage techniques (Blessing and others 2008). Limits of detection and linearity should be determined, and runs should be examined for carryover effects as these could cause significant differences for the isotope ratio values (Mottram and Evershed 2003).

Isotopes—Natural Abundances and Ratios

Some discussion of isotopes and their natural abundances is essential in order to understand the principles of the GC-C-IRMS instrument and its applications. The term "isotope" was first introduced by Soddy in 1913 as a condensed version of "They are chemically identical, and save only as regards the relatively few physical properties that depend on atomic mass directly, physically identical also" (Soddy 1913). The word "isotope" is derived from the Greek, "isos" meaning "equal" and "topos" meaning "place," that is the chemical element (same atomic number) with different atomic weights (different number of neutrons), resides in the same position on the periodic table. Isotopes exist in 2 forms, stable and

Table 1–Mean natural abundance of the stable isotopes of the bioelements.

Element	Stable isotope	Mean natural abundance (%)
Hydrogen	^{1}H	99.99
	^{2}H (D)	0.01
Carbon	^{12}C	98.89
	^{13}C	1.11
Nitrogen	^{14}N	99.63
	^{15}N	0.37
Oxygen	^{16}O	99.76
	^{17}O	0.04
	^{18}O	0.20
Sulfur	^{32}S	95.00
	^{33}S	0.76
	^{34}S	4.22
	^{36}S	0.02

unstable. The bioelements H, C, N, O, and S exist in nature as 2 or more stable isotopes (Table 1).

Isotopes of an element have the same electronic structure, and therefore, very similar chemical reactivity. Nonetheless, isotopes show different natural abundance, through processes that may be collectively termed "fractionation." Fractionation results in either enrichment or depletion of an isotope away from its mean natural abundance (Table 1). For example, since $H_2{}^{18}O$ is heavier than $H_2{}^{16}O$, evaporation of water from the earth's oceans will result in more $H_2{}^{16}O$ in the atmosphere, and consequently the atmosphere is depleted in $H_2{}^{18}O$ relative to the ocean (Craig and Gordon 1965).

Fractionation processes are governed by kinetic and thermodynamic effects and are due to the isotope's physical and chemical properties that often are proportional to their mass differences and that can affect either the rate of a process (kinetic isotopic effect) or the energy state of a system (thermodynamic isotopic effect) (Galimov 1985). As will be described in more detail below, fractionation processes that occur during the biosynthesis of the components of foods or beverages can be used in authenticity and traceability studies. Small changes in the ratios of isotopes such as $^{13}C/^{12}C$ and/or $^{15}N/^{14}N$ can provide a fingerprint that provides a unique identifier for the origin of particular organic compounds within a food or beverage. By determining these ratios with GC-C-IRMS, and therefore, in specific food components, much information can be derived about foods and beverages, which can be used to ensure that they meet consumer and regulatory requirements.

Carbon

Variations in the $^{13}C/^{12}C$ ratio in natural systems were discovered in 1939 (Nier and Gulbransen 1939) and are associated with the manner of their formation, beginning from the primary C sources; atmospheric CO_2. The $\delta^{13}C$ of atmospheric CO_2 is decreasing progressively, albeit very slowly with time, from −7.92‰ at the end of 1998 to −8.22‰ at the end of 2003 over the central Indian ocean (Longinelli and others 2005). Some major processes that incur isotopic fractionation in the inorganic and organic carbon cycles are the exchanges between the atmosphere and the hydrosphere that generate sedimentary carbonate deposits enriched in ^{13}C (+5‰ to −5‰) and biological processes cause biospheric carbon to be enriched in ^{12}C (typical ratios from −15‰ to −35‰) (Galimov 1985).

The fractionation of carbon isotopes within a biological vegetal system occurs through photosynthetic pathways, Calvin (C3), Hatch-Slack (C4), and the Crassulacean acid metabolism (CAM). The C3 plants (tomato, potato, wheat, rice, and grape) that utilize the Calvin cycle to fix CO_2 have a $^{13}C/^{12}C$ ratio more depleted in ^{13}C than the C4 plants (such as corn and sugarcane [Winkler 1984]) that utilize the Hatch-Slack cycle (Smith and Epstein 1971) (Figure 2). The reason for this is that the Calvin cycle fixes atmospheric CO_2 in the 1st step with a carboxylation reaction utilizing the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) (Galimov 1985; Ting 1985) and this step is rate-determining, irreversible, and discriminates against ^{13}C causing fractionation (O'Leary 1988). This carboxylation step leads to 2 molecules of phosphoglycerate containing 3 carbons and hence the term "C3" given to plants implementing the Calvin cycle (Krueger and Reesman 1982; Galimov 1985; O'Leary 1988). The Hatch-Slack cycle, however, fixes atmospheric CO_2 via carboxylation, aided by the enzyme phosphoenolpyruvate (PEP), carboxylase into oxaloacetic acid that is then converted into molecules of either malate or aspartate, both of which contain 4 carbons, and hence the term "C4" used for these plants (Krueger and Reesman 1982; Galimov 1985; O'Leary 1988). The molecule is then relocated to the bundle sheath cells where it will become a molecule of pyruvate (Galimov 1985) and a molecule of CO_2 is released (Krueger and Reesman 1982; Galimov 1985; O'Leary 1988). The CO_2 molecule is then incorporated into the Calvin cycle. As the limiting step in this photosynthetic cycle is the diffusion through the stomata (O'Leary 1988), and the carboxylation reaction via PEP carboxylase is efficient and irreversible, the molecules generated do not vary greatly from the atmospheric CO_2 (Krueger and Reesman 1982; O'Leary 1988); hence, fractionation is not as great as for the Calvin cycle. As a consequence, the $\delta^{13}C$ values for 80% to 90% of C3 plants are between −24‰ to −34‰ (Krueger and Reesman 1982), whereas those for C4 plants are between −10‰ and −16‰ (Winkler 1984). CAM plants (vanilla, pineapple, and cacti) tend to have ratios (−12‰ to −30‰, Winkler 1984), similar to C3 and C4 plants as they are able to utilize both forms of photosynthetic pathways depending on the conditions they are subjected to (Osmond and others 1973).

Secondary carbon metabolism induces kinetic isotope effects due to metabolic and branching reactions, including the extent of reversibility of these reactions (Schmidt and Kexel 1997; Schmidt 1999). These metabolism reactions tend to cause further fractionation, and therefore, their products are generally more depleted in $\delta^{13}C$, for example, the reaction of carbohydrates to proteins (2‰ depletion of $\delta^{13}C$) and to lipids (5‰ depletion of $\delta^{13}C$) (DeNiro and Epstein 1977; Winkler 1984).

Factors such as temperature, water availability, relative humidity, CO_2 concentration, fertilization, salinity, light intensity, and photorespiration (O'Leary 1981) also impact the $\delta^{13}C$ value of plants and their products because they have an effect on stomatal aperture and CO_2 diffusion into the leaf (Farquhar and others 1982; O'Leary 1995; Scheidegger and others 2000).

The fractionation processes described above mean that $\delta^{13}C$ can be used to detect authenticity of food when adulterated with compounds derived from plants with a different photosynthetic cycle (such as unpermitted addition of cane sugar to wine). When the adulteration has been made with compounds derived by plants with the same photosynthetic cycle, adulteration is more difficult to detect. One approach is to use the isotope ratio of a compound of known origin, which can be used as standard reference. Further challenges arise from the use of synthetic compounds as adulterants. Such compounds, derived from coal and petroleum originating from reservoirs of carbon formed from ancient C3 plants, have $\delta^{13}C$ generally ranging from −25‰ to −30‰ very similar

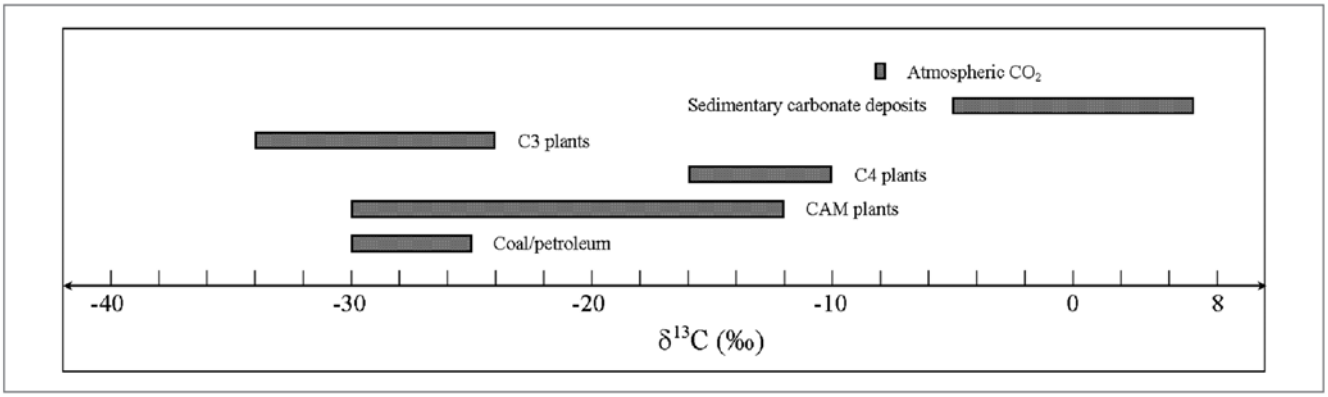

Figure 2–Carbon isotope ratio natural abundance ranges (ratio ranges are based on various literature data presented in this review).

to those of C3 plants today (Krueger and Reesman 1982). These synthetic compounds may be used as food additives to enhance or replace naturally derived compounds (Krueger and Reesman 1982). Detection of this substitution by isotope ratio monitoring may be difficult and may require the use of complementary analytical techniques.

Nitrogen

The natural environmental cycle of nitrogen is slightly more complicated than for carbon as it progresses through the atmosphere, plant and microbes and can occur in reduced and oxidized forms, and there are more factors causing isotope fractionation. Atmospheric nitrogen is utilized as an international standard for $\delta^{15}N$ measurements, since isotope ^{15}N in the air remains constant with a natural abundance of 0.366% (Ehleringer and Rundel 1989). Nitrogen fixation by biological or abiotic processes incurs a slight fractionation when atmospheric N_2 is converted to ammonia. This process occurs through bacteria, physical processes that produce high temperatures such as lightning and fire, and also human activities including the use of fertilizers and the production of energy (Fogel and Cifuentes 1993) (Figure 3).

The $\delta^{15}N$ of soil can vary significantly on the basis of the processes occurring in the soil such as mineralization, nitrification, volatization, and nitrate reduction or denitrification, each incurring an isotopic fractionation (Mariotti and others 1981; Mariotti and others 1982; Choi and others 2006; Tang and Maggi 2012). The extent of these processes depends on factors such as soil depth, vegetation type, and climate, and therefore, the ^{15}N natural isotope abundance ranges vary (Persson and Wiren 1995; Malchair and others 2010). For cropped soils, the main factor affecting $\delta^{15}N$ is fertilizing practices. Synthetic fertilizers produced by the Haber process have ^{15}N values in the range of −6‰ to +6‰, while organic fertilizers (enriched in ^{15}N) have ^{15}N values in the range of +0.6‰ to 36.7‰ (manure between +10‰ and +25‰) (Bateman and Kelly 2007).

Plants display $\delta^{15}N$ values that are linked to ammonia and nitrates in the soils and also associated with isotope effects due to the assimilation of these compounds into the plant (Werner and Schmidt 2002). Factors such as proximity to sea and water stress can induce enrichment in ^{15}N (Heaton 1987). Leguminous and nitrogen-fixing plants are an individual case as they can fix nitrogen directly from the air, and therefore have $\delta^{15}N$ values around 0‰ (Martinelli and others 1992). Organic fertilizers such as manure also affect δ15N values as the nitrogen in the manure has been through various trophic levels (that is where an organism occurs in the food chain, for example, plant, herbivore, microbe, and so on).

The $\delta^{15}N$ analysis can be used for food authenticity, for example, to verify the use of organic instead of synthetic fertilizers, as required by law for organic food production. $\delta^{15}N$ analysis of bulk samples has proven to be useful in the differentiation of organic and conventional produce such as potato tubers (Camin and others 2007), wheat (Schmidt and others 2005; Senbayram and others 2008), carrots (Bateman and others 2005), tomatoes (Schmidt and others 2005; Bateman and others 2007), oranges (Rapisarda and others 2010; Camin and others 2011), peaches and strawberries (Camin and others 2011), though most of these techniques require, for absolute authentication, other complementary techniques. This application of $\delta^{15}N$ could be improved by analyzing, using GC-C-IRMS, single components, or an array of components, such as amino acids.

Isotopic fractionation within natural systems occurs via a multitude of processes and it provides an isotopic fingerprint for each biological system. Analytical tools are vital in investigating such systems and certifying these fingerprints. The GC-C-IRMS is one such tool that has the potential to be implemented in fingerprinting.

Application of GC-C-IRMS in foods and beverages

GC-C-IRMS is increasingly being used within the food and beverage industries to assure product authenticity and curb product adulteration (Takeoka and Ebeler 2011). GC-C-IRMS can measure separately the ratio of stable isotopes of C and N of different compounds within a single matrix; this technique is known as compound-specific isotope analysis (CSIA). CSIA may be advantageous in detecting adulteration compared to elemental analyzer-isotope ratio mass spectrometry (EA-IRMS), which is utilized in bulk sample analysis. In EA-IRMS, the entire sample is combusted, not just a single component as in CSIA, and hence compound-specific data are lost in EA-IRMS.

CSIA can focus on a single compound within a matrix (such as a fatty acid in olive oil) that provides more detailed information on the final composition of the sample. In addition, the CSIA approach can be extended to monitor classes of molecules that may be of interest, for example, terpenes. As mentioned above, there are many ways in which the authenticity of foods or beverages may be compromised by substitution or addition of specific compounds and CSIA is a powerful technique in addressing these.

As well as detecting single compounds that may indicate food/beverage fraud, it is also useful to consider the isotopic authenticity range of a particular product. This range is determined

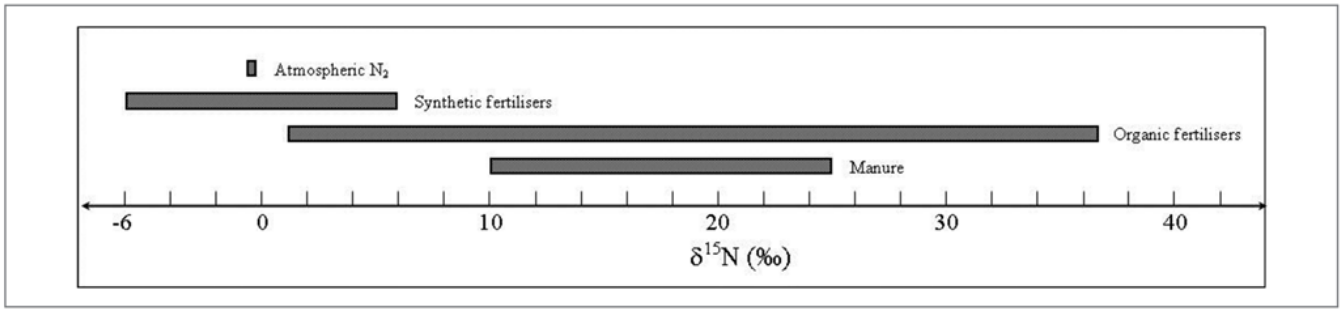

Figure 3–Nitrogen isotope ratio natural abundance ranges (ratio ranges are based on various literature data presented in this review).

by measuring a large number of authentic samples for compounds found specifically within that product, providing minimum, maximum, and/or 95% of variability (a range) per compound. Compilation of the compound-specific data facilitates the production of a profile for the specific product. To determine whether a sample is authentic, the sample must be analyzed and its values compared to the isotopic authenticity range previously established. If the sample has 1 or more compounds outside the "range" then that sample may be adulterated. Often an internal isotopic standard (i-IST) is used as part of the isotopic fingerprint to remove the underlying and predominant fractionation caused by CO_2 fixation in plants, allowing for the fractionation due to secondary metabolic synthesis to be revealed (Braunsdorf and others 1993b). The compound chosen to be an i-IST requires the following: be in sufficient quantity for analysis; be genuine; no isotopic fractionation should occur during sample preparation or analysis; should be biogenetically related to the other compounds in question; should not be sensorially important; and also be stable during storage (Braunsdorf and others 1993b). Thus, GC-C-IRMS in conjunction with an i-IST can be used to study plant metabolic processes in their own right, as well as being applied to the detection of food/beverage fraud.

Another consideration for the implementation of GC-C-IRMS is the potential for fractionation to occur during the sample preparation stage of the analysis. Schumacher and others (1995) investigated fractionation during different extraction procedures (dynamic headspace, simultaneous distillation, and solvent extraction) for the analysis of volatile components from strawberries. Also studied were the effects of the additives ammonium sulfate and calcium chloride (typically added to the strawberry pulp during crushing to avoid enzyme activity) on the volatile component, with ammonium sulfate showing slight fractionation for 1 of the internal standards. Schumacher and others (1995) were able to provide $\delta^{13}C$ data for selected strawberry volatiles such as butanoic acid, 2-methylbutanoic acid, hexanoic acid, γ-decalactone, and γ-dodecalactone. Further examples of the application of GC-C-IRMS to traceability and authenticity of foodstuffs, beverages, and related plant metabolic processes are discussed below and are summarized in Table 2. In the following discussion, we report some data for δ^2H and $\delta^{18}O$ compound-specific analysis using GC-P-IRMS (Sessions 2006), considering only the studies when this technique was combined with GC-C-IRMS. The δ^2H and $\delta^{18}O$ values are expressed in per mil relative to the international reference material VSMOW (Vienna Standard Mean Ocean Water) (Coplen 1996).

Aroma compounds in fruit

Seasonal variation in rainfall, amount of sunlight, and so on, can cause the quality and/or yield of food products to vary greatly (Siebert 2002). The addition of flavor and aroma compounds is widely implemented to achieve the desired food and beverage products that will sell on the market. Natural flavors are often expensive to produce in comparison to flavors produced synthetically and could lead to possible fraudulent activity to increase profits to the producer. To guard and inform consumers, labeling requirements were adopted for food products (as stated previously) to include more specific labeling to assign the use of flavors in foodstuffs as "natural" products, as opposed to synthetic, artificial, or nature-identical (the latter term can mean compounds derived from microorganisms or enzyme treatments of natural products). For example, vanillin can be derived from a plant, made synthetically, or even biotechnologically derived (Greule and others 2010). It would appear from the literature that, as yet, there is no standard terminology for the different ways flavor/aroma compounds can be derived and as such, we will use the terminology as reported in the original literature.

Discrimination between natural and synthetic aromas

Due to the requirement for baseline separation in GC-C-IRMS, generally, the compounds selected for study are characteristic or key aroma compounds of the fruit or fruit product that can be isolated chromatographically. In the case of pear, 7 compounds were investigated by gas chromatography-combustion/pyrolysis-isotope ratio mass spectrometry (GC-C/P-IRMS) to evaluate $\delta^{13}C$ (combustion) and δ^2H (pyrolysis), namely, 1-butanol, hexyl acetate, 1-hexanol, butyl acetate, methyl *E,Z*-2,4-decadienoate, ethyl *E,Z*-2,4-decadienoate, and ethyl *E,E*-2,4-decadienoate (Kahle and others 2005). These compounds were determined in a variety of matrices such as juices, brandies, and baby food, and additionally synthetic and "natural" (biotechnologically acquired) compounds were also analyzed. The synthetic samples ($\delta^{13}C$: −25.0‰ to −31.3‰ δ^2H −63‰ to −159‰) could be distinguished from "natural samples" ($\delta^{13}C$: −12.4‰ to −42.4‰ δ^2H −140‰ to −346‰), fruit ($\delta^{13}C$: −29.7‰ to −42.7‰ δ^2H: −48‰ to −255‰), and fruit products ($\delta^{13}C$: −27.2‰ to −41.9‰ δ^2H −46‰ to −262‰), thus enabling this technique to be used as an authenticity tool.

Similar considerations and methodology have been applied to the study of typical aroma compounds of pineapple fruits including methyl hexanoate, ethyl hexanoate, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, and 2,5-dimethyl-4-methoxy-3[2H]-furanone (Preston and others 2003). The C and H isotopic ratios of these compounds were measured in fruits (CAM plant; $\delta^{13}C$: −12.6‰ to −28.6‰; δ^2H: −118‰ to −263‰), recovery aromas/water phases ($\delta^{13}C$: −15‰ to −29.4‰; δ^2H: −150‰ to −265‰) and "natural" ($\delta^{13}C$: −11.8‰ to −32.2‰; δ^2H: −242‰ to −323‰) and synthetic ($\delta^{13}C$: −22.8‰ to −35.9‰; δ^2H: −49‰ to −163‰) versions of the compounds.

Table 2–Food and beverage analysis by GC-C-IRMS.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Fruit					
Pear	Natural and synthetic aromas	1-Butanol, hexyl acetate, 1-hexanol, butyl acetate, methyl *E,Z*-2,4-decadienoate, ethyl *E,Z*-2,4-decadienoate, ethyl *E,E*-2,4-decadienoate	Naturally derived and synthetic compounds could be distinguished; authenticity range for various products (fruit, juice, brandy, and baby food)	$\delta^{13}C$, $\delta^{2}H$	Kahle and others (2005)
Pineapple	Natural and synthetic aromas	Methyl hexanoate, ethyl hexanoate, 2-methylbutanoate, ethyl 2-methylbutanoate, 2,5-dimethyl-4-methoxy-3[2H]-furanone	GC-C/P-IRMS could distinguish natural and synthetic aromas	$\delta^{13}C$, $\delta^{2}H$	Preston and others (2003)
Raspberry	Natural and synthetic aromas	*E*-$\alpha(\beta)$-Ionone	MDGC-C/P-IRMS could distinguish the naturally derived compound from the synthetic one	$\delta^{13}C$, $\delta^{2}H$	Sewenig and others (2005)
Strawberry, raspberry, pineapple, passionfruit, plum, coconut	Natural and synthetic	γ-Decalactone	Enantiomeric distribution and isotopic ratios in isolation were not capable of proving the genuineness of a flavour but when combined and with more isotopic data this could be achieved	$\delta^{13}C$, bulk	Nitz and others (1991)
Apricot, blueberry, guava, mango, passionfruit, peach, pineapple, plum, strawberry	Natural and synthetic	γ-Decalactone	MDGC and HRGC-IRMS were used for analysis. Synthetic compounds, microbial compounds, stone fruits and strawberry could be differentiated based on the isotopic ratio of γ-decalactone for each	$\delta^{13}C$, bulk	Bernreuther and others (1990)
Cactus pear (*Opuntia ficus indica*)	Authenticity assessment	1-Hexanol, *E*-2-hexenol, *E*-2-nonenol, *E,Z*-2,6-nonadienol	The isotopic results clearly showed that these compounds originated from CAM plants, which could be useful for flavor authenticity	$\delta^{13}C$	Weckerle and others (2001)
Blackberry,	Authenticity assessment	Heptan-2-ol, *trans*-linalool oxide (furanoids)	Enantioselctive analysis and MDGC-C/P-IRMS in combination could be used for authenticity assessment. The jams, jelly, juices, and yogurts tested were not in the limit of quantitation for IRMS analysis due to insufficient quantities of the blackberry constituents	$\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$	Greule and Mosandl (2008)
Raspberry, *Litsea cubeba*, lemongrass	Authenticity assessment	α-Ionone, β-ionone, α-ionol	Synthetic compounds were differentiated from the natural compounds in raspberry. The compounds synthesized from C4 plants had a more enriched isotopic ratio	$\delta^{13}C$, $\delta^{2}H$, bulk	del Mar Caja and others (2007)

(*Continued*)

Table 2–Continued.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Strawberry	Origin of aromas—from strawberry or other fruit	Methyl butanoate, ethyl butanoate, *E*-2-hexenal, methyl hexanoate, butyl butanoate, ethyl hexanoate, hexyl acetate, linalool, hexyl butanoate, octyl isovalerate, γ-decalactone, and octyl hexanoate	Volatiles from different sources could be distinguished as natural, synthetic (nature-identical) or artificial strawberry	$\delta^{13}C$	Schipilliti and others (2011b)
Banana	Fruit origin and ripening stage	Pentan-2-one, isobutyl acetate, 2-methyl propan-1-ol, isoamyl acetate, isobutyl butyrate, isoamyl alcohol, isoamyl butyrate, isoamyl isovalerate, eugenol	GC, GC-MS, and GC-C-IRMS used to determine that there are different aroma compositions for the different ripening stages of the fruit. Isotopes can be used to authenticate the fruit aromas and determine their biosynthetic pathways	$\delta^{13}C$	Salmon and others (1996)
Peach (*Prunus persica* var. *persica*), apricot (*Prunus armeniaca*), and nectarine (*Prunus persica* var. *nectarina*)	Natural, synthetic, and biotechnological aromas	γ-Decalactone and δ-decalactone	GC-C/P-IRMS enabled differentiation of fruit-derived compounds from the synthetic and biotechnological-derived compounds	$\delta^{13}C$, $\delta^{2}H$	Tamura and others (2005)
Apple	To determine if processing can modify the isotopic ratios of apple juice aroma; to preserve the qualitative apple aroma throughout production	*E*-2-Hexenal, 1-hexanol, *E*-2-hexenol	The C isotopic ratios for these aroma compounds remained constant though for H there were slight isotopic changes (not great enough effect to influence authenticity assessments)	$\delta^{13}C$, $\delta^{2}H$	Elss and others (2006)
Acerola berries, camu-camu fruits (tropical fruits)	Determination of origin	Ascorbic acid	A combination of ^{13}C SNIF-NMR, EA-IRMS, GC-C-IRMS, and multivariate analysis was used to determine the origin of ascorbic acid, either natural or industrial	$\delta^{13}C$, bulk	Albertino and others (2009)
Strawberry	Method development	Butanoic acid, 2-methylbutanoic acid, hexanoic acid, γ-decalactone, γ-dodecalactone	A method was developed for strawberry volatiles without fractionation	$\delta^{13}C$	Schumacher and others (1995)
Essential oils					
Fennel and anise oils	Natural and synthetic	*trans*-Anethole	Combination of GC-C- and GC-P-IRMS established an authenticity range for naturally derived *trans*-anethole	$\delta^{13}C$, $\delta^{2}H$	Bilke and others (2002)
Lemon essential oil, *Citrus limon* (L.) Burm.	Natural and synthetic	α-Pinene, β-pinene, limonene, α-terpineol, neral, geranial, neryl acetate, geranyl acetate, β-caryophyllene, *trans*-α-bergamotene, β-bisabolene, norbornanol, campherenol, α-bisabolol	GC-C-IRMS together with Es-GC, GC-FID, and CD can be used in combination for the control of authenticity	$\delta^{13}C$	Schipilliti and others (2012)
Mandarin essential oil (*Citrus reticulata* Blanco)	Natural and synthetic, blending of citrus products, origin assignment	Methyl *N*-methylanthranilate, methyl anthranilate	GC-C-IRMS established that essential oils from Italy have different isotopic ratios to Greece, Brazil, and Argentina	$\delta^{13}C$, $\delta^{15}N$	Faulhaber and others (1997a)
Orange oil	Natural and synthetic	Octanal, nonanal, decanal, dodecanal, neral, geranial	Differentiation of synthetic compounds from natural was possible	$\delta^{13}C$	Braunsdorf and others (1992)

(*Continued*)

Table 2–Continued.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Lemon oil	Natural and synthetic	β-Pinene, limonene, β-terpinene, nerol, geraniol, neral, geranial, neryl acetate, geranyl acetate	Lemon oils could be authenticated using GC-C-IRMS with the use of an internal standard against neral and geranial	$\delta^{13}C$	Braunsdorf and others (1993b)
Melissa, lemon (*Citrus limon*), lemongrass, citronella, *Litsea cubeba*, *Lippia citriodora*, lemon myrtle (*Backhousia citriodora*), and lemon gum (*Eucalyptus citriodora*)	Natural and synthetic	Citral (geranial + neral), citronellal	Combination of GC-FID for enantioselective analysis and GC-C/P-IRMS was essential for the determination of the adulteration of essential oils with cheaper oils or synthetic additions. PCA was also a useful statistical tool	$\delta^{13}C$, $\delta^{2}H$	Nhu-Trang and others (2006)
Lemon balm (*Melissa officinalis* L.)	Natural and synthetic	Citral (geranial and neral), citronellal, β-caryophyllene, germacrene D, caryophyllene oxide	Not all differences between natural and synthetic compounds established, but 4 of the lemon balms were shown to be citronella oils	$\delta^{13}C$, bulk	Wagner and others (2008)
Coriander (*Coriandrum sativum* L.)	Natural and synthetic	Limonene, γ-terpinene, *p*-cymene, linalool, geraniol, myrcene, geranyl acetate, β-pinene, camphene, terpinolene, sabinene	An isotopic fingerprint was determined with use of an i-IST. Also, enantio-GC-IRMS was used for the isomers of linalool as an additional tool that could be used for authenticity assessment	$\delta^{13}C$	Frank and others (1995)
Cinnamon oil, *C. ceylanicum* (ceylon), *C. cassia* (cassia), and *C. burmanii* (cassia vera)	Natural and synthetic	Cinnamaldehyde	The combination of GC-C/P-IRMS enabled the establishment of an authenticity technique to differentiate cinnamaldehyde of different origin	$\delta^{13}C$, $\delta^{2}H$	Sewenig and others (2003)
Basil extracts, (*E*)-methyl cinnamate (various sources), commercial strawberry, and blueberry aromas	Natural and synthetic	(*E*)-Methyl cinnamate, methanol and cinnamic acid	The differences between the isotopic data were great enough to allow for differentiation between synthetic and natural alcohol (methanol)	$\delta^{13}C$, $\delta^{2}H$	Fink and others (2004)
Estragole; methyl eugenol; tarragon oil; sweet basil oil; pimento oil; laurel leaf oil; *Tagetes lucida* oil	Natural and synthetic	Estragole, methyl eugenol	The methods developed were not sufficient enough to determine isotopic differences between natural and synthetic samples due to the synthetic samples being produced from natural starting products	$\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$	Ruff and others (2002)
Lavender oil	Natural and synthetic	Linalool, linalyl acetate	A combination of techniques was necessary to determine authenticity of lavender oils; multielement/multicomponent IRMS and (GC) enantioselective analysis	$\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$	Jung and others (2005)
Vanilla planifolia, *Vanilla tahitensis*	Natural and synthetic	Vanillin, 4-hydroxybenzyl alcohol, vanillic acid, 4-hydroxybenzaldehyde, anisic alcohol, anisic acid, and 4-hydroxybenzoic acid	GC-C IRMS profiles could distinguish different vanilla species and the origin of synthetic vanillin	$\delta^{13}C$	Kaunzinger and others (1997)

(*Continued*)

Table 2–Continued.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Vanillin	Natural and synthetic, origin determination	Vanillin	Simultaneous on-line analysis of vanillin using GC-C/P-IRMS. Synthetic and biotechnologically derived vanillin and natural vanillin could be differentiated.	$\delta^{13}C$, $\delta^{18}O$	Hener and others (1998)
Vanilla ice cream	Natural and synthetic aromas	Vanillin	There was no isotopic difference between the vanillin in the ice cream and the vanillin flavoring used	$\delta^{13}C$	Fayet and others (1995)
Vanilla, *V. Planifolia, V. tahitensis, V. pompon*	Natural and synthetic	Vanillin	A reliable and rapid method for authentication of vanillin	$\delta^{13}C$, $\delta^{2}H$	Greule and others (2010)
Vanilla, mint (*Mentha arvensis*)	Natural and synthetic	Vanillin, *cis*-3-hexenol	The method developed allowed for small quantities to be measured without purification and therefore no fractionation. The method was able to determine natural and synthetic aromas	$\delta^{13}C$	Breas and others (1994)
Mandarin essential oil (*Citrus reticulata* Blanco)	Develop an authenticity profile	α-Sinensal, limonene, γ-terpinene, α-thujene, β-pinene/sabinene, myrcene, terpinolene, MNM, linalool, and octanal	An authenticity profile was developed, which could be used to evaluate the authenticity of commercial mandarin oils	$\delta^{13}C$	Faulhaber and others (1997b)
Mandarin essential oil	Authenticity assessment	α-Thujene, α-pinene, β-pinene, myrcene, limonene, γ-terpinene, terpinolene, terpinen-4-ol, α-terpineol, decanal, thymol, MNM, α-farnesene, and α-sinensal	GC-C-IRMS together with Es-GC, GC-FID, and HPLC are a useful combination for the control of adulteration; GC-C-IRMS especially for the detection of the addition of thymol, MNM, and α-sinensal to commercial products	$\delta^{13}C$	Schipilliti and others (2010)
Neroli oil, petitgrain oil, bergamot oil	Authenticity assessment	α-Pinene, β-pinene/sabinene, limonene, myrcene, linalool, linalyl acetate, neryl acetate, caryophyllene, γ-terpinene	The combination of data from enantio-MDGC and GC-C-IRMS is useful in the determination of adulterated citrus oils.	$\delta^{13}C$	Mosandl and Juchelka (1997)
Lemon petitgrain oil, mandarin petitgrain oil	Composition, authenticity assessment	α-Thujene, α-pinene, β-pinene, myrcene, α-terpineol, neral, geranial, neryl acetate, geranyl acetate, β-caryophyllene, MNM	The isotopic ratios determined provided a terpene fingerprint for these oils. The results generated could be used to defend the authenticity of these oils	$\delta^{13}C$	Schipilliti and others (2013b)
Bergamot oil, *Citrus bergamia* Risso	Authenticity assessment	Linalool, linalyl acetate	Able to detect adulterants, the nature of the adulterant and also determine geographic origin using an isotopic authenticity range	$\delta^{13}C$	Schipilliti and others (2011a)
Bergamot oil	Authenticity assessment and characterization of cold-pressed and processed oils	α-Thujene, α-pinene, β-pinene, myrcene, limonene, γ-terpinene, linalool, linalyl acetate, α-terpinyl acetate, neryl acetate, geranyl acetate, β-caryophyllene, *trans*-α-bergamotene, β-bisabolene, norbornanol, campherenol, α-bisabolol, nootkatone	An authenticity range was developed and used to prove genuineness of the oils. Characterization of these oils was done using a variety of techniques, GC-FID, GC-MS, GC-C-IRMS, Es-GC, MDGC, HPLC, and HPLC-MS-ion trap-TOF	$\delta^{13}C$	Dugo and others (2012)

(*Continued*)

Table 2–Continued.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Egyptian Neroli oil (*Citrus aurantium* L.)	Authenticity assessment, geographic origin	β-Pinene, myrcene, limonene, linalool, terpinen-4-ol, α-terpineol, nerol, neryl acetate, geranyl acetate, (*E*)-caryphyllene, (*E*)-nerolidol, (2*E*,6*E*)-farnesol	Along with data obtained from GC-FID, GC-MS, Es-GC, and HPLC, GC-IRMS was used to develop an isotopic fingerprint with small variation and therefore could be used to determine authenticity and geographic origin	$\delta^{13}C$	Bonaccorsi and others (2011)
Peppermint oil (*M. piperita*)	Authenticity assessment	Isomenthone, menthyl acetate, menthone, menthol, 1,8-cineole	i-IST enabled an isotopic fingerprint of authentic peppermint oil. Addition of menthyl acetate was able to be detected in adulterated samples. The use of a chiral column enabled isotopic ratio determination of the isomers of menthyl acetate and also enabled determination of the amount of true menthyl acetate in the adulterated sample	$\delta^{13}C$	Faber and others (1995)
Lime essential oils (*C. aurantifolia* Swingle, and *C. latifolia* Tanaka)	Authenticity assessment	α-Pinene, β-pinene, limonene, α-terpineol, neral, geranial, β-caryophyllene, *trans*-α-bergamotene, germacrene B	MD-GC and GC-C-IRMS provided a more reliable approach to detect adulteration. Use of an i-IST helped with determining the authenticity ranges for each oil	$\delta^{13}C$	Bonaccorsi and others (2012)
Dill, *Anethum graveolens* L.	Biochemical pathways in monoterpene synthesis	Limonene, carvone, dill ether, α-/β-phellandrene	Pathways for monoterpenes were identified via GC-C-IRMS	$\delta^{13}C$	Faber and others (1997)
Orange oil	Effect of technological processing	Octanal, nonanal, decanal, dodecanal	Isotopic ratio of C was not affected/influenced by sample clean-up during technological processing	$\delta^{13}C$	Braunsdorf and others (1993a)
Edible oils and fats					
Olive oils, food fats	Authenticity assessment, origin assessment	Glycerol, fatty alcohols	The developed method serves as a tool for assessment of origin for food fats and oils. An authenticity range was developed for samples of C3 origin and glycerol from fats and oils was significantly different to synthetic glycerol	$\delta^{13}C$, $\delta^{18}O$	Jung and others (2007)
Olive oil	Authenticity assessment	Palmitic acid, oleic acid	Bulk and fatty acids can be used to identify oil source. Blending may also be detected in combination with GC-MS data	$\delta^{13}C$, bulk	Spangenberg and others (1998)
Maize oil	Authenticity assessment	Palmitic acid, oleic acid, linoleic acid, sterols, tocopherols	The combination of isotopic ratios for fatty acids and minor components (sterols and tocopherols) allowed adulteration of maize oil to be detected at 5% (mass/mass)	$\delta^{13}C$	Mottram and others (2003)
Maize oil, rapeseed oil, groundnut oils	Authenticity assessment	Palmitic acid, oleic acid, linoleic acid	Detection of adulterant of above 5% in maize oil was possible	$\delta^{13}C$	Woodbury and others (1995)
Maize oil and other vegetable oils	Authenticity assessment	Palmitic acid, oleic acid, linoleic acid	The data generated in this study can help to identify adulterations to maize oil using the major fatty acids found in this oil	$\delta^{13}C$	Woodbury and others (1998a)
Groundnut oil, rapeseed oil, palm oil, sunflower oil	Authenticity assessment	Palmitic acid, stearic acid, oleic acid, linoleic acid	Sole use of fatty acids to determine adulterations in single seed oils is not possible but could be used in conjunction with other techniques	$\delta^{13}C$	Kelly and others (1997)
Olive oil, pumpkin seed oil, sunflower oil, soybean oil, sesame oil, maize oil, and rapeseed oil	Authenticity assessment	Palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid	Vegetable oils can be classified using the isotopic ratios of the bulk oil, the fatty acids, and also the composition of the fatty acids	$\delta^{13}C$, bulk	Spangenberg and Ogrinc (2001)

(*Continued*)

Table 2–Continued.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Cooking oils, commercial vegetable oils, animal oils, illegal swill-cooked oils	Authenticity assessment	Myristic acid, palmitic acid, stearic acid	Isotopic ratios can determine the origin of the oil, though the method is not 100% reliable as there is some overlap of the isotopic ranges	δ^{13}C, bulk	Liu and others (2012)
Cocoa butter, Cocoa butter counterparts	Authenticity assessment	Bulk fat, stearic acid, palmitic acid, oleic acid	Cocoa butter and its counterparts can be differentiated based on their isotopic ratios (bulk and fatty acids) and blending of more than 15% of cocoa butter counterparts to cocoa butter is able to be detected	δ^{13}C, bulk	Spangenberg and Dionisi (2001)
Olive oil, hazelnut oil, sunflower oil, soybean oil, maize oil	Authenticity assessment	Palmitic acid, oleic acid, linoleic acid	Information on geographical, botanical, and temporal characteristics of the oils can form the basis for an authenticity check	δ^{13}C	Royer and others (1999)
Olive oil	Authenticity assessment	Phytol, geranyl geraniol, citrostadienol, docosanol, tetracosanol, hexacosanol	The analysis is able to detect a 3% adulteration of olive oil with pomace oil based on the isotopic ratio of the aliphatic alcohols	δ^{13}C	Angerosa and others (1997)
Rapeseed oil (*Brassica napus*), flax and false flax oils, poppy, sunflower, and safflower oils	Adulteration with refined or less expensive oils, geographical origin	Palmitic acid, oleic acid, α-linoleic acid, linoleic acid	The fatty acid carbon ratios were different for the rape, flax, and poppy oils, and differences in ratios were also observed within each species	δ^{13}C, bulk	Richter and others (2010)
Camelina sativa Oil	Adulteration with refined or less expensive oils, geographical origin	Fatty acids; $C_{16:0}$, $C_{18:0}$, $C_{18:1n9}$, $C_{18:1n7}$, $C_{18:2n6}$, $C_{18:3n3}$, $C_{20:0}$, $C_{20:1n9}$, $C_{20:3n3}$, $C_{22:1n9}$	PCA was able to separate the oil samples from different continents; the correlation between $\delta^{13}C_{18:2n6}$ and $\delta^{13}C_{18:3n3}$ would be able to show a suspected impurity or adulteration	δ^{13}C, bulk	Hrastar and others (2009)
Olive oil	Geographical origin	Methyl palmitoleate, methyl palmitate, methyl oleate	Use of 3 FAME peaks enabled greater differentiation between samples of different geographic origin compared to using the isotopic ratios of the bulk oils	δ^{13}C	Baum and others (2010)
Beverages					
Coffee beans (*Coffea arabica* L. and *Coffea canephora* var. *robusta*)	Natural and synthetic	Alkylpyrazines such as 2-ethyl-3-methylpyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine 2,6-dimethylpyrazine, 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine	GC-C/P-IRMS. For some of the alkylpyrazines, the isotopic values of the natural compounds were differentiated from the synthetic ones	δ^{15}N, δ^{2}H	Richling and others (2005)
Black and green tea (*Camellia sinensis*), Rooibos tea (*Aspalathus linearis*)	Natural and synthetic	(±)-Dihydroactinidiolide	The synthetic and natural educts reflected their origin in their isotopic ratios. The Rooibos tea was shown to be slightly more enriched than the black and green teas allowing for differentiation	δ^{13}C, δ^{2}H	del Mar Caja and others (2009)
Wine	Natural and synthetic	Ethanol, 2-methylpropan-1-ol, 2- and 3-methylbutan-1-ol, butan-2,3-diol, and 2-phenyl-1-ethanol	The developed method could be used to determine the addition of exogenous alcohols	δ^{13}C	Spitzke and Fauhl-Hassek (2010)

(*Continued*)

Table 2–Continued.

Food and beverage	Purpose of study	Compounds	Outcome	Isotopic ratio	Reference
Apple juice, sugar syrups	Authenticity assessment	Hexamethylenetetramine	The combination of $\delta^{13}C$ and $\delta^{2}H$ data was proven to be reliable in detecting the addition of commercial beet and cane sugar syrups to the apple juice	$\delta^{13}C$, $\delta^{2}H$	Kelly and others (2003)
Wine	Authenticity assessment	Glycerol	GC-C/P-IRMS was able to distinguish wine grape glycerol from synthetic glycerol and glycerol produced from cane sugar. Also depending on amount and origin, addition of foreign glycerol detection is possible. Origin assessment is possible too	$\delta^{13}C$, $\delta^{18}O$	Jung and others (2006)
Citrus liquers; limoncello, bargamino, mandarinetto	Authenticity assessment	α-Pinene, β-pinene, myrcene, limonene, γ-terpinene, linalool, terpinene-4-ol, α-terpineol, decanal, neral, geranial, linalyl acetate, neryl acetate, MNM, β-caryophyllene, trans-α-bergamotene, β-bisabolene,	Isotopic ratios Es-GC-C-IRMS was able to prove genuineness of the citrus liquers and could be used to prove adulteration	$\delta^{13}C$	Schipilliti and others(2013a)
Bottled waters; sparkling, still, flavored	Authenticity assessment, origin determination	Dissolved inorganic carbon (dissolved CO_2, carbonic acid, bicarbonate, carbonate)	Bottled waters from various origins, bottling and processing procedures could be differentiated	$\delta^{13}C$	Brencic and Vreca (2007)
Black Ceylon, Assam, and Darjeeling teas	Adulteration detection implementing isotopic fingerprinting	Linalool, *trans*-2-hexenal, *cis*-3-hexenol, *cis*-linalool oxide (fur.), *trans*-linalool oxide (fur.), methyl salicylate, geraniol	Adulteration of teas with methyl salicylate was detected by use of an internal standard and isotopic fingerprinting	$\delta^{13}C$	Weinert and others (1999)
Wine	Endogenous and exogenous glycerol and characterization	Glycerol, ethanol	GC-C-IRMS enabled correlations between ethanol and glycerol but the parameters may not be enough to determine geographical origin or adulteration	$\delta^{13}C$, bulk	Calderone and others (2004b)
Sparkling wine, beer, carbonated water, carbonated drinks	Origin of CO_2, authenticity assessment	CO_2, ethanol	Discrimination between natural C3- and C4-derived CO_2 and technological CO_2 was possible, but may not be enough for authenticity assessment	$\delta^{13}C$, $\delta^{2}H$	Caledrone and others (2007)
Distillates from fermented carbohydrates, distillates of fermented apple and grape juice, apple products	Biosynthetic pathways	2-Methylbutanol, 3-methylbutanol	Determined that C3 and C4 products were able to be differentiated	$\delta^{13}C$	Schumacher and others (1999)
Vinegars					
Japanese vinegars	Origin determination, control of quality	Acetic acid	Intramolecular carbon isotope distribution could be used to determine origin of acetic acid in food.	$\delta^{13}C$	Hattori and others (2011)
Rice vinegar, apple vinegar, grape vinegar, tomato vinegar, pineapple vinegar, wheat vinegar, lychee vinegar	Botanical origin	Acetic acid	HS-SPME combined with GC high-temperature conversion-C-IRMS enabled the acetic acid to be discriminated by botanical origin	$\delta^{13}C$, $\delta^{2}H$	Hattori and others (2010)

The authors concluded that GC-C/P-IRMS may be a useful tool for recognizing authenticity of pineapple products, but would require a larger data bank of samples, including those from more regions such as the Philippines, Indonesia, and Brazil, to be used definitively.

Prunus fruits such as peaches, apricots, and nectarines were analyzed by Tamura and others (2005) for the isotopic ratios of γ-decalactone and δ-decalactone (δ^{13}C ranges of γ-decalactone and δ-decalactone for fruits: −34.0‰ to −38.4‰; "natural": −27.7‰ to −30.1‰; and synthetic: −27.4‰ to −28.3‰ and δ^2H ranges of both compounds for fruits: −160‰ to −228‰; "natural": −185‰ to −286‰; and synthetic: −151‰ to −184‰). Berries also have been subjected to isotope analysis, for example, del Mar Caja and others (2007) analyzed raspberry for α-ionone, β-ionone, and α-ionol (δ^{13}C ranges of α-ionone, β-ionone, and α-ionol for fruits: −30.3‰ to −36.6‰; "natural": −9.1‰ to −28.0‰; and synthetic: −24.5‰ to −29.0‰ and δ^2H ranges of both compounds for fruits: −176‰ to −225‰; "natural": −43‰ to −257‰; and synthetic: −26‰ to −184‰). Both studies were able to differentiate between fruit, "natural," and synthetic-derived compounds, proving that the GC-C/P-IRMS is useful for authenticity assessments.

In gas chromatograms where the compound of interest cannot be baseline-resolved, it may be helpful to employ MD chromatography to achieve better separation of the target compound. This was the approach of Sewenig and others (2005), who used a constant-flow MD gas chromatography-combustion/pyrolysis-isotope ratio mass spectrometry (MDGC-C/P-IRMS) method to authenticate *(E)*-$\alpha(\beta)$-ionone in raspberries and raspberry products. This technique allowed the sample to be passed through a precolumn and the target compounds to be "cut" from the rest of the chromatogram and introduced to the main column for further separation and purification (Figure 4). The method was able to distinguish between fruit (δ^{13}C: −28.3‰ to −35.1‰; δ^2H: −190‰ to −214‰), "natural" (δ^{13}C: −14.9‰ to −15.6‰; δ^2H: −155‰ to −204‰), and synthetic (δ^{13}C: −21.6‰ to −25.8‰; δ^2H: −28‰ to −213‰) *(E)*-$\alpha(\beta)$-ionones, and the authenticity of the raspberry flavor was validated in a number of different products (Sewenig and others 2005).

Even more information about the origin of compounds can be found by combining results from enantioselective GC, multidimensional GC, and GC-IRMS. For example, Bernreuther and others (1990) were able to differentiate the origin of γ-decalactone from various natural sources including fruits (−28.2‰ to −40.9‰) and synthetic products (−24.4‰ to −26.9‰), while Nitz and others (1991) analyzed γ-lactones in strawberry, raspberry, pineapple, passion fruit, plum, and coconut. More recently, Greule and Mosandl (2008) investigated blackberry flavors by the combined techniques for authentication purposes.

Other applications of GC-C-IRMS for the study of aroma compounds

Apart from authenticity (natural or synthetic) testing, GC-C-IRMS has been used to determine the origin of bananas and derived products, and the ripening stage of bananas used in those products by studying volatile compounds in banana aroma (Salmon and others 1996). Bananas from 3 different areas (Ivory Coast, Martinique, and Central America) could be differentiated based on the concentrations of selected aroma compounds and their ^{13}C isotope ratios. Similarly, bananas at 2 ripening stages—early and late—could also be differentiated, confirming that banana nectar is made from very ripe fruit. The study also discovered that natural banana flavor could be distinguished from synthetic flavor.

Schipilliti and others (2011b) implemented SPME combined with GC-C-IRMS to determine ^{13}C isotope ratios in selected volatile compounds from strawberry. The compounds studied were chosen as contributors to the overall aroma of strawberries and their carbon isotope ratios were determined from fresh organic strawberries that established an authenticity range that included the use of an i-IST. The results showed that this technique can be used to determine food authenticity, and several products tested (including strawberry yogurts, strawberry flavored lolly ice) were shown to contain aroma compounds that were not derived from strawberries. Moreover, it was possible to distinguish between different fruits (pineapple, peach) containing volatile compounds common to strawberries, even when the volatile compounds were derived from plants sharing a common photosynthetic pathway (both strawberry and peach are C3 plants). The authors noted that "This result appears useful to evaluate mixed-fruits flavor with common volatiles among their aroma active ones. However, more detailed studies should be carried out in this direction."

As previously discussed, a combination of GC-C-IRMS with chiral chromatography can provide useful information on substitution of 1 natural product for another to achieve a similar aroma/flavor. Thus, Weckerle and others (2001) provided isotopic and chiral data for the cactus pear (*Opuntia ficus indica*) flavor for future work in authenticity determinations, as products seeking a melon-like aroma could be enhanced with this extract.

Technological processing techniques such as heating, distillation, and vaporizing can cause isotopic fractionation of aroma compounds. Elss and others (2006) studied the effects of processing on the isotopic values and the quantitative profile of apple aromas. The aroma profiles for authentic apple juice, apple concentrate, and apple fruit were different and could be attributed to cultivar differences, storage, bottling, pasteurization, and packaging, but the profiles during the industrial processing were determined to be sustained (Elss and others 2006). The isotopic ratios of C and H were measured for 3 aroma compounds of apple (*E*-2-hexenal, 1-hexanol, and *E*-2-hexenol) to determine if isotopic fractionation occurred during processing. The results showed that for δ^{13}C, with all the compounds, there was no fractionation, though for *E*-2-hexenal and 1-hexanol, the δ^2H ratios were slightly depleted indicating that fractionation did occur. Authenticity assessment was not affected by this fractionation as it was too small.

Aroma compounds in essential oils

Discrimination between natural and synthetic aromas. Essential oils can be used as flavorings in foodstuffs, but their production is very expensive in comparison with the synthetic counterparts and, for this reason, fraudulent activity, such as stating the product is natural when it is synthetic, often occurs. A characteristic flavor compound of fennel and anise oils is *trans*-anethole and it is extracted as a flavoring additive for beverages and foodstuffs (Newberne and others 1999; Bilke and Mosandl 2002). *trans*-Anethole is also produced synthetically and could be potentially used as a substitute for the natural extract. Bilke and others (2002) developed an authenticity range for *trans*-anethole based on self-extracted fennel and anise oils implementing a combination of GC-C-IRMS and GC-P-IRMS, as separately these methods could not definitively determine authenticity. Bilke and Mosandl (2002) were able to show that "natural" fennel oil and anise oils that were commercially available (δ^{13}C: −24.2‰ to −29.6‰; δ^2H: −71‰ to −99‰) fell in the authenticity range (δ^{13}C: −25.3‰

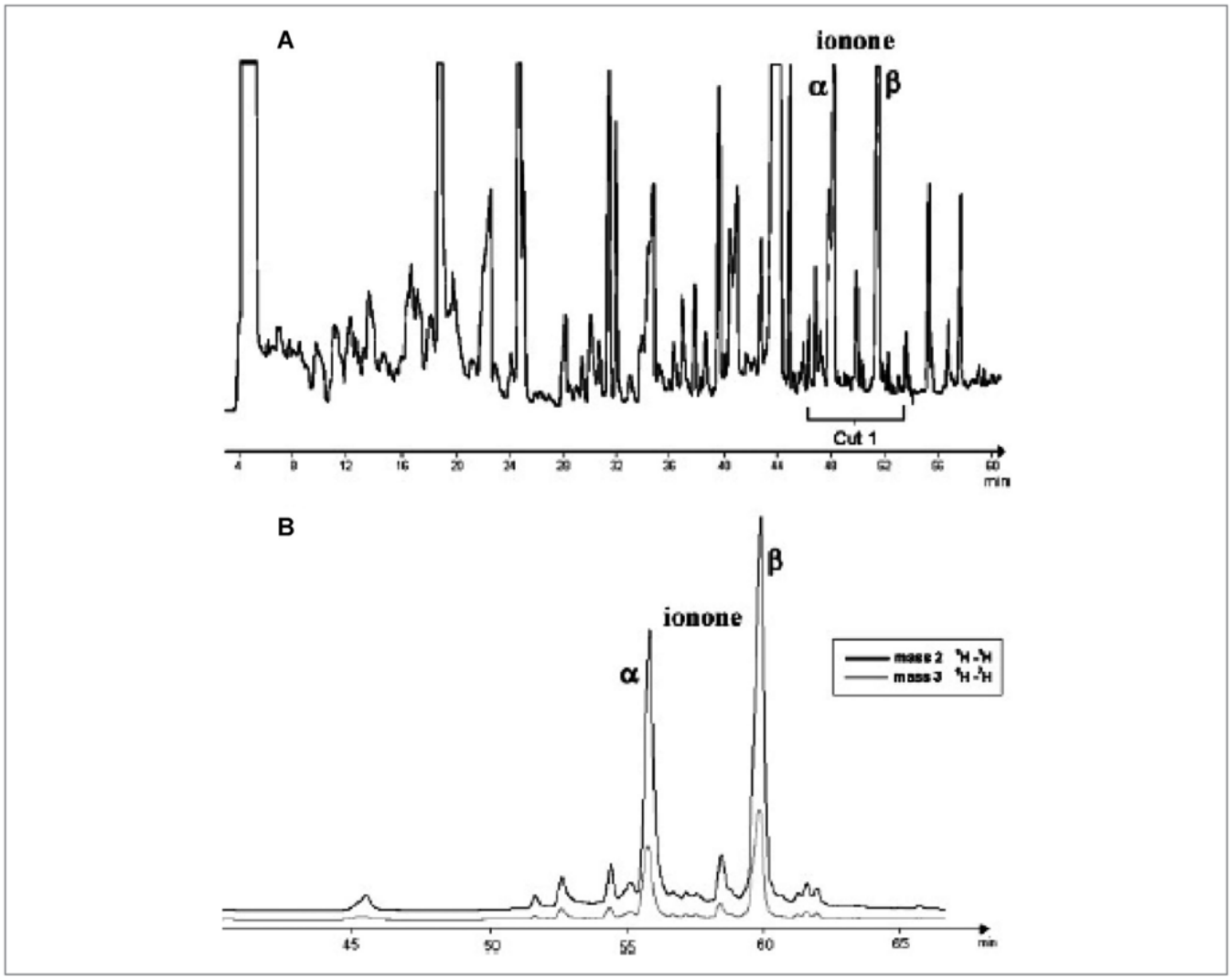

Figure 4–Precolumn (A, FID) and main column (B, SIM detection) chromatogram of a raspberry extract (Sewenig and others 2005).

to −28.3‰; δ^2H: −46‰ to −84‰) and that most of the synthetic compounds (δ^{13}C: −29.6‰ to −32.1‰; δ^2H: −20‰ to −79‰) and 1 commercial fennel oil sample were outside this range, therefore enabling this method to be used for authentication.

Fink and others (2004) investigated (*E*)-methyl cinnamate from basil and other sample extracts, several commercial natural aromas, and a synthetic compound by a combination of δ^{13}C and δ^2H ratios. They were able to measure a difference between synthetic (*E*)-methyl cinnamate (δ^{13}C: −29.5‰ to −31.4‰; δ^2H: +328‰ to +360‰) and that derived from basil (δ^{13}C: −28.9‰ to −29.0‰; δ^2H: −126‰ to −133‰), as well as showing that some of the commercial natural aromas (δ^{13}C: −25.7‰ to −28.5‰; δ^2H: −85‰ to −191‰) were incorrectly labeled.

Frank and others (1995) used the combination of enantioselective analysis, δ^{13}C and an i-IST, for the measurement of essential oils in coriander samples. The use of the i-IST allowed for the development of fingerprints using the terpenes found in the oils. Fingerprint matching between authentic and commercial oils identified 1 commercial sample as natural. Another commercial sample had a different fingerprint with respect to γ-terpinene and geraniol, which suggests that these compounds were not natural in this commercial sample.

Citrus fruits and their essential oils have been studied at great length by GC-C-IRMS owing to the fact that these aromas/essential oils are used in many food and beverage products. Examples include studies on lemons (Braunsdorf and others 1993b; Nhu-Trang and others 2006; Schipilliti and others 2012), oranges (Braunsdorf and others 1992, 1993a), mandarins (Faulhaber and others 1997a; Schipilliti and others 2010), and also lemon balms (and while not a citrus, its leaves have a lemon aroma and it is a C3 plant) (Nhu-Trang and others 2006; Wagner and others 2008). In the work of Nhu-Trang and others (2006), the focus was the terpenes citral (a mixture of neral and geranial, *E*/*Z* isomers of 3,7-dimethyl-2,6-octadien-1-al, respectively) and citronellal (3,7-dimethyl-6-octenal, which occurs as 2 enantiomers). Thus, they employed chiral GC with GC-C-IRMS to determine enantiomer and ^{13}C and ^{2}H isotopic ratios for these compounds genuinely derived from different plants having the "lemon" aroma. The natural citral had δ^{13}C ratio of *ca.* −26‰ and synthetic citral had a δ^{13}C range from −30‰ to −28‰, whereas the δ^2H composition of natural citral (*ca.* −244‰) was very different from that of synthetic citral (−6‰ to 63‰). In addition to identifying synthetic and natural differences, other plant types (C3/C4) were also identified by isotope ratios of citral.

Vanillin extracted from the bean of the CAM plant vanilla is another flavoring agent of enormous economic significance. Early studies on vanillin by GC-C-IRMS were undertaken in 1994 by Breas and others (1994). Then, a GC-C-IRMS method was used by Fayet and others (1995) to determine if the $\delta^{13}C$ of extracted vanillin flavoring from ice cream was the same as the $\delta^{13}C$ of the vanillin flavoring from the supplier or from a different source. The results showed that the $\delta^{13}C$ of the vanillin flavoring from the supplier was similar to the $\delta^{13}C$ of the vanillin found in the ice cream. Then, in 1998, Hener and others (1998) measured various sources of vanillin using GC-C-IRMS for $\delta^{13}C$ in comparison with GC-P-IRMS for $\delta^{18}O$. The values for $\delta^{13}C$, when comparing methods, were very similar except for 2 samples, one of biotechnological origin and the other from Mexico. The $\delta^{13}C$ values easily separated vanillin samples from different origins; biotechnologically derived (−37‰), synthetic (−27.4‰), and natural (−20.2‰ to −18.7‰).

In order to develop a more comprehensive analysis of compounds derived from vanilla, Kaunzinger and others (1997) implemented an isotopic fingerprint based on 4-hydroxy benzyl alcohol, vanillic acid, 4-hydroxybenzaldehyde, and vanillin. This enabled them to show that the $\delta^{13}C$ values are stable from year to year and that for different varieties of vanilla the $\delta^{13}C$ values for vanillin are quite different (*V. tahitensis*: −15.5‰; *V. planifolia*: −19‰), though for 4-hydroxybenzaldehyde the $\delta^{13}C$ are similar (approximately −16.5‰). Further studies of vanillin undertaken by Greule and others (2010) employed GC-C-IRMS for the authentication of vanillin in conjunction with an analysis for $\delta^{2}H$ using the pyrolysis technique. Correlation plots of the isotopic bulk analysis data (synthetic – $\delta^{13}C$: −28.17‰ to −29.74‰; $\delta^{2}H$: 54.6‰ to 79.7‰; lignin – $\delta^{13}C$: −32.46‰; $\delta^{2}H$: −61.7‰; *vanilla* pods – $\delta^{13}C$: −14.58‰ to −18.52‰; $\delta^{2}H$: −47.4‰ to −58.5‰), and the isotopic values of the methoxyl group of vanillin for $\delta^{13}C$ and $\delta^{2}H$ were able to differentiate between vanillin produced by various sources such as guaiacol (synthetic; methoxyl group—$\delta^{13}C$: −29.73‰ to −52.24‰; $\delta^{2}H$: −84.5‰ to −169.4‰) and lignin (semisynthetic; methoxyl group—$\delta^{13}C$: −37.15‰; $\delta^{2}H$: −235.6‰), as opposed to vanillin produced naturally from tropical orchid *vanilla* pods (methoxyl group—$\delta^{13}C$: −7.08‰ to −24.08‰; $\delta^{2}H$: −149.0‰ to −181.6‰). Gruele and others (2010) conclude that the method could also be used to differentiate between the various varieties of vanilla (in line with Kaunzinger and others above), though this would require a much larger database of vanillin samples, and also that with this method adulterated vanillin samples could be detected.

However, not all studies on essential oils have been able to detect a difference between natural and synthetic aroma compounds. For the case of estragole and methyl eugenol, the differentiation between natural and synthetic products was not discernible due to the synthetic version being produced from natural compounds (Ruff and others 2002), and therefore, this technique cannot be used for authentication purposes in this instance, although application of other complementary techniques may have assisted.

Aroma compounds in essential oils—adulteration. Another type of fraud commonly used for essential oils is blending the authentic high-value oil with another low-cost oil (or oils). Development of an isotope ratio authenticity range for the major compounds that are found in the essential oil is a reliable method to detect fraud (Sewenig and others 2003; Hrastar and others 2009; Greule and others 2010). For example, mandarin oil was shown to be adulterated by less expensive oils such as orange peel oil (Faulhaber and others 1997b), and cassia cinnamon oil is often used in the adulteration of ceylon cinnamon oil (Sewenig and others 2003). A component typical of mandarin oil, methyl *N*-methylanthranilate (MNM), is not found in any other citrus peel oil and for this reason can be implemented in the detection of adulterants. More sophisticated adulterations have added synthetic MNM, which means that the detection method requires additional complementary methods to appropriately identify adulteration.

Such complementary methods can include HPLC, GC-FID, and enantioselective GC (Es-GC) and Schipilliti and others (2010) employed these and GC-C-IRMS to investigate 5 commercial mandarin essential oil samples. While all techniques provided useful information, GC-C-IRMS enabled the detection of synthetic and natural compounds that were added to 4 of the samples to enhance the overall aroma of the oils (Schipilliti and others 2010).

Bergamot (Dugo and others 2012; Mosandl and Juchelka 1997; Schipilliti and others 2011a), neroli (Bonaccorsi and others 2011), and lime oils (Bonaccorsi and others 2012) are further examples of citrus oils with which fraud by blending occurs. To assess these oils for authenticity, several techniques have been employed such as MDGC, GC-FID, GC/MS, Es-GC, HPLC, and GC-C-IRMS. It has been found that GC-C-IRMS is the most sensitive to the slightest adulteration when compared to the other techniques, although it is recommended to use more than 1 technique for reliability, efficiency, comparison, sensitivity, and confirmation of the GC-C-IRMS results. For example, Schipilliti and others (2011a) used GC-FID and Es-GC in comparison with GC-C-IRMS and were able to determine that a number of the commercial bergamot oil samples analyzed were adulterated either due to the addition of adulterants or blending with a different oil into bergamot oil.

For lemon essential oils, Schipilliti and others (2012) used a number of techniques, such as Es-GC, GC-FID, and spectrophotometry (CD), together with GC-C-IRMS to prove the viability and reliability of GC-C-IRMS for authenticity assessments. An isotopic fingerprint was developed using 30 genuine lemon essential oils and an i-IST for the analysis of commercial lemon essential oils and distilled oils. The authors showed that a sample from Brazil and a sample from Argentina had been adulterated as the values for certain targeted compounds were out of the authenticity range. The enantiomeric distributions of compounds within the samples were also outside of the normal range for those from genuine samples. The authors (Schipilliti and others 2012) concluded that the Brazilian sample was made up of a combination of sweet orange oil terpenes and distilled lemon oil, by combining results from the different techniques used.

The use of 3 isotopic ratios, $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$, together with enantioselective analysis has also been employed in the analysis of essential oils. For example, Jung and others (2005) analyzed lavender oils and key aroma compounds, linalool and linalyl acetate, and were able to determine that 2 commercial lavender oil samples did not originate from lavender. They concluded that "Owing to the variety of adulterations of lavender oils multielement/multicomponent IRMS and the consideration of $\Delta\delta$ values as well as enantioselective analysis are necessary for comprehensive authenticity assessment." The comprehensive nature of the analyses required illustrates the degree of challenge in reliably detecting adulteration for some compounds and essential oils.

Aroma compounds in essential oils—biochemical pathways. Analysis of essential oils by GC-C-IRMS can be used to identify biochemical pathways within plants. Faber and others (1997) found that the composition of the essential oil is very different

during the various stages of plant development in dill (*Anethum graveolens* L.). Of interest were the biochemical pathways of monoterpenes (enantiomers of limonene, carvone, dill ether, and α-/β-phellandrene) in the plant as their biological purpose is uncertain. Faber and others (1997) were able to postulate a pathway for the above-mentioned monoterpenes using the results gained by GC-C-IRMS and enantioselective analysis. By comparing $\delta^{13}C$ in different plant parts during different stages of development, the authors were able to discover that different enzymes were responsible for the biosynthesis of limonene in dill herb to those in the buds, flowers, and seeds. Monoterpenes generally have a specific aroma and are vital for the food and beverage industries to enhance flavors. Therefore, fundamental knowledge of their biosynthesis could be of benefit for developing new and more sophisticated adulteration detection methods.

Edible fats and oils

Assessment of authenticity. Edible oils such as olive oil and vegetable oils are often adulterated with cheaper oils (Angerosa and others 1997; Lees 1998; Mottram and others 2003) and IRMS on bulk oil (the "whole" oil) was proposed as a possible means to detect this type of fraud over 30 y ago (Gaffney and others 1979). As well as bulk oil, various fractions of oil, such as aliphatic alcohols (Angerosa and others 1997), can be submitted to isotope ratio analysis to determine whether adulteration has occurred. The first use of GC-C-IRMS for the detection of fraudulent oils was published by Woodbury and others (1995) who looked at isotope ratios of individual fatty acids in maize oil (a C4 plant) and compared them to isotope ratios for fatty acids in rapeseed oil (a C3 plant). This group went on to publish a comprehensive database of $\delta^{13}C$ values of the major fatty acids of more than 150 vegetable oils (Woodbury and others 1998a) and each particular oil was shown to have its own unique isotopic fingerprint. Arising from this work, the authors noted that variability in $\delta^{13}C$ values was related to the geographical origin of the oil, year of harvest, and the particular variety of oil (Woodbury and others 1998a). This suggests that both environmental and genetic factors contribute to the observed isotope ratios. One particular interest was the bimodal distribution of $\delta^{13}C$ values depending on whether the oils originated in the northern or southern hemispheres.

GC-C-IRMS has also been applied to the analysis of fatty acids in false flax oil (*Camelina sativa* oil) (Hrastar and others 2009), olive oil (Spangenberg and others 1998; Royer and others 1999; Baum and others 2010), vegetable oils (Kelly and others 1997; Woodbury and others 1998b; Spangenberg and Ogrinc 2001; Mottram and others 2003), and cooking oils (Liu and others 2012) for the purposes of authentication and determination of origin. A method using GC-C-IRMS in combination with bulk isotope ratio analysis has been developed by Richter and others (2010) to combat against possible frauds and adulterations of rapeseed oil. The bulk measurements determined the isotope ratios of C, H, and O giving details on geographical origin, whereas GC-C-IRMS measured the fatty acid components in the rapeseed oil. The results were that individual fatty acids can be used to differentiate between different species of C3 plants such as flax, poppy, and rapeseed, but this by itself would not be enough to determine fraud; bulk IRMS analysis would also be required.

Cocoa butter is a lipid-rich food ingredient that is prone to fraud due to its high value. Bulk IRMS and GC-C-IRMS were used to investigate cocoa butter of different origins, suppliers, and varieties and equivalents (Illexao 30-61, Illexao 30-71, Illexao 30-96, Choclin, Coberine, Chocosine-Illipé, Chocosine-Shea, Shokao, Akomax, Akonord, and Ertina) (Spangenberg and Dionisi 2001). The aim of this study was to determine if fatty acids (bulk fat, palmitic acid [16 : 0], stearic acid [18 : 0], oleic acid [18 : 1], and linoleic acid [18 : 2]) could be differentiated by bulk and molecular isotopic ratios. Principal component analysis (PCA) showed that the addition of vegetable fats or cocoa butter equivalents of 15% was able to be detected, the exception being illipé fat, which was not possible to be detected in the cocoa butter. Spangenberg and Dionisi (2001) suggested that an improvement in precision of the method would be to use other qualitative and quantitative methods in combination with isotopic ratios.

Jung and others (2007) developed a method to detect glycerol and fatty alcohols of olive oil and other edible fats and oils by means of reductive ester cleavage, with the GC-C/P-IRMS for ^{13}C and ^{18}O. The correlation of $\delta^{13}C$ and $\delta^{18}O$ ratios for glycerol provided an edible fats and oils authenticity assessment and was able to prove that ratios from synthetic glycerol and glycerol from other sources were different from the glycerol derived from edible fats and oils. For fatty alcohols, the analysis of $\delta^{18}O$ was not possible with GC-P-IRMS as the concentration of oxygen in the fatty alcohols was not enough to be measured. Therefore, only the $\delta^{13}C$ ratios for fatty alcohols was measured with GC-C-IRMS and were determined to be useful for the authenticity assessment of edible fats and oils.

Beverages

GC-C-IRMS has been implemented to characterize flavor and aroma compounds and to detect fraud and adulterations through the analysis of $\delta^{13}C$ in a range of beverages such as teas (Weinert and others 1999; del Mar Caja and others 2009), spirits (Schumacher and others 1999), apple juice (Kelly and others 2003), and carbonated drinks (Calderone and others 2007). Weinert and others (1999) have evaluated the carbon isotope ratios of the characteristic flavor compounds (*trans*-2-hexenal, *cis*-3-hexenol, *trans*/*cis*-linalool oxides [furanoids], linalool, methyl salicylate, and geraniol) found in specific black teas (Ceylon, Assam, and Darjeeling) to differentiate between authentic teas and adulterated teas. Methyl salicylate is a major component of black teas and it is often added as an adulterant to enhance the overall flavor/aroma thereby increasing consumer sales (Abraham and others 1976; Weinert and others 1999). The authors (Weinert and others 1999) were able to produce isotopic fingerprints for each of the specific black teas studied and were able to prove that adulteration by methyl salicylate could be detected through the use of an i-IST. Although this method worked well, it was not perfect, with about 6% of all Ceylon black teas investigated unable to be classified. Weinert and others (1999) stated that the use of another isotopic measurement (namely, $\delta^{18}O$ ratios by GC-P-IRMS) could help overcome this problem.

Alkylpyrazines, derived from roasting, are very important for the typical aroma of roasted coffee. Richling and others (2005) analyzed alkylpyrazines for $\delta^{15}N$ and $\delta^{2}H$ implementing GC-C/P-IRMS. Synthetic compounds and compounds claimed as "natural" were also analyzed for comparison to determine the origin of commercial samples as a check to deter possible adulterations. The results for some of the alkylpyrazines showed differentiation between synthetic and/or natural compounds and the alkylpyrazines from coffee. For example, 2-methylpyrazine synthetic references had a $\delta^{15}N$ range of 0.3‰ to −1.2‰ and $\delta^{2}H$ range of 15‰ to 40‰, "natural" references had a $\delta^{15}N$ range of −0.9‰ to −2.4‰ and $\delta^{2}H$ range of −102‰ to −117‰, whereas the coffee samples studied had a $\delta^{15}N$ range of 2.5‰ to 2.8‰ and $\delta^{2}H$ range

of −13‰ to −77‰ clearly showing differentiation between the different references and the coffee samples. This study provided new isotopic data of N and H for alkylpyrazines and it has proven that isotopic methods could be used for authenticity assessment of products containing coffee, though more data will be required before the method is used in commercial situations.

Schumacher and others (1999) studied the fusel alcohols 2-methylbutanol and 3-methylbutanol in alcoholic apple beverages. These alcohols are formed during fermentation from metabolism of isoleucine and leucine, respectively. The authors wanted to determine whether these alcohols had different $\delta^{13}C$ values depending on the source of carbohydrate used in fermentation (of C3 or C4 plants,) and whether there was a difference in $\delta^{13}C$ values between apple juice and processed alcoholic beverages. The $\delta^{13}C$ isotopic values for 2-methylbutanol in distillates from C3 plants (−17.0‰ to −26.1‰) were clearly separated from those of C4 plants (−7.7‰ to −13.4‰). Similarly, apple-derived alcoholic beverages had $\delta^{13}C$ isotopic values for 2-methylbutanol (−24.5‰ to −27.0‰, with one exception of −19.0‰) different from apple juices (−38.1‰ to −39.3‰). Further differentiation was evident for $\delta^{13}C$ isotopic values for 2-methylbutanol among C4 plants as maize starch and glucose syrup had $\delta^{13}C$ values of −7.7‰, which were much higher than those for cane sugar −13.4‰ to −13.9‰. Similar trends were observed for 3-methylbutanol. Additionally, $\delta^{13}C$ values were 4‰ to 5‰ lower than for 2-methylbutanol from the same product, typical for fermented products (Schumacher and others 1999).

In the EU, properties of CO_2 (origin, concentration, and purity) for addition to beverages are regulated and, hence, methodologies to ensure adherence to these regulations are required. The origin of CO_2 is either from industrial, botanical, or geological sources, which generate a wide range of $\delta^{13}C$ values (Calderone and others 2007) that can be used for authentication purposes. Calderone and others (2007) have utilized GC-C-IRMS for the analysis of CO_2 in the headspace of sparkling and carbonated drinks and found that naturally carbonated waters had a $\delta^{13}C$ range of −4.5‰ to 1.05‰ which matched the natural CO_2 vent sources from which the products were derived. On the other hand, industrial carbonation of water produced more depleted carbon isotope values (−29.24‰ to −54.15‰). A study by Brencic and Vreca (2007) showed that bottled waters from different origins (bottling and processing procedures) could be differentiated based on their ^{13}C isotopic ratios of dissolved inorganic carbon. The average $\delta^{13}C$ value for sparkling water produced naturally was −3.3‰, whereas sparkling water produced artificially had a mean value $\delta^{13}C$ of −36.5‰. The average $\delta^{13}C$ ratio for still (−10.0‰) and flavored (−11.0‰) waters was similar. Beers could be distinguished in light of their manufacturing, whereby those produced with C4 plant ingredients had a $\delta^{13}C$ range for CO_2 of −17.5‰ to −21.8‰ and double malt beers as well as a beer that was industrially carbonated had $\delta^{13}C$ values of less than −30‰ (Calderone and others 2007). Similarly, sparkling wines could be differentiated based on CO_2 stable isotope ratios due to the type of sugar (C3 or C4) added during the 2nd fermentation. The CO_2 $\delta^{13}C$ range for sparkling wine with C3 sugar added was −19.23‰ to −21.12‰ and for the sparkling wine with C4 sugar added the result was −9.7‰ (Calderone and others 2007).

Still wines and distillates are also subjected to strict rules and regulations, especially in countries such as Italy and France and where certain alcoholic beverages (such as grappa, cognac, vodka, tequila, and so on) are of great economic importance. Protected designation of origin (PDO) assignments of these products are fundamental to protect and regulate wines and distillates from distinct regions. Fraudulent production of PDO beverages includes mislabeling/misdescription and adulteration such as addition of sugar (sugar cane and sugar beet) and addition of artificial/synthetic flavors and dilution with water (Dordevic and others 2013). Spitzke and Fauhl-Hassek (2010) developed a GC-C-IRMS method that could be used to determine the isotopic ratios of wine ethanol and other alcohols, for example, 2-methylpropan-1-ol, 2- and 3-methylbutan-1-ol; butan-2,3-diol, 2-phenyl-1-ethanol, and glycerol. They were able to make correlations of the $\delta^{13}C$ of the higher alcohol compounds (such as 2-/3-methylbutan-1-ol) with $\delta^{13}C$ wine ethanol ($R^2 = 0.829$), for authenticity analysis of wines and distillates. Other groups (Calderone and others 2004a; Jung and others 2006) have also investigated wine ethanol and glycerol by GC-C-IRMS for alternative techniques to determine adulteration of wines.

Wines and distillates are generally evaluated by their perceived aroma by consumers, and this fact could lead producers to adulterate their products with added flavor compounds to increase profits. Previous studies (Bauer-Christoph and others 1997, 2003; Engel and others 2006; Winterova and others 2008) have measured the concentrations of flavor compounds found in distillates in combination with isotopic data of bulk ethanol to provide an authenticity profile of the distillate type (fruit spirits, wines, whiskeys, and so on), but the isotopic values of the flavor compounds were not investigated. A recent study undertaken by Schipilliti and others (2013a) employed headspace SPME (HS-SPME) coupled to a GC-C-IRMS to determine the authenticity of Italian liqueurs. They were able to measure the $\delta^{13}C$ of typical flavor compounds found in 3 types of Italian liqueurs, "limoncello," "mandarinetto," and "bergamino" and then apply an authenticity range using an i-IST. This technique was successful in determining adulterations such as the addition of essential oil fractions, blending with artificial flavors, and the addition of other citrus oils.

Miscellaneous applications of GC-C-IRMS

Hattori and others (2010, 2011) proposed 2 methods to analyze acetic acid in vinegar. One method analyzed the ferments of raw materials such as rice, tomatoes, and apple juice for C and H by HS-SPME-GC-IRMS (Hattori and others 2010). The method was able to differentiate the origins of the vinegars, especially between those derived from C3 and C4 plants. For example, a sample labeled as apple vinegar had $\delta^{13}C$ values in the range of a C4 plant, yet apple is a C3 plant. The 2nd method of Hattori and others (2011) analyzed the $\delta^{13}C$ of the methyl carbon and carboxyl carbon of acetic acid to determine whether these values could provide more information on the origin of samples. The results of this work showed that in all cases but one (determined to be not 100% pure acetic acid derived from *Acetobacter*), the methyl C was more depleted than the carbonyl C and that the samples did show some discrimination of the isotopic differences ($\delta^{13}C_{carboxyl} - \delta^{13}C_{methyl}$) between origin, C3 (2.1‰ to 6.7‰), CAM (18.2‰), and C4 (11.6‰) plants. Hattori and others (2011) proposed that the data generated from this method were not sufficient for origin differentiation and that more samples from the different botanical origins were required to improve the database.

To this point in the review, the main isotope of interest has been ^{13}C with various applications also drawing on ^{2}H and ^{18}O. There is, however, growing utilization of ^{15}N in food authentication, especially with respect to organic produce. For example, organic fertilizers must be used and mineral or synthetic fertilizers are prohibited, which has led to investigations of N uptake by plants.

Understanding the variation in $\delta^{15}N$ values in plants as a result of synthetic or organic fertilizer use requires knowledge of $\delta^{15}N$ values in soil, how they change as the plants assimilate nitrogen, and how metabolic processes within a plant subsequently affect $\delta^{15}N$ values; as well as isotopic ratios in the applied fertilizer. Mineral or synthetic fertilizers have $\delta^{15}N$ values around −6 to +6‰ as they originate from atmospheric $\delta^{15}N$ and the subsequent industrial processes induce slight, if any, fractionation (Bateman and Kelly 2007b). On the other hand, in the production of organic fertilizers such as manure and composts, there are more processes occurring that incur trophic shifts, and therefore isotopic fractionation is greater. Thus, organic fertilizers have much higher $\delta^{15}N$ values, from +1‰ to + 37‰ (Bateman and Kelly 2007b) than mineral fertilizers.

Soil $\delta^{15}N$ values primarily reflect the added fertilizer, though other factors such as the type of soil, altitude, moisture, and processes like denitrification, mineralization, nitrogen assimilation, and leaching are also involved. The $\delta^{15}N$ values in the plants derive mainly from the uptake of nitrogen in the soil and can be influenced by external factors such as the timing of fertilizer application to the soil (Choi and others 2002), soil water availability (Choi and others 2003), nature of the fertilizer, such as liquid or pellet form (Evans and others 1996), and internal factors such as plant traits and metabolic processes (Senbayram and others 2008).

The main assimilation forms of inorganic nitrogen by plant roots are NO_3^- and NH_4^+. Nitrate is transported to the chloroplasts, where it is reduced to NH_4^+, rapidly distributed to various organelles, and converted into organic forms by the glutamine synthetase reaction. According to Winkler (1984), plants assimilating nitrate to form NH_4^+ have an increase in $\delta^{15}N$ of about 10‰ therefore when the NH_4^+ is converted to proteins and amino acids, enrichments in ^{15}N (Winkler 1984) again occur. Isotopic differences for amino acid components of organic and conventional produce, as determined by GC-C-IRMS, could therefore be used to assess authenticity.

The metabolism of plant nitrogen was studied by Styring and others (2014) in barley, wheat, broad beans, and peas through an investigation of the $^{15}N/^{14}N$ ratios of amino acids. The results showed that different anabolic and catabolic processes in the plants cause isotopic fractionation, and therefore, the amino acids have different $\delta^{15}N$ values. The amino acids involved in the metabolism of nitrogen in barley and wheat had similar $\delta^{15}N$ values suggesting that the pathways were similar, whereas for the broad beans and pea seeds, the $\delta^{15}N$ values were different, indicating different pathways for development. It was shown by utilizing $\delta^{15}N$ values that amino acids follow known metabolic pathways. This information could be useful for more in-depth studies on N cycling within a plant and to determine the effect of external factors on plant metabolism.

Additional evidence that GC-C-IRMS could be implemented to study amino acids in plants comes from Bol and others (2002) who studied amino acid isotopic composition in relation to the available sources of N and uptake systems in temperate grasslands. Their results illustrated that the amino acids histidine and phenylalanine could be utilized to differentiate between plant species. Also, it was noted that the different isotopic N ratios were due to the diverse uptake mechanisms of N by the plants.

Thus, while $\delta^{15}N$ values of amino acids show promise for utilization in food authenticity and traceability studies, to date no such studies utilizing GC-C-IRMS have appeared (to the best of the authors' knowledge). This represents a potentially fruitful area of research to complement the well-recognized applications of $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ to detect fraudulent activity in the food industry.

Conclusion

Consumers around the world want to understand and be informed as to what they are eating, where it comes from, and how it was made. There is increasing information easily obtainable through electronic sources on healthy eating, types of produce, organic or conventional farming, and food processing protocols. Products that contain labels "organically grown" or are PDO, command premium prices and hence there is a great need to be able to test foods and beverages to verify that all the contents are genuine (in terms of ingredients, origin, and so on). As demonstrated in this review, GC-C-IRMS is an analytical technique that has the potential to be applied across a wide range of foods, beverages, and ingredients to detect fraudulent activity. While the foundation of this technique can be traced to the very beginnings of MS, modern-day applications require sophisticated, precisely calibrated instrumentation, and careful attention to detail in terms of sample preparation. Nevertheless, the tremendous versatility of GC-C-IRMS is evident from the numerous applications documented in this review and we anticipate that it will become a central technique in the detection of fraud either alone or in combination with other analytical methods.

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van Leeuwen was responsible for sourcing, reading, analyzing, and collating the literature as part of her PhD program. Prenzler, Ryan, and Camin were responsible for editing, synthesizing, and structuring the review in their role as PhD supervisors for van Leeuwen.

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LITERATURE REVIEW UPDATE

Since submission of van Leeuwen and others (2014), various articles implementing GC-C-IRMS for analysis of food and beverages have been published. Topics include the analysis of essential oils, edible oils and fats, beverages, vinegars and grains (Table 1)

These topics match those presented in van Leeuwen and others (2014), and show that GC-C-IRMS continues to be an active area or research. In terms of this thesis, however, it is important to ensure that no other studies have been conducted that detract from the novelty of the work that I have undertaken. As will be demonstrated, below, there have been some studies that are aligned with my work, but none that impact on my contribution of new knowledge to the field.

Articles of particular interest in relation to my research (see Chapter 1, Aims & Objectives) are $\delta^{13}C$ analysis of vanillin in foodstuffs for authenticity (Schipilliti and others 2017); bulk analysis ($\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$) of milk powder and compound specific ($\delta^{13}C$, $\delta^{2}H$) analysis of fatty acids in feed and farm water and their impact on dairy milk composition for traceability (Ehtesham and others 2015); and the analysis of amino acid $\delta^{15}N$ values for grains, wheat and legumes and $\delta^{13}C$ for wheat for differentiation of conventional and organic farming systems (Styring and others 2014; Paolini and others 2015).

Starting with the work of Schipilliti and others (2017), this study reported on identifying vanillin in baked products and used solid phase microextraction (SPME) to extract headspace vanillin, prior to GC-C-IRMS. In my work, SPME was not feasible due to the high concentration of ethanol in the distillates interfering with analyte absorption on the

fibre. Thus, a liquid-liquid extraction method needed to be developed and validated for use with distillates. Moreover, my work is the first report for the $\delta^{13}C$ analysis of authentic vanillin in distillates. This allowed differentiation of vanillin derived from the wood of the storage barrel and adulterations by the addition of synthetic vanillin in distillates.

The research by Ehtesham and others (2015) confirmed that the isotopic ratios of farm water and grass fed to cows are reflected in the animal (deNiro and Epstein 1978) through analysis of its milk, which could enable the milk to be traced back to its geographic source. The research that I undertook also confirmed that the diet is reflected in the animal, but my study went a step further to determine the effect certain feed types had on the production of fatty acids in the animal. This knowledge facilitates greater insight on how to influence the animal's diet for the production of 'healthier' quality meat for human consumption.

The amino acid analysis for wheat and grains (Styring and others 2014; Paolini and others 2015) to differentiate between organic and conventional farming systems implemented the measurement of $\delta^{15}N$ values (Styring and others 2014; Paolini and others 2015) and $\delta^{13}C$ values (Paolini and others 2015) to trace the amino acid uptake in the plant. The focus of my research was on tomato fruits grown under the organic and conventional farming regimes, but with comprehensive analysis of $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$ and $\delta^{34}S$ for the whole tomato and $\delta^{13}C$ and $\delta^{15}N$ for single amino acids to enable differentiation between the two systems.

Note: Table 1 and references in the text are in the style of the journal: Comprehensive Reviews in Food Science and Food Safety.

Table 1 - Food and beverage analysis by GC-C-IRMS.

Food and Beverage	Purpose of Study	Compounds	Outcome	Isotopic ratio	Reference
Essential Oils					
Orange, orange flavoured beverages	Assessment of authenticity	Myrcene, limonene, nonanal, decanal, linalool, terpinene-4-ol, geranial	An authenticity range was developed and used to prove genuineness of the oils.Characterization of these oils was done using GC-FID, GC-MS and HS-SPME-GC-C-IRMS	$\delta^{13}C$	Schipilliti & others (2015)
Vanillin, cake, chocolate bars, cookies, wafers, snacks, coffee	Natural and synthetic	Vanillin	HS-SPME-GC-C-IRMS was implemented successfully for the discrimination of vanillins of different origin	$\delta^{13}C$	Schipilliti & others (2017)
Vanillin	Authenticity and traceability	Vanillin	*Vanilla planifolia* and *Vanilla tahitensis* was able to be differentiated from each other as well as from vanilla produced in other ways using GC-C/P-IRMS	$\delta^{13}C$, $\delta^{2}H$	Hansen & others (2014)
Edible Oils and Fats					
Olive oil	Geographical origin	Stearic acid, oleic acid, palmitic acid, linoleic acid	Implementation of bulk analysis with FAME analysis combined with statistics (PCA & PLS-DA) enabled differentiation by pedoclimatic and geographic parameters	$\delta^{13}C$	Faberi & others (2014)
Vegetable oil	Geographical origin	Palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid	Bulk analysis and compound specific analysis of plant FA was combined with statistical analysis to determine geographical origin of vegetable oil	$\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$	Spangenberg & others (2016)
Beverages					
Coffee beans, *Coffea arabica* L.	Geographical origin	Caffeine	GC-MS, GC-C-IRMS & ^{2}H-NMR were implemented to obtain classification results to discriminate coffees from different regions. Analysis by NMR was best for predictive modelling	$\delta^{13}C$	Arana & others (2016)

Table 1 - Continued.

Food and Beverage	Purpose of Study	Compounds	Outcome	Isotopic ratio	Reference
Wine	Tracer study	Acetic acid, isobutyric acid, decanoic acid, isoamyl hexanol, hexanoic acid, isobutyl alcohol, octanoic acid, ethyl octanoate, ethyl lactate, 1-butanol, ethyl hexanoate, ethyl 3-hydroxybutyrate, ethanol, isoamyl acetate, hexyl acetate, methionol, β-phenylethanol, phenylethyl acetate, 1-hexanol	Volatile compounds were traced during fermentation of juice to wine with uniformly labeled [U-^{13}C]-glucose	$\delta^{13}C$	Nisbet & others (2014)
Milk	Traceability and geographical origin	Fatty acids; C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C18:1, C18:2, C18:3	It was determined that $\delta^{2}H$ was more reliable for authentication than the other measured isotopes and that the isotopic nature of the milk sample was compiled from the water and feed source	$\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, $\delta^{18}O$, bulk	Ehtesham & others (2015)
Vinegars					
Vinegar	Isotopic fractionation related to organic acid (bio)transfromations	Acetic acid	SPME-GC/Py-GC-C-IRMS was used for the simultaneous determination of the intramolecular and molecular carbon-isotopic composition of acetic acid	$\delta^{13}C$, bulk	Nimmanwudipong & others (2015)
Grains					
Barley, bread wheat grains and rachis, broad bean seeds, pea seeds	Metaboloic pathways of amino acids	Alanine, aspartate/asparagine, glutamate/glutamine, glycine, leucine,phenylalanine, proline, serine and valine	The influence of manure and unmanured soil on the metabolic pathway for amino acids was found for different species of crops and parts of plants, using GC-C-IRMS	$\delta^{15}N$	Styring & others (2014)
Wheat	Authenticity	Alanine, aspartate, glutamate, glycine, isoleucine, leucine, phenylalanine, proline, threonine and valine	Bulk analysis and GC-C-IRMS, with statistics was used to determine isotopic values of amino acids in wheat which was able to differentiate between conventional and organic growing conditions	$\delta^{13}C$, $\delta^{15}N$	Paolini & others (2015)

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CHAPTER THREE

INTRODUCTORY COMMENTS

RESEARCH PAPER

Paper 2 van Leeuwen, K. A., Prenzler, P. D., Ryan, D., Paolini, M., & Camin, F. (2017). Differentiation of wood derived vanillin from synthetic vanillin in distillates implementing GC-C-IRMS for $\delta^{13}C$.

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INTRODUCTORY COMMENTS

Vanillin is an important flavour and aroma compound in distillates such as whiskey and brandy and it is a quality marker for the beverage. To improve the quality of sub-standard beverages and therefore sell them at a higher price, adulterations via the addition of synthetic compounds, such as vanillin, ensues. This chapter focuses on the isotopic $\delta^{13}C$ analysis of vanillin by GC-C-IRMS for the authentication of distillates.

Differentiation of wood derived vanillin from synthetic vanillin in distillates implementing GC-C-IRMS for $\delta^{13}C$

Short title: $\delta^{13}C$ of vanillin in distillate using GC-C-IRMS

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Abstract

Rationale: Typical storage in oak barrels release in distillates different degradation products such as vanillin, which plays an important role in their flavour and aroma. The addition of vanillin, as well as other aroma compounds, of different origin is prohibited by European laws.

As vanillin from different sources have different $\delta^{13}C$ values, $\delta^{13}C$ could be used to determine whether the vanillin is authentic (lignin-derived), or if it has been added from another source (e.g. synthetic).

Methods: The $\delta^{13}C$ values for vanillin derived from different sources including natural, synthetic, and tannins, were measured by gas chromatography-combustion-isotope ratio mass spectrometry, after diethyl ether addition and/or ethanol dilution. A method for analysing vanillin in distillates after dichloromethane extraction was developed. Tests were undertaken to prove the reliability, reproducibility and accuracy of the method with standards and samples. Distillate samples were run to measure the $\delta^{13}C$ values of vanillin and to compare them to values for other sources of vanillin.

Results: $\delta^{13}C$ values were determined for: natural vanillin extracts (-21.0‰ to -19.3‰, 16 samples); vanillin ex-lignin (-28.2‰, 1 sample); and synthetic vanillin (-32.6‰ to -29.3‰, 7 samples). Seventeen tannin samples were found to have $\delta^{13}C$ values of -29.5‰ to -26.7‰, which were significantly different ($p<0.05$) from the natural and synthetic vanillins. The vanillin measured in distillates (-28.9‰ to -25.7‰) were in the tannin range, however one spirit (-32.5‰) was found to have synthetic vanillin.

Conclusions: The results show that synthetic vanillin added to a distillate was able to be differentiated from that of vanillin derived from oak barrels by $\delta^{13}C$ values. The GC-C-IRMS method could be a useful tool in the determination of adulteration of distillates.

Keywords

Vanillin; authentication; compound-specific; GC-C-IRMS; distillate

1. INTRODUCTION

Distilled and fermented beverages, “distillates”, such as whisky, brandy, rum and grappa are commonly aged in oak barrels [1, 2]. Generally, the storage of distillates in oak barrels can impart a more complex palette of flavour and aroma to the beverage [1, 3] compared to those that are unoaked. Adulteration of distillates with synthetic or bio-synthetic aroma compounds is prohibited by the European Commission (EC) Regulation No. 110/2008, which states that rum, whisky, grain spirit, wine spirit, grape marc spirit and fruit marc spirit cannot be sweetened or flavoured, though plain caramel can be added for colour enhancement and any additions to the spirits need to be declared. As such, adulteration is fraudulent and constitutes a major economic problem, as manufacturers seek to improve the quality and monetary value of their product through the unauthorised addition of key aroma compounds to their beverages.

An important step in the production of distillates is storage in oak barrels, commonly derived from American or French oak [4, 5]. The oaking process imparts important aroma and flavours to the distillate depending on: species and origin of the wood; seasoning of the oak for barrel making; toasting of the oak and at different levels (light, medium and heavy); the length of time the distillate is in contact with the wood; and the barrel storage environment [1]; but the factor that influences the overall sensory aspect of the distillate from the wood is heat [6]. As the lignin, cellulose, hemicellulose and other extractives in the wood of the barrel break down, due to the toasting process, degradation products such as volatile phenols [7], furan aldehydes [8] and vanillin [7, 9] are released into the distillate [3, 7]. In the first few months of maturation these compounds are released into the distillate quite readily as they are extremely soluble in combined liquids of alcohol and water [1].

Further into the maturation process of the distillates, lignin is slowly degraded by a process known as ethanolysis (reaction of lignin with the distillate ethanol to produce an ethanol-lignin soluble compound which is then oxidised) releasing more compounds into the distillate [1, 10-12]. Furan aldehydes, such as furfural, 5-methyl furfural and 5-HMF, which derive from the degradation of hemicelluloses and celluloses [5] , have been shown to increase as the length of maturation time increases [8], imparting caramel, toasty and honey flavours [5, 13] , and may also create the sensation of "hotness" to the beverage [13, 14].

It is well known that vanillin (4-hydroxy-3-methoxybenzaldehyde) plays an important role in the flavour and aroma of distillates [1, 15-17] such as brandy [18], rum [19] and whiskey, [1] and it defines the overall quality of the beverage [18]. Vanillin is reported to have a "vanilla" and "sweet" aroma [19, 20] with an aroma threshold in 40% ethanol of 22 μg/L [19]. According to Maga, [21] vanillin has a taste threshold of 100 μg/L in 40% ethanol which means that a minute quantity of vanillin is required to fraudulently alter the flavour of a distillate. The cost of natural vanillin from *Vanilla spp.* is estimated to be USD 1500/kg compared to the much cheaper synthetic vanillin that costs between USD 10 – 20/kg (http://www.evolva.com/vanillin/#sthash.ztwstm6p.dpuf). Because of this significant price difference, adulteration of natural vanillin with biosynthetic and synthetic vanillin has been reported in several types of foodstuffs [22-25].

Fortunately, vanillin derived from different sources such as natural, synthetic, biosynthetic and extracted from lignin may be discriminated on the basis of the stable isotope ratio of carbon, namely $^{13}C/^{12}C$ expressed in $\delta^{13}C$. Natural vanillin derived from the tropical orchid, *Vanilla* (mainly the species *V. planifolia*, *V. tahitensis* and *V. pompon)*, is produced via the CAM photosynthetic pathway and has a $\delta^{13}C$ ratio between

-22‰ to -14‰ [23, 26-38], whereas biosynthetic (ex-ferulic acid and turmeric) and synthetic (lignin and guaiacol precursors) vanillin have significantly lower $\delta^{13}C$ values (-38‰ to -29‰ and -30‰ to -27‰, respectively) [23].

Analysis of compound specific stable isotope ratios by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) offers a highly sensitive and selective approach for the detection of adulteration, however very little work on the adulteration of distillates has been conducted. Mostly the analysis of distillates by GC-C-IRMS has focused on the analysis of ethanol and the higher alcohols by GC/MS and IRMS, [39-41] however there is one report using GC-C-IRMS on volatile components of citrus liqueurs[42]. In the current study, a method for measuring $\delta^{13}C$ of vanillin found in distillates has been developed. In order to determine the source of vanillin in the distillates, a range of sources of vanillin were also subjected to GC-C-IRMS and their $\delta^{13}C$ values are reported.

2. EXPERIMENTAL

2.1 Standards and reagents

The reagents: diethyl ether, dichloromethane, and potassium metabisulfite were purchased from Sigma Aldrich, Milan, Italy. A synthetic vanillin (99% purity), termed “vanillin standard” (Table 1) was used for method development purposes and was purchased from Sigma Aldrich, Milan, Italy. Ethanol was purchased from Fluka, Milan, Italy. Sodium sulfate was bought from Carlo Erba, Milan, Italy. Hydrogen chloride and sodium chloride were purchased from Merck, Milan, Italy.

2.2 Samples

Commercial edible samples containing vanillin (including 4 powders of natural extract and sugar, 1 natural extract and sugar syrup, 1 essence aroma for sweets, 1 powder aroma for sweets) were bought from local stores in Italy and France (Table 1). Three synthetic reagents of vanillin were purchased from Carlo Erba (99%), one from Fluka (Milan, Italy) and two from Sigma Aldrich (99%) and all were purchased in Milan (Italy). Sixteen commercial industrial food vanillin (ethanol/water) extracts were acquired in Italy. Seventeen commercial tannin samples were bought in Italy. Borregaard EuroVanillin Supreme ex. Lignin (termed "vanillin ex-lignin"), 99.6% purity, was supplied by Eigenmann & Veronelli, Italy. Distillate samples comprising 20 Scotch Malt Whisky, 3 Cognac, 4 Bourbon, and 3 Rum were provided by The Scotch Whisky Research Institute (Edinburgh, UK). Two distillate samples, a Grappa and a Brandy, were purchased locally in Italy. For method development purposes (see below), a young Grappa (no detectable vanillin), was purchased locally.

2.3 Sample preparation

The vanillin standard, other synthetic vanillins, and the vanillin ex-lignin were made up as 1 mg/mL solutions in ethanol. For repeatability measures, a 10 ppm vanillin solution was made in model distillate (water and ethanol) as follows: ethanol (60 mL) was spiked with 100 μL of 1 mg/mL vanillin standard and made to 100 mL with deionised water. For the limit of adulteration test, volumes of vanillin standard and vanillin ex-lignin (Table 2) were added to 25 mL of the young grappa.

2.3.1 Edible commercial powders and extracts

Commercial samples (powder: 1 g; extract/essence: 2 mL) were prepared in ethanol (2.5 mL; 1 mL). Samples were then vortexed, and (deionised) water (powder: 2.5 mL; extract:

3 mL) was added and the samples were vortexed again to obtain a homogenous solution. Diethyl ether (5 mL) was then added, the samples were mixed, and layers were allowed to separate. The diethyl ether layer was removed and concentrated to dryness under N_2 at room temperature. The concentrates were dissolved in ethanol (1 mL) and vortexed, then diluted 8-fold with a series of 3 two-fold dilutions with ethanol. The samples were transferred to a GC/MS vial for analysis.

2.3.2 Natural vanillin extracts

Diethyl ether (3 mL) was added to the vanillin extract (3 mL) and the sample was shaken for approx. 2 min before the layers were allowed to separate. The diethyl ether layer was removed and concentrated to dryness under N_2 at room temperature. The concentrate was dissolved in ethanol (1 mL) and then vortexed. The sample was diluted 8-fold as above and then injected into the GC-C-IRMS.

2.3.3 Tannin samples

Ethanol (0.9 mL) was added to the tannin sample (100 mg) and vortexed to help dissolve the sample. Deionised water (2.1 mL) was then added to completely dissolve the sample and the solution was again vortexed. Diethyl ether (3 mL) was added, the sample shaken by hand and the layers were allowed to separate. The diethyl ether layer was removed and retained. The water layer was re-extracted with diethyl ether (1 mL) and the ether extracts were combined and then concentrated to dryness under N_2 at 30 °C. The sample was dissolved in ethanol (0.3 mL) and vortexed, transferred to an insert in a GC/MS vial prior to analysis.

2.3.4 Distillate samples

The distillate (30 mL) was adjusted to pH 1 with aqueous HCl (1M). The ethanol was then removed via rotary evaporation at 50 psi and 30 °C using a Heidolph rotary evaporator and filtered using a 45 µm Cellulose Acetate filter (Minisart NML, hydrophilic, non-sterile, Sartorius, Germany). The sample was then extracted with dichloromethane (3 x 3 mL), dried under sodium sulfate, concentrated to dryness under N_2 at 30 °C and dissolved in 0.2 mL of dichloromethane. The sample was then transferred to an insert in a GC/MS vial prior to analysis.

For those distillates where 5-HMF co-eluted with vanillin, 50 mg potassium metabisulfite was added to 25 mL distillate. The mixture was shaken to dissolve the distillate, then extracted as above, and the extract was analysed by GC-C-IRMS.

2.4 Instrumental methods

2.4.1 Elemental analysis (EA) of the vanillin standard, vanillin ex-lignin and 5-hydroxymethylfurfural (5-HMF)

The $\delta^{13}C$ values of the vanillin standard, vanillin ex-lignin and 5-HMF were measured implementing an elemental analyzer (Flash EA 1112, Thermo Scientific, Bremen, Germany), furnished with an autosampler (Finnigan AS 200, Thermo Scientific) and interfaced to a DELTA V isotope ratio mass spectrometer (Thermo Scientific) through a ConFlo IV dilutor (Thermo Finnigan, Bremen, Germany) (see Fig. S1, Supporting information). The temperature of the combustion reactor was 910 °C and for the reduction reactor the temperature was 680 °C, the post reactor GC-column temperature was 45 °C. The He carrier gas had a flow rate of 120 mL/min, the reference gas flow rate was 150 mL/min and the oxygen flow rate was 250 mL/min. The cycle (run time) was 320 s, with a sampling delay of 15 s and an oxygen injection end of 5 s. For EA-IRMS measurements,

tin capsules (SÄNTIS analytical AG, Teufen, Switzerland) were used. Samples and standards (2 different casein standards, one used as the working standard and the other as a control within the sequence) were weighed (0.8 mg) in replicate. A blank sample was run first in the sequence and then the working standard (x2), the samples in replicate, the control (x2) and at the end of the sequence, the working standard (x2). Daily calibration checks were undertaken using the calibrated working casein standard (MRI 64) and controlled with the other casein standard (MRI 63).The isotopic values were calculated against two in-house standards and calibrated against the international reference materials: L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), mineral oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA). The values were expressed in δ‰ against the international standard (Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$). The uncertainty of measurements (2σ) was ±0.3‰.

2.4.2 GC-C-IRMS analysis of samples for $\delta^{13}C$ of vanillin

$\delta^{13}C$ and retention time for the vanillin standard was determined by GC-C-IRMS and identified by GC/MS with selected ions (m/z: 152, 151, 81, 109, 123) and compared with the NIST library (NIST Standard Reference Database 1A NIST/EPA/NIH Mass Spectral Library (NIST 08) and NIST Mass Spectral Search Program (Version 2.0f)).

A Trace GC Ultra (GC Isolink + ConFlo IV, Thermo Scientific, Bremen, Germany) furnished with an autosampler (Triplus, Thermo Scientific) and a ZB-FFAP column (30 m x 0.25 mm i.d. x 0.25 μm film thickness, Phenomenex, Milan, Italy) installed. The GC was interfaced to an IRMS (DELTA V, Thermo Scientific, Bremen, Germany) and a single quadrupole MS (ISQ Thermo Scientific, Bremen, Germany) by an open split device.

The commercial vanillin samples, synthetic samples, vanillin extract and some distillate samples were analysed with the following method ('short' method); the initial oven temperature was 65 °C and was held for 1 min and then increased to the final oven temperature of 250 °C at a rate of 20 °C/min and held at this temperature for 16 min. The method of analysis for the tannin samples was as for the commercial vanillin samples except that the final hold time was 26 min. The amount of sample and standard injected in splitless mode was 0.4 μL with a splitless time of 2 min. Helium was used as the carrier gas and the flow rate was 1.4 mL/min. For the distillate samples ('long' method) the initial oven temperature was 50 °C and held for 10 min, it was then increased at a rate of 3 °C/min to 90 °C and held for 2 min, and increased again to 250 °C at a rate of 6 °C/min and then held at this temperature for 20 min. The flow rate of He was 1.2 mL/min. The sample was injected in splitless mode with a splitless time of 2 min and the volume injected was 0.6 μL (for the standard the volume injected was 0.4 μL). The injector temperature for all samples was 260 °C and the transfer line temperature was 200 °C. Electron impact (EI) was the mode of ionization at 70 eV.

Compounds eluting from the GC column were split 90:10, IRMS:MS, respectively. Before the eluent flows via an open split to the IRMS it is first combusted to CO_2 and H_2O in a combustion reactor which consists of an alumina tube (320 mm length) comprising three braided wires of nickel oxide, copper oxide and platinum (0.125 mm diameter, 240 mm length) centered within the tube. A Nafion® membrane inside a water trap removed water vapor from the eluent/sample. The combustion reactor was operated at a temperature of 1030 °C.

The performance of the instrument was monitored using the vanillin standard $\delta^{13}C$ value, which was measured by GC-C-IRMS before, within, and at the end of each run. Three

direct injections of CO_2 were similarly undertaken at the beginning and end of each sample run. Each sample was measured in triplicate and the isotopic ratio was expressed in δ‰ relative to V-PDB (Vienna – Pee Dee Belemnite) for $\delta^{13}C$ according to the notation developed by Brand and Coplen [43]. A correction was applied to the instrumental data to account for the $\delta^{13}C$ value difference between the measurement of the vanillin standard by EA-IRMS and that of GC-C-IRMS.

The accuracy of the GC-C-IRMS $\delta^{13}C$ values for the vanillin standards, 5-HMF and the vanillin ex-lignin was established by plotting the $\delta^{13}C$ values against the $\delta^{13}C$ values determined by EA-IRMS. The $\delta^{13}C$ values obtained by EA-IRMS were the mean of at least two measurements. The $\delta^{13}C$ values obtained by GC-C-IRMS were the mean of at least three measurements. The $\delta^{13}C$ values were shown to be linearly correlated thus demonstrating the integrity of the method (see Fig. S2).

The repeatability (1σ) of the 'short' and 'long' method for GC-C-IRMS analysis was evidenced by analyzing 10 times (average of three measurements each) a vanillin standard, a vanillin extract, a grappa spiked with vanillin ex-lignin and the model distillate. For the 'short' method it was ±0.1‰ for the vanillin standard, ±0.2‰ for the vanillin extract and ±0.3‰ for the model distillate. For the 'long' method it was ±0.5‰ for $\delta^{13}C$ of the vanillin standard, ±0.4‰ for grappa and ±0.1‰ for model distillate. For a distillate (Scotch Malt Whisky) sample the standard deviation of two replicates (average of three measurements each) for the GC-C-IRMS analytical runs was ±0.4‰ for $\delta^{13}C$.

Reliable measurements of the isotope ratios were achieved when the amplitude of the m/z ion 44 was greater than 350 mV. This occurred for vanillin concentrations ≥ ~63 µg/mL

as shown in Table S1 and Figure S3 (Supporting Information). At this concentration, the standard deviation of the $\delta^{13}C$ of vanillin was ≤ 0.30, which was deemed acceptable to give reliable isotope ratios.

Statistical analysis

Basic statistics of the samples, such as mean, standard deviation and linear correlation were done in excel. The results were statistically analysed by one-way ANOVA, Tukey's Honest Significant Difference (HSD) test, and a boxplot combined with a scatterplot using the R program (R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (URL https://www.R-project.org/).

3. RESULTS AND DISCUSSION

3.1 Sample selection

This work required measuring $\delta^{13}C$ values in various sources of vanillin in order to establish typical ranges. As this study was interested in oaked distillates, 17 tannin samples and one ex-lignin sample were selected to provide sufficient $\delta^{13}C$ values to cover the range expected from this source. In addition, seven synthetic vanillin samples were tested to provide an indication of $\delta^{13}C$ values expected if adulteration of the distillates occurred. In terms of the distillates, 32 samples were chosen covering a variety of starting plant products – barley/wheat (Scotch malt whisky); grapes (cognac, grappa, brandy), corn (bourbon) and sugar cane (rum).

The report is divided into three sections. First, typical $\delta^{13}C$ ranges were measured in sources of vanillin as described above. This was followed by development of a GC-C-IRMS method capable of determining $\delta^{13}C$ values in distillates. As will be discussed

below, the complexity of the aroma profile arising from some distillates meant that there was co-elution of other volatile compounds with vanillin. Attempts to resolve this issue are covered below. The third section reports the $\delta^{13}C$ values found in the distillates, with evidence presented that one distillate was adulterated with synthetic vanillin.

3.2 GC-C-IRMS of vanillin from tannins, from vanilla and synthetic vanillin

Vanillin in distillates derives from wood lignin and therefore measuring the $\delta^{13}C$ values of this type of vanillin was required. As well as wood lignin, vanillin can be derived from the use of tannin coadjuvants, which is permitted in distillate production. Thus the $\delta^{13}C$ of vanillin ex-lignin and of authentic vanillin from 17 samples of commercial tannins were determined. The $\delta^{13}C$ values range between -29.5‰ and -26.7‰ and mainly agree with those found in the literature: from -28.2‰ to -26.8‰ [23, 26, 30, 32, 33, 37, 44, 45]. The $\delta^{13}C$ values also partially overlap with those of synthetic vanillin (ex-guaiacol): from -36.2‰ to -27.4‰ [23, 26-28, 30, 33-35, 37] and biosynthetic (ex-turmeric): from -29.3‰ to -28.7‰ [23] (Fig.1).

For comparison, samples of natural vanilla extracts, commercial food powder and food extracts and synthetic vanillin were analysed. The natural vanillin extracts (-21.0‰ to -19.3‰) had values of $\delta^{13}C$ within the range found in the literature for natural vanillin (-21.8‰ to -14.6‰) [23, 26-38]. They also have a higher $\delta^{13}C$ than for the tannin and the vanillin ex-lignin (mentioned above) which is expected, as their formation follows different photosynthetic pathways (CAM vs C3). The synthetic vanillin/commercial reagents of vanillin (-32.6‰ to -29.3‰) showed that their $\delta^{13}C$ values were within the range for synthetic vanillin in the literature (mentioned above) and are lower than $\delta^{13}C$ values for natural vanillin, but slightly overlap the lower end of the tannin $\delta^{13}C$ range.

A one-way ANOVA comparing the means of the differently sourced vanillin confirmed that the $\delta^{13}C$ ratios for synthetic vanillin, tannin extracted vanillin and natural vanillin were significantly different ($p<0.05$). Furthermore, Tukey's HSD test showed that the synthetic vanillin, tannin extracted vanillin and natural vanillin were different from each other, whereas the tannin extracted vanillin and the vanillin ex-lignin were not different, which was expected.

The analysis of commercial food vanillin, as a powder or an extract, to be added to baked goods such as cakes and biscuits, resulted in 5 of the products (-22.5‰ to -18.9‰) with their $\delta^{13}C$ value in the range of natural vanillin and 2 of the products (-31.7‰ & -31.4‰) in the range of synthetic vanillin. Indeed these 2 products had labels with the words 'aroma for sweets' and they therefore weren't labelled as 'natural', providing further confirmation of the validity of the method.

3.3 Development of the method for analysis of $\delta^{13}C$ of vanillin in distillates

As mentioned above, distillates are aged in oak barrels, sometimes up to 20 years or longer, and there is the possibility that the process of aging changes the isotopic ratios of compounds over time through fractionation. This would mean that $\delta^{13}C$ values could not be used to identify sources of vanillin. Therefore an experiment was undertaken to confirm that the $\delta^{13}C$ values of vanillin do not change during the extraction and aging process of the distillate. The vanillin isotope ratio for a grappa stored in a new oak barrel was compared to that of the same grappa stored in a seven year old oak barrel. As expected, the concentration of vanillin was much higher in the grappa from the old oak barrel than for the new barrel, because vanillin is leached from the barrel over time. However, the $\delta^{13}C$ values did not change: that of vanillin for the new oak barrel was -

28.0‰ (0.37 SD) whereas that of vanillin for the old barrel was -27.6‰ (0.51 SD), indicating that there was no fractionation.

Another factor to consider in the method was the point at which the addition of synthetic vanillin to a distillate was noticeable. To determine this we spiked a sample of young grappa (containing no vanillin) with vanillin ex-lignin, and synthetic vanillin was added in increments and $\delta^{13}C$ values measured. Based on the results (Table 2), it is clear that an addition of more than 120 μL of synthetic vanillin in 25 mL of young grappa (i.e. 60% of the total vanillin present in the distillate is synthetic vanillin and 40% is ex-lignin standard), gives a $\delta^{13}C$ value outside the tannin range (-29.5‰ to -26.8‰). This provides the lower limit at which adulteration can be detected by isotope ratios – below 60% adulteration, the $\delta^{13}C$ value would fall in the expected range for vanillin derived from the barrel. Although this may seem quite high, it would be difficult to achieve in practice, since it would require a very precise measurement of the concentration of vanillin occurring in the original distillate.

It also was of interest to determine at what point adulteration by natural vanillin could be detected. Although this is unlikely to occur due to the high cost of natural vanillin, nevertheless, such an exercise presents an opportunity to further understand the system. In this case isotope ratios were simulated via calculation and the $\delta^{13}C$ values are presented in Table 3. As can be seen, addition of only 20% natural vanillin gives a $\delta^{13}C$ value outside the tannin range.

Having established some basic parameters for measuring $\delta^{13}C$ values, further method development was achieved by optimising the gas chromatographic separation of flavour and aroma compounds. As mentioned above, the interactions between the wood barrel

and the distillate ensure a highly complex matrix, which is challenging to analyse chromatographically. Indeed co-elution was a major issue and in this instance the co-elution of vanillin with other compounds, particularly 5-HMF was observed (see below).

Separation of the analytes was achieved using two different temperature programs. A 'short' method (26 mins) was implemented successfully for some distillates, but for other more complex distillates many of the compounds in the matrix co-eluted and therefore, a longer run (60 mins) was developed. Even with the longer method there were still issues with vanillin co-elution, primarily with 5-HMF, and particularly when the concentration of 5-HMF was overloaded, which can occur with rum. To try to remove/reduce the amount of 5-HMF in the sample we trialled the use of potassium metabisulfite additions to distillates.

A reduction in the amount of 5-HMF was observed, but also in the amount of vanillin. Potassium metabisulfite will react with the aldehyde groups of both 5-HMF and vanillin to form sulfonic acids, which are expected to be less volatile than the original aldehyde and therefore not detected by GC/MS and IRMS. However in adding metabisulfite, it was hoped to reduce the peak due to 5-HMF enough so that the vanillin peak was separated from the 5-HMF tail, but unfortunately this did not occur. Isotopic fractionation of vanillin did not occur with the removal of 5-HMF with potassium metabisulfite.

The influence of the co-eluting 5-HMF on the $\delta^{13}C$ value of vanillin was observed in a whisky sample where an isotopic ratio of -23.6‰, was recorded. This is much higher than $\delta^{13}C$ values for vanillin derived from tannins (Fig. 1).

Depending on its origin (barrel, addition of caramel), 5-HMF may have a different $\delta^{13}C$ value to vanillin and therefore could affect the $\delta^{13}C$ ratio of vanillin if the peaks co-elute. A synthetic standard of 5-HMF gave a $\delta^{13}C$ value of -23.7‰ by EA-IRMS. 5-HMF from

the whisky sample had a $\delta^{13}C$ value of -7.4‰ by GC-C-IRMS, and is clearly not of synthetic origin. A high $\delta^{13}C$ value such as this suggests the 5-HMF is derived from cane sugar, since sugar cane implements the C4 photosynthetic pathway giving $\delta^{13}C$ values between -16 and -10‰ [46] for sugar. Caramel is a permitted additive to whisky and contains 5-HMF, therefore it appears that this whisky had caramel derived from cane sugar added to it. Nonetheless, the $\delta^{13}C$ value for 5-HMF in this sample is quite high for a C4 plant. According to a study undertaken by González-Pérez [47] 5-HMF has on average a 3‰ higher $\delta^{13}C$ value than the sugar it was derived from; therefore, the value of -7.4‰ is possible given the value of the sugarcane is around -10‰.

The results from this analysis of 5-HMF have implications for the accurate measurement of $\delta^{13}C$ values for vanillin in distillates. That is, co-elution of 5-HMF with vanillin will alter the $\delta^{13}C$ value of vanillin. In the example described above, the high $\delta^{13}C$ value for 5-HMF will raise the apparent $\delta^{13}C$ value for vanillin, making it appear that the distillate may have been adulterated with natural vanillin (which also raises the $\delta^{13}C$ value, see above).

Apart from 5-HMF, there was another compound that was detected by GC/MS as co-eluting with vanillin. Implementing the NIST library, this compound was tentatively assigned as 4-ethoxy-3-anisaldehyde (4-ethoxy-3-methoxybenzaldehyde; m/z: 151, 152, 180, 109, 123, 181, 153); structurally similar to vanillin (an ethoxy instead of a hydroxy group). As the compounds both derive from the same process and barrel, their $\delta^{13}C$ values were deemed to be the ‘same’ and therefore for our purposes we analysed the peaks as one.

$\delta^{13}C$ of vanillin of commercial distillates

A survey of 32 spirits (Scotch Malt Whisky, Cognac, Bourbon, Rum, Grappa, Brandy) on the market showed that the majority of spirits contained lignin derived vanillin (Table 4). The $\delta^{13}C$ values of vanillin for these distillates ranged from -28.9‰ to -25.7‰, which mostly lie within the values determined for vanillin derived from tannins, and therefore come from the barrel (Fig. 2). One spirit was found to have a comparatively high concentration of vanillin (the peak dominated the chromatogram, almost to the point of being overloaded, though it was still within the linearity range) and the vanillin was determined to be synthetic based on its $\delta^{13}C$ value (-32.5‰, SD: 0.05). Another spirit, brandy, was found to have ethyl vanillin (determined by comparing ions with the NIST library), which is a synthetic compound, as it cannot be made in nature: thus, this spirit was not measured (for ethyl vanillin or vanillin) as it co-eluted with the vanillin peak in the sample. There were also some distillates for which the vanillin peak eluted in the tail of the 5-HMF peak or the concentration of vanillin was too low to be measured accurately by GC-C-IRMS and therefore mentioned in the table as not detected (nd).

CONCLUSIONS

In this study, $\delta^{13}C$ values for 48 samples of vanillin were determined by GC-C-IRMS. Isotope ratio ranges found for vanillin ex-lignin, synthetic and natural vanillin were consistent with those reported in the literature. The method was able to distinguish between natural and synthetic vanillin in commercial flavouring ingredients.

In applying the GC-C-IRMS method to distillates, first the $\delta^{13}C$ range for tannin-derived vanillin was established and found to be consistent with the literature. Issues to do with barrel aging and the limit at which adulteration could be detected were also investigated. Of the 32 distillate samples analysed, one was clearly shown to have had synthetic vanillin

added. Co-elution of 5-HMF was problematic, particularly in rum samples. Further work could investigate the possibility of multidimensional GC coupled to C-IRMS to assist in separating the two compounds.

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Table 1: Sample Information

Sample type	Description	No. of samples
Synthetic vanillin	Sigma Aldrich	1*
	Sigma Aldrich	2
	Fluka	1
	Carlo Erba	3
Natural vanillin	ethanol/water extract	16
Commercial vanillin	powder – natural extract	4
	liquid – natural extract	1
	powder – aroma for sweets	1
	essence – aroma for sweets	1
Tannin	powder – extracted from wood	17
	Borregaard EuroVanillin Supreme ex. Lignin	1
Distillates	Scotch Malt Whisky	20
	Cognac	3
	Bourbon	4
	Rum	3
	Grappa	1
	Brandy	1

*used as the standard for method development

Table 2: Addition of synthetic vanillin to a young grappa spiked with vanillin ex-lignin

Vol. of synthetic vanillin added (μL)	Vol. of vanillin ex-lignin spiked (μL)	$\delta^{13}C$ (‰)	Std Dev (n=3)
200	0	-32.3	0.4
160	40	-30.7	0.5
120	80	-29.7	0.3
80	120	-28.9	0.4
40	160	-28.1	0.3
0	200	-27.9	0.5
0	0	no vanillin	NA

Table 3. Simulation of addition of natural vanillin to a distillate spiked with vanillin ex-lignin

Vanillin added (μL)	% of natural vanillin	Vol. of vanillin ex-lignin spiked (μL)	Simulated $\delta^{13}C$ (‰)*
200	100	0	-20**
160	80	40	-21.6
120	60	80	-23.1
80	40	120	-24.7
40	20	160	-26.2
0	0	200	-27.8
0	0	0	no vanillin

* calculated e.g.: 20% of natural vanillin + 80% of lignin vanillin

** generalised value for natural vanillin

Table 4. $\delta^{13}C$ values of vanillin in distillates

Type of Spirit	Average $\delta^{13}C$ (‰)	Std Dev. (n≥3)
Scotch Malt Whisky	-27.6	1.1
Scotch Malt Whisky	-26.7	0.3
Scotch Malt Whisky	nd[a]	na
Scotch Malt Whisky	-26.7	0.6
Scotch Malt Whisky	-27.2	0.4
Scotch Malt Whisky	-28.9	1.0
Scotch Malt Whisky	-27.7	0.2
Scotch Malt Whisky	-27.9	0.2
Scotch Malt Whisky	-28.2	0.9
Scotch Malt Whisky	-27.2	0.3
Scotch Malt Whisky	-26.2	0.3
Scotch Malt Whisky	-27.9	0.2
Scotch Malt Whisky	-26.6	1.0
Scotch Malt Whisky	-26.0	0.5
Scotch Malt Whisky	-27.9	0.2
Scotch Malt Whisky	-26.2	0.4
Scotch Malt Whisky	-26.6	0.3
Scotch Malt Whisky	-26.4	0.3
Scotch Malt Whisky	-26.4	0.6
Scotch Malt Whisky	-28.1	0.3
Cognac	-26.0	0.1
Cognac	-27.5	0.4
Cognac	-25.7	0.2
Bourbon	-27.4	0.4
Bourbon	-27.6	0.2
Bourbon	-27.8	0.2
Bourbon	-28.2	0.3
Rum	-32.5	0.1
Rum	nd[b]	na
Rum	nd[b]	na
Grappa	-27.3	0.5
Brandy	ethyl vanillin	na

[a] concentration of vanillin too low for $\delta^{13}C$ value to be determined accurately
[b] $\delta^{13}C$ value for vanillin could not be determined due to co-elution with 5-HMF

Figure 1. Boxplot of synthetic, natural and tannin vanillin combined with a scatterplot of the commercial vanillin and vanillin ex-lignin

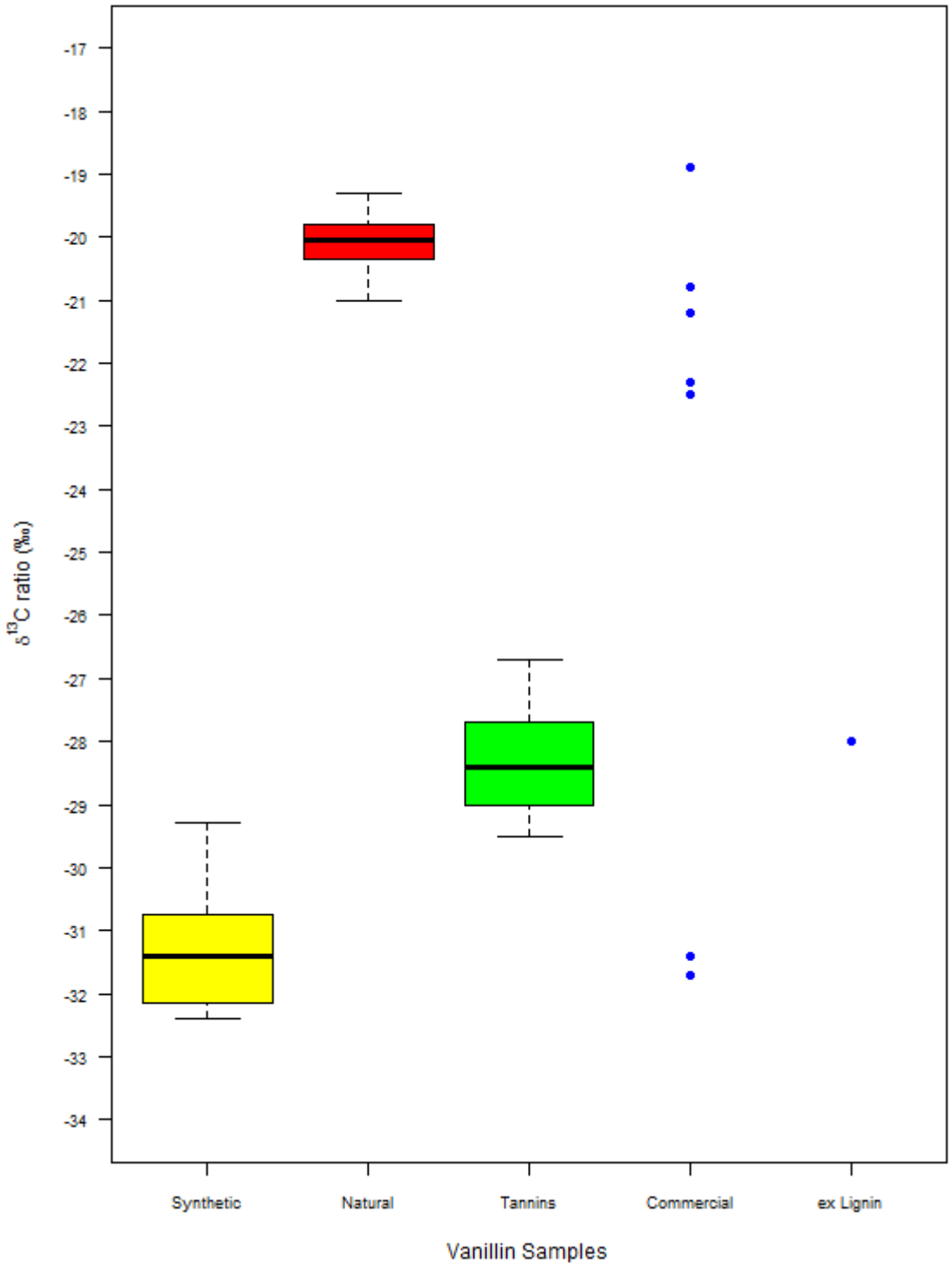
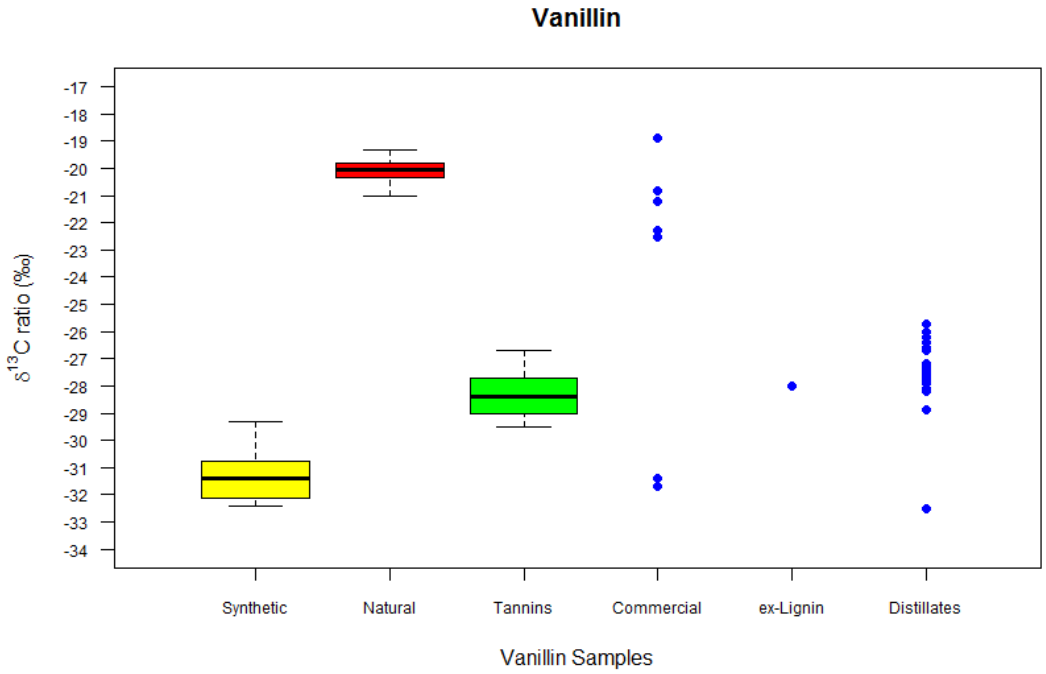


Figure 2. Boxplot of synthetic, natural and tannin vanillin combined with a scatterplot of the commercial vanillin, vanillin ex-lignin and distillate samples

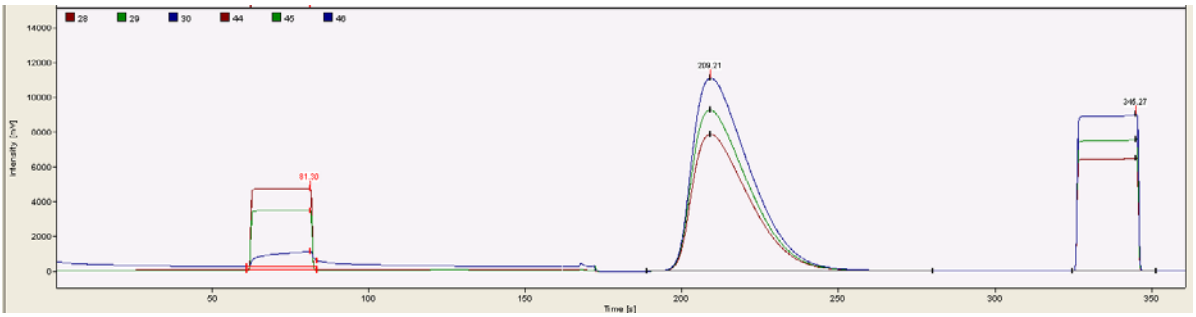


Supporting information for Differentiation of wood derived vanillin from synthetic vanillin in distillates implementing GC-C-IRMS for $\delta^{13}C$, by van Leeuwen et al.

Table S1. Calibration curve results for the vanillin standard by GC-C-IRMS

Mean Amplitude of ion: m/z 44 (mV)	**Concentration of vanillin standard (mg/mL)**	**Std Dev (n=3)**	**Corrected $\delta^{13}C$ ratio (‰)**
53	0.0078	1.16	-28.116
84	0.016	0.90	-29.128
200	0.03	0.55	-28.798
359	0.0625	0.30	-28.699
766	0.125	0.25	-28.934
1950	0.25	0.22	-29.551
3994	0.5	0.17	-29.490
8155	1.0	0.05	-29.287
11009	1.5	0.04	-29.153
14478	2.0	0.05	-29.119
17001	2.5	0.06	-29.176

Figure S1. EA-IRMS chromatogram of a vanillin standard for carbon. The red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



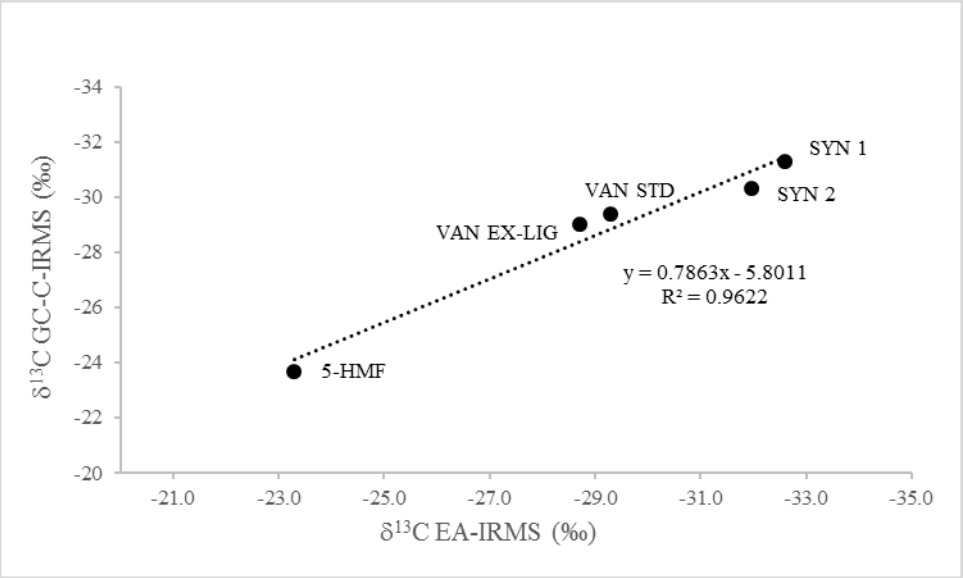
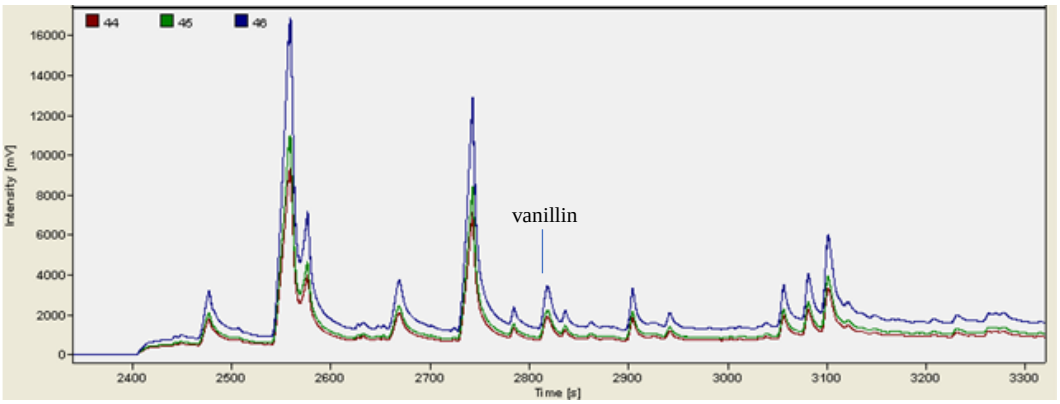


Figure S2. $\delta^{13}C$ values of vanillin (ex-lignin, & synthetic) and 5-HMF implementing GC-C-IRMS ($n \geq 3$) versus EA-IRMS ($n \geq 2$).

Figure S3. Carbon GC-C-IRMS chromatogram of a distillate sample with vanillin as the target compound. The red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



CHAPTER FOUR

INTRODUCTORY COMMENTS

RESEARCH PAPER

Paper 3 van Leeuwen, K.A., Camin, F., Jerónimo, E., Vasta, V., Prenzler, P. D., Ryan, D., & Bessa, R. J. B. (2017). Dietary effects on stable carbon isotope composition of polar and neutral fatty acids in the intramuscular fat of lambs.

Submitted to Journal of Agricultural and Food Chemistry: 29 Jun 2017
DOI:10.1021/acs.jafc.7b02999.

Presented in the style of the journal: Journal of Agricultural and Food Chemistry.

This paper was accepted after the submission date of the thesis and the final version is slightly different to that presented in Chapter Four.

INTRODUCTORY COMMENTS

Ruminant meat nutritional value is currently a hot topic in food science. In particular, researchers have studied ways to enhance animal feed with polyunsaturated fatty acids (PUFA) so as to reduce the level of saturated fatty acids in the resultant meat.

In a study by Jeronimo (2010) lambs were fed a diet supplemented with oil and a tannin source with the observation of an increase in the intramuscular fat. Given that neither of these dietary regimes, tannin or oil supplementation, should influence the fatty acids (FA) accumulation in muscles, it is not clear how this takes place. Understanding whether this phenomena occurs via *de novo* synthesis of FA or from dietary preformed FA is of paramount importance to further studies on intramuscular fat deposition and to optimise meat nutritional quality.

Carbon isotopic ratios ($\delta^{13}C$) can be used to distinguish between FA from the diet and those resulting from the *de novo* synthesis. This chapter presents a GC-C-IRMS method to measure the $\delta^{13}C$ of the main FA present in neutral (NL) and polar (PL) intramuscular lipids of meat samples from lambs fed with 4 different dietary regimes namely a control diet, a diet supplemented with oil, a diet supplemented with *Cistus ladanifer* and a diet supplemented with *Cistus ladanifer* and oil. This research was of increased complexity to that of the work of vanillin (single compound analysis) as it focused on the analysis of multiple analytes belonging to one class of structurally related compounds, i.e. FA.

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Dietary effects on stable carbon isotope composition of polar and neutral fatty acids in the intramuscular fat of lambs

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ABSTRACT

In this study we measured the $\delta^{13}C$ of the main fatty acids (FA) present in neutral and polar intramuscular lipids of meat samples from 24 lambs, fed with 4 different diets supplemented with oil and the tanniferous shrub *Cistus ladanifer* L. The objective was to understand if the increase in intramuscular fat observed in the lambs fed simultaneously *C. ladanifer* and oil was explained mostly by incorporation of diet derived FA or by increased *de novo* FA synthesis. *De novo* FA synthesis was evaluated by the ^{13}C enrichment (‰) of 16:0 in tissues compared to bulk diet or compared to dietary 16:0. The oil reduced the ^{13}C enrichment of 16:0 in lipid, but had no effect when the diet included *C. ladanifer* (*P* value < 0.01). Thus the inclusion of *C. ladanifer* in the diets blocked the inhibitory effects of lipid supplementation on the *de novo* FA synthesis.

INTRODUCTION

Currently there is a lot of research aiming to improve the nutritional value of ruminant meats by supplementing the diets with lipid sources rich in polyunsaturated fatty acids (PUFA) in order to reduce the hypercholesterolemic saturated fatty acids (SFA) and increase the conjugated linoleic acid (CLA) isomers [1-3]. Moreover, condensed tannins have been proposed as modulators of PUFA biohydrogenation in the rumen, reducing the completeness of those pathways and thus increasing the availability of health beneficial biohydrogenation intermediates like 18:1 *trans*-11 and 18:2 *cis*-9,*trans*-11[4]. In a previous study [5] the effect of two dietary condensed tannin sources (*Cistus ladanifer* L. and grape seed extract) and oil (sunflower and linseed oil blend) supplementation on lamb meat fatty acid (FA) composition was explored. The authors found that diet supplemented by oil and *C. ladanifer* increased significantly the deposition of intramuscular FA compared to the other diets. Intramuscular fat deposition is one of the major determinants of meat quality affecting positively its tenderness, flavor and juiciness. As neither the lipid supplementation of diets nor the inclusion of tanniferous dietary sources are expected to increase intramuscular FA deposition *per se* the reason why simultaneous inclusion of oil and *C. ladanifer* in the diets of lambs increased the intramuscular FA deposition remains unclear and deserves to be further investigated.

Oleic (18:1 *cis*-9), palmitic (16:0), stearic (18:0) and less abundantly linoleic (18:2*n*-6) acids comprise the overwhelming majority (~70 to 85% of total FA) of FA present in lamb tissues. Linoleic acid is an essential FA and thus must be supplied exogenously through dietary sources, but the 18:1 *cis*-9, 18:0 and 16:0 present in tissue lipids might derive either from *de novo* synthesis or from preformed FA derived from diet. Dietary lipid supplementation of ruminants increases the metabolic availability of preformed FA derived from diet but strongly depresses the *de novo* FA synthesis pathways in tissues [6], and thus does not usually promote fat deposition in muscles. Tanniferous shrubs, like *C.*

ladanifer, have low nutritive value and thus do not tend to increase the availability of metabolizable energy necessary to favor *de novo* FA synthesis in tissues. Nevertheless, as condensed tannins form complexes with dietary protein, they might reduce the protein/energy ratio of nutrients absorbed from the digestive tract and induce intramuscular fat deposition as well described in other species [7]. Information on which proportion of FA present in animals muscles derive from *de novo* synthesis or from dietary derived preformed FA would help to define targets for further investigation on intramuscular fat deposition.

When animals assimilate FA from the diet, their stable isotope ratios of carbon (expressed as $\delta^{13}C$ values) are generally identical to those of dietary FA [8-9]. However, FA resulting from *de novo* synthesis present lower $\delta^{13}C$ values than those derived from the diet and from whole tissue components due to depletion of the ^{13}C isotope mediated mostly by the pyruvate dehydrogenase enzyme [10]. Thus, the analysis of $\delta^{13}C$ of FA present in animal tissues using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) has potential to discriminate the metabolic origin of FA present in animal tissues and provide new insight on the study of intramuscular fat deposition in ruminants.

In the current investigation, we implemented a GC-C-IRMS method to measure the $\delta^{13}C$ of the main FA present in neutral (NL) and polar intramuscular lipids (PL) of meat samples from lambs. Lambs were fed with 4 different feed treatments (control diet: C0, diet supplemented with oil: C6, diet with *Cistus ladanifer*: CL0 and diet supplemented with both oil and *Cistus ladanifer*: CL6) as described in Jerónimo, et al. [5]. NL, comprised mostly of triacylglycerols, are the main lipids accumulated in muscle when intramuscular fat increases, while PL or phospholipids are structural components of cell membranes and concentrations remain fairly constant independent of total lipid deposition on muscle [1]. The main objective was to apply GC-C-IRMS to understand whether the increase in NL

of intramuscular FA observed in lambs fed oil and *C. ladanifer* was explained mostly by incorporation of diet derived preformed FA or by increased *de novo* FA synthesis.

MATERIALS & METHODS

Animal treatments and sample preparation.

The lipid samples were derived from a lamb production experiment described in detail by Jerónimo, et al. [5]. Briefly, 24 Merino Branco ram lambs were allocated to one of 4 diets: C0) a basal diet containing 900 g/kg of dry matter (DM) of dehydrated lucerne and 100 g/kg of DM of wheat bran; CL0) the basal diet with incorporation of 250 g/kg of *C. ladanifer*; C6) the basal diet supplemented with 60 g/kg of DM of an oil blend; CL6) the basal diet and 250 g/kg of DM of *C. ladanifer* and oil blend 60 g/kg of DM. The oil blend consisted of sunflower and linseed oils (1:2, v/v) and *C. ladanifer* was composed of leaves and soft stems of the plant. The lambs were fed every day for 6 weeks and then slaughtered. *Longissimus dorsi* muscle samples were collected on the third day after slaughter, freeze-dried and stored vacuum packed at -80 °C until required for lipid analysis. Intramuscular lipids were extracted with dichloromethane and methanol (2:1 v/v) and separated in NL and PL, using a solid-phase extraction with silica gel cartridges (LiChrolut® Si, 40-63 μm, 500 mg/mL, Standard, Merck KGaA, Darmstadt, Germany) as previously described by Jerónimo, et al. [11]. Transesterification of the NL and PL portions were undertaken in a two-step process which involved sodium methoxide in methanol and then hydrochloric acid in methanol as described by Raes, et al. [12].

Materials.

Methyl pentadecanoate (me-15:0; 99.5 %, Fluka), methyl palmitate (me-16:0; 99.0 %, Sigma), methyl heptadecanoate (me-17:0; 99.5 %, Fluka), methyl stearate (me-18:0; 99.5 %, Fluka), methyl oleate (me-18:1 *cis*-9; 99.0 %, Fluka), methyl linolenate (me-18:3*n*-3;

99.0 %, Sigma), methyl linoleate (me-18:2*n*-6; 99.0 %, Sigma) and methyl *cis*-11-octadecenoate (me-18:1 *cis*-11; ≥ 99.0 %, Fluka) reference standards were purchased from Sigma Aldrich (Milan, Italy) and stored at 4 °C or at – 18 °C.

The international reference materials used for calibrating the working in-house standards for elemental analysis (EA) were L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6. For GC-C-IRMS analysis carbon dioxide (CO_2) with 99.998% purity was used (Rivoira, Italy).

The reagents used for the preparation of FAME were *n*-hexane (GC-grade, Merck, Portugal), toluene (anhydrous, 99.8%, Sigma Aldrich, Spain), sodium methoxide in methanol (sodium methoxide solution, ACS reagent, 0.5 M CH_3ONa in methanol (0.5 N) Sigma Aldrich, Spain), hydrochloric acid (ACS reagent 37% HCl, Sigma Aldrich, Spain), dichloromethane (GC-grade, Merck, Portugal) and methanol (GC-grade, Merck, Portugal). For analysis the solvent used was *n*-hexane (ACS – For analysis, Carlo Erba, Milan, Italy).

For elemental analysis-isotope ratio mass spectrometry (EA-IRMS) measurements executed in tin capsules (SÄNTIS analytical AG, Teufen, Switzerland).

Stable Isotope Ratio Analysis (SIRA)

1. EA-IRMS

Separately and in duplicate, the fatty acid methyl ester (FAME) reference standards (0.4 mg) were weighed (Satorious Pro II Micro Balance) in tin capsules and then transferred to an autosampler (Finnigan AS 200 Thermo Scientific, Bremen Germany) to be

introduced to the Elemental Analyser (EA Flash 1112 Thermo Scientific, Bremen, Germany), connected to a ConFlo III dilutor (Thermo Scientific, Bremen, Germany) and interfaced with an Isotope Ratio Mass Spectrometer (IRMS Delta Plus XP Thermo Scientific, Bremen, Germany) to determine their $\delta^{13}C$ (Supporting Information (SI 1)). The temperature of the combustion reactor was 910 °C and for the reduction reactor the temperature was 680 °C, the post reactor GC-column temperature was 45 °C. The He carrier gas had a flow rate of 120 mL/min, the reference gas flow rate was 150 mL/min and the oxygen flow rate was 250 mL/min. The cycle (run time) was 320 s, with a sampling delay of 15 s and an oxygen injection end of 5 s. Samples and standards (2 different casein standards, one used as the working standard and the other as a control within the sequence) were weighed (0.8 mg) in replicate. A blank sample was run first in the sequence and then the working standard (x2), the samples in replicate, the control (x2) and at the end of the sequence, the working standard (x2). Daily calibration checks were undertaken using the calibrated working casein standard (MRI 64) and controlled with the other casein standard (MRI 63).

The isotopic composition was denoted in delta [13] in relation to Vienna Pee Dee Belemnite (VPDB), according to the following general equation [14] :

$$\delta^{i}\,E = \frac{\left(i_{R_{SA}} - i_{R_{REF}}\right)}{i_{R_{REF}}}$$

where ^{i} is the mass number of the heavier isotope of element E (^{13}C);

R_{SA} is the respective isotope number ratio of a sample (number of ^{13}C atoms/number of ^{12}C atoms or as an approximation $^{13}C/^{12}C$);

R_{REF} is the relevant internationally recognized reference material such as VPDB for CO_2.

The delta values are multiplied by 1000 and are expressed in units “per mil” (‰).

2. GC-C-IRMS

The FAME reference standards were also analyzed separately to determine retention time with a 6890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) furnished with an autosampler (GC-PAL, GC Analytics AG, Zwingen, Switzerland) and with a BPX-70 capillary column (60 m × 0.32 mm i.d. × 0.25 μm film thickness, SGE, Rome, Italy) installed. The injector temperature was 250 °C throughout the run with the injector mode set on splitless. The initial oven temperature was 50 °C and maintained for 4 min, then ramped to 170 °C at a rate of 30 °C/min, then increased to 200 °C at a rate of 2 °C/min, then ramped again to 220 °C at a rate of 1 °C/min, then increased to 250 °C at a rate of 5 °C/min and maintained for 8 min. The carrier gas was He (Rivoira, purity: 99.999 %) with a constant flow of 1 mL/min. The same conditions were also adopted for the FAME samples. After separation in the GC the sample then flowed through an oxidation reactor which consisted of three braided wires of 0.125 mm diameter inside an 32 cm alumina tube (1 × nickel oxide, 1 × copper oxide, 1 × platinum), kept at 940 °C and housed in the GC whereby it was quantitatively combusted to CO_2 and H_2O (removed via a Nafion® membrane). The GC-C was coupled via an open split interface to an isotope ratio mass spectrometer (IRMS Delta V, Bremen, Germany).

A FAME reference standards mix was prepared (SI 2), in which the compounds’ concentrations were made to match the relative concentration for those corresponding compounds found, in general, in the samples (pentadecanoic acid (15:0): 12.6 μg/mL; 16:0: 51.1 μg/mL; heptadecanoic acid (17:0): 15.3 μg/mL; 18:0: 77.16 μg/mL; 18:1 *cis*-9: 140.9 μg/mL; *cis*-11-octadecenoic acid (18:1 *cis*-11): 18.79 μg/mL; 18:2*n*-6: 52.6 μg/mL and α-linolenic acid (18:3*n*-3): 28.5 μg/mL). This was done to try to get a more

accurate $\delta^{13}C$ value for each compound. Calibration curves were completed, implementing the peak heights (in mV, amplitude 44 for C), for the FAMEs measured to determine which $\delta^{13}C$ values should be accepted.

The FAME samples were diluted with *n*-hexane (approximately 2 mg/mL) before injection and were measured at least 3 times by GC-C-IRMS. To calculate their $\delta^{13}C$ values, the mixture of FAME reference standards was analyzed before and after each FAME sample. The samples were run in separate batches, with each run batch including 8 injections of a standard mix and two samples injected three times each. The specifics of each injection batch were as follows: standard mix (×3), sample 1 (×3), standard mix (×2), sample 2 (×3) and, finally the standard mix (×3). Each batch followed this procedure to account for $\delta^{13}C$ drift within the run. The instrumental data for each sample were corrected on the basis of the difference existing between the $\delta^{13}C$ value of the pure compound in GC-C-IRMS (mean of the first three mix results and mean of the last three mix results) and that in EA-IRMS.

The FAMEs analyzed in the samples were 16:0, 18:0, 18:1 in NL and PL and finally 18:2*n*-6 (in PL only) (SI 3 and SI 4). The FAME which had peaks too small to be analyzed were 15:0, 17:0, and 18:3*n*-3. The 18:1 *cis*-11 and 18:1 *cis*-9 co-eluted and so these FAMEs were analyzed as one peak and will be denoted as 18:1. To normalize the data the EA value (-29.8 ‰) for 18:1 *cis*-9 was used as this peak was much stronger than the 18:1 *cis*-11 peak which had an EA value slightly lower (-30.2 ‰).

The repeatability for the FAME mix run 10 times consecutively was determined for 16:0 (mean ± standard deviation (SD): -29.5 ‰ ± 0.6 ‰) 18:0 (mean ± SD: -23.2 ‰, ± 0.3 ‰), 18:1 (mean ± SD: -29.9 ‰, ± 0.4 ‰) and 18:2*n*-6 (mean ± SD: -30.6 ‰, ± 1.5 ‰) (SI 5).

The overall repeatability over time for FAME in the control mixes for the entire batch of samples is for 16:0 (mean ± SD: -29.8 ‰, ± 1.0 ‰), 18:0 (mean ± SD: -23.2 ‰, ± 0.6 ‰), 18:1 (mean ± SD: -29.7 ‰, ±0.3 ‰) and 18:2*n*-6 (mean ± SD: -29.8 ‰, ± 1.0 ‰) (SI 6). These values were linearly correlated against the EA values for the same FAME (SI 7).

A sample (feed group CL0; NL; 16:0 (mean ± SD: -28.2 ‰, ± 0.8 ‰); 18:0 (mean ± SD: -28.4 ‰, ± 0.8 ‰); 18:1 (mean ± SD: -28.4 ‰, ± 0.7 ‰)) was run 9 times over time to check repeatability over a period of time (SI 8). The data have not been corrected for the added C introduced by the FA conversion to FAME as the difference in values is small (≤ 0.8 ‰, (SI 9)).

Statistical analysis

Data were analyzed using the MIXED procedure of SAS according to a model that considered the 2×2×2 factorial arrangement of factor where the oil (0 vs. 6 g/kg DM) and *C. ladanifer* (0 vs. 250 g/kg) in the diet and lipid fractions (PL vs. NL) were the main effects. The animals were the experimental units and muscle lipid fractions were included in the model considering the covariance of both fractions collected within the same animal using the unstructured (UN) covariance option with the repeated statement of Proc MIXED. Least square means and standard error of means (SEM) are presented in Table 2. When significant interaction effects were detected in the model, the least square means were compared using the Tukey-Kramer method for correcting for multiple comparisons. Significance was accepted for $P < 0.05$.

RESULTS

The FA content (g/kg DM) and the $\delta^{13}C$ values (‰) of the diets are presented in Table 1 [5]. The diets that included the sunflower and linseed oil blend present higher content of all FA reported, but reflecting on the composition of oils the increase was particularly larger for 18:2*n*-6 and 18:3*n*-3. Diets containing *C. ladanifer* presented more FA content (+11 g/kg) than diets without *C. ladanifer*.

The $\delta^{13}C$ value of whole (bulk) diet C0 (-31.3 ‰) was lower than that of the other diets which ranged from -29.0 to -28.8 ‰. The same trend of lower $\delta^{13}C$ values in C0 than in the other diets is observed for the $\delta^{13}C$ values of 16:0, 18:0 and 18:1 but not for 18:2*n*-6 and 18:3*n*-3. Within each diet, $\delta^{13}C$ values of 16:0 and 18:0 were always lower than the respective bulk diet, but this pattern is less clear for the 18:1, 18:2*n*-6 and 18:3*n*-3. Inclusion of oil in the diets had no consistent effects on $\delta^{13}C$ values of individual FA and of whole diet and the same was observed for the inclusion of *C. ladanifer*.

The FA content and profile present in NL and PL fractions of meat from lambs fed the experimental diets are presented in Table 2 [5]. The total FA from PL ranged from 15 to 20 mg/g of meat DM whereas the total FA from NL ranged from 60 to 86 mg/g of meat DM. Only the C6 diet presented a slightly, but significant, lower amount of FA from PL than the other diets. The major FA in PL was the 18:1 *cis*-9 (~23% FA) in lambs fed diet not supplemented with oil and 18:2*n*-6 (~22% FA) in lambs fed oil supplemented diets. The CL6 produced the highest content of FA from NL, and significant interaction ($P = 0.005$) between oil and *C. ladanifer* was observed. The 18:1 *cis*-9 was always the major FA present in NL although its proportion decreased ($P < 0.05$) with oil supplementation, which increases the proportion of 18:2*n*-6. For all FA, the effect of oil supplementation and lipid fraction were highly significant ($P < 0.001$) but also the interaction between oil supplementation and lipid fraction was highly significant ($P < 0.01$). In fact, the response

to oil supplementation observed for 18:0, 18:1 and 18:2*n*-6 in the PL was much larger than that observed in NL, whereas for 16:0 the effect of oil supplementation was larger for NL than in PL. The inclusion of *C. ladanifer* in the diet had no effects on FA proportion except for 16:0 in NL, where the inclusion of *C. ladanifer* increased the 16:0 from 22.9 to 24.5% FA.

The $\delta^{13}C$ values of FA present in NL and PL fractions are also presented in Table 2 and in Figures 1 to 3. It was not possible to determine the $\delta^{13}C$ values for 18:2*n*-6 present in NL, due to its lower concentration in this fraction. The $\delta^{13}C$ values for 16:0 and 18:1 in PL were lower ($P < 0.01$) than those in NL (Figure 1). The differences (mean difference ± SE) of $\delta^{13}C$ values between NL and PL averaged -0.96 ‰ ± 0.269 ($P = 0.003$) and -0.59 ‰ ± 0.163 ($P = 0.001$), respectively for 16:0 and 18:1. The same general pattern was observed for 18:0, as it also presented lower $\delta^{13}C$ values in PL than NL. However, the differences of 18:0 $\delta^{13}C$ values between lipid fractions were larger when diets included oil than when no oil was added (Figure 2A, interaction lipid fraction×oil, $P = 0.03$) or when diets did not include *C. ladanifer*, than when *C. ladanifer* was included (Figure 2B, interaction lipid fraction × *C. ladanifer*, $P = 0.02$). The differences of $\delta^{13}C$ values for 18:0 in NL and PL averaged -0.52 ‰ ± 0.219 ($P = 0.028$) and -1.23 ‰ ± 0.219 ($P < 0.001$) for no oil and 6 % oil diets respectively, and averaged -1.27 ‰ ± 0.219 ($P < 0.001$) and -0.48 ‰ ± 0.219 ($P = 0.042$) for diets without and with *C. ladanifer* respectively. Inclusion of oil in the diets had no effect on the $\delta^{13}C$ value of 18:1 but decreased the $\delta^{13}C$ values of 16:0 (-0.55 ‰ ± 0.259, $P = 0.048$) and 18:2*n*-6 (-1.33 ‰ ± 0.375, $P = 0.002$) in muscle lipids, irrespective of the lipid fraction (Figure 3A). The effect of dietary oil supplementation on $\delta^{13}C$ value of 18:0 were dependent of the lipid fraction (Figure 2A), decreasing more in PL (-1.09 ‰ ± 0.309, $P = 0.002$) than in NL (-0.38 ‰ ± 0.309, $P = 0.057$).

Inclusion of *C. ladanifer* in the diets had no effect on the $\delta^{13}C$ value of 18:2*n*-6 but increased the $\delta^{13}C$ values of 16:0 (+1.85 ‰ ± 0.259, $P < 0.001$) and 18:1 (+1.23 ‰ ± 0.274, $P = 0.002$) in muscle lipids, irrespective of the lipid fraction (Figure 3B). The effect of *C. ladanifer* in the diets on $\delta^{13}C$ value of 18:0 were dependent of the lipid fraction (Figure 2B) as although it increased the $\delta^{13}C$ value of 18:0 in both lipid fractions, that increase was significantly larger ($P < 0.02$) in PL (+1.94 ‰ ± 0.309, $P < 0.001$) than in NL (+1.15 ‰ ± 0.189, $P < 0.001$). No interactions between oil and *C. ladanifer* inclusion were observed for $\delta^{13}C$ values of the FA present in muscle lipids.

Diet C0 was more depleted in ^{13}C than the other diets (Table 1) and this introduced a variation source that was not accounted for in the previous data analysis. Computing the ^{13}C enrichment (‰) of 16:0 present for NL and PL as the difference of $\delta^{13}C$ values of 16:0 in each lipid fraction minus the $\delta^{13}C$ value of bulk diets (Figure 4, left panels) allowed us to detect a significant interaction ($P = 0.0002$) between oil and *C. ladanifer*. In fact, oil supplementation clearly reduced the ^{13}C enrichment of 16:0 present in NL and PL when no *C. ladanifer* was included in the diet but had no effect when the diet included *C. ladanifer*. The same response was observed when the ^{13}C enrichment (‰) of 16:0 present for NL and PL was computed as the difference of $\delta^{13}C$ values of 16:0 in each lipid fraction minus the $\delta^{13}C$ value of dietary 16:0 (Figure 4, right panels). The interaction between oil and *C. ladanifer* is also highly significant ($P = 0.003$) and follows the same pattern as described above.

DISCUSSION

In animal science, GC-C-IRMS has been mainly used to differentiate animals on the basis of their diet with the purpose of food authentication [15] and only very few reports were focused on ruminant lipid metabolism [16-18]. Intramuscular fat deposition is one of the

major determinants of meat quality affecting its sensorial traits as well its FA profile [19]. Dietary manipulations to improve FA composition of meat usually involved lipid supplementation [1]. Lipid supplementation increases the metabolic availability of exogenous (diet derived) FA and usually depresses the *de novo* synthesis of FA in tissues [6] and thus the balance in terms of intramuscular fat deposition tends to be neutral [20]. The development of analytical tools to evaluate the metabolic origin of FA deposited in muscle can be very useful to evaluate the nutritional strategies to simultaneously increase intramuscular fat deposition and improve its FA profile. We used samples from a lamb feeding experiment where lipid supplementation either had a neutral or positive effect on intramuscular fat deposition, depending on the inclusion of a tanniferous shrub in the diet. Our aim was to evaluate the potential of $\delta^{13}C$ FA analysis to discriminate the metabolic origin of the FA deposited in neutral and polar fractions of muscle lipids.

The 16:0 is the FA with better potential to allow the discrimination between its metabolic origins (diet vs. *de novo* synthesis) as already recognized by Richter, et al. [16]. In fact, the 16:0 is not a major dietary FA, particularly of the oil-supplemented diets. Moreover, the 16:0 is the major product of FA synthase and thus most of 16:0 deposited in muscle NL should be derived from *de novo* synthesis. $\delta^{13}C$ of dietary 16:0 ranged from -34.8 to -31.6 ‰, whereas the $\delta^{13}C$ of NL 16:0 ranged from -29.3 to -27.5 ‰, which indicates a ^{13}C enrichment relative to dietary 16:0 and to whole diet (Figure 4). Consistently, in diets without *C. ladanifer*, as lipid supplementation increases, the availability of exogenous FA increases and depletion of ^{13}C of muscle FA occurs. This enrichment of *de novo* synthesized FA apparently contradicts the well-established concept that *de novo* FA synthesis presents lower $\delta^{13}C$ values than those derived from the diet due to depletion of the ^{13}C isotope mediated mostly by pyruvate dehydrogenase enzyme [10]. Ruminant digestive physiology and metabolism is quite complex, as it involves an extensive microbial anaerobic metabolism in the rumen and extensive use of rumen derived acetate

for lipid *de novo* FA synthesis in the animal. The $\delta^{13}C$ volatile FA produced in the rumen is similar to the fermented biomass when C3 plant material is used and contrasts with strong depletion observed in the methane production [21]. It follows that the rumen-derived acetate used for *de novo* FA synthesis were not as depleted as could be predicted from the simple pyruvate dehydrogenase depletion described by DeNiro and Epstein [10]. A ^{13}C enrichment of 16:0 associated to *de novo* FA synthesis was also reported by Richter et al.[17] in milk fat and by Richter, et al. [16] in the adipose tissue, in which 16:0 presented $\delta^{13}C$ values similar to the bulk diet and more than 4 ‰ relative to dietary 16:0.

The oil supplementation also decreased the $\delta^{13}C$ of 18:0 but not of 18:1. Interpretation of $\delta^{13}C$ changes of both 18:0 and 18:1 is complicated by the fact that besides *de novo* synthesis, they can be derived from the diet either directly or indirectly through rumen biohydrogenation of dietary 18:1 *cis*-9, 18:2*n*-6 or 18:3*n*-3, which differ from each other in $\delta^{13}C$ values. Moreover, the 18:1 peak analyzed by GC-C-IRMS might include minor 18:1 isomers.

The main objective of this work was to apply GC-C-IRMS to understand whether the increase in NL of intramuscular FA observed in lambs fed oil and *C. ladanifer* was explained mostly by incorporation of diet derived preformed FA or by increased *de novo* FA synthesis. Data presented in Figure 4 showing the ^{13}C enrichment of 16:0 in muscle lipids of CL6 lambs compared to C6 clearly indicates that animals fed diets containing both *C. ladanifer* and oil increased the intramuscular fat content by a sustained *de novo* FA synthesis. This clearly contrasts with the animals fed diets without *C. ladanifer* where, as expected, oil supplementation clearly inhibits the *de novo* FA synthesis (Fig. 4). The reasons for such an effect are puzzling considering the low nutritive value of *C. ladanifer* [22]. The possibility that dietary tannins sources might somehow modulate lipogenic gene expression has been reported [23-25]. Our data clearly indicate that the inclusion of *C.*

ladanifer in the diets was able to block the inhibitory effects of lipid supplementation on *de novo* FA synthesis. The elucidation of the exact mechanism of action would be of great importance to the development of more effective nutritional strategies to increase intramuscular fat in beef and lamb.

In general, FA present in PL presented lower $\delta^{13}C$ values than those in NL. The $\delta^{13}C$ differences between NL and PL were for 16:0 (-0.96 ‰ ±0.269), 18:0 (-0.87 ‰ ±0.151) 18:1 (-0.59 ‰ ±0.163). This might indicate that PL incorporate exogenous FA in a larger extent than NL. Indeed NL are mostly accumulated in adipocytes and PL are on membranes of all cells, including those that do not have as a main vocation to synthesize FA (i.e. myocites). Moreover, muscle PL might have a higher C turnover than NL as demonstrated by Harrison, et al. [26]. Thus, larger utilization of exogenous FA and larger FA turnover on muscle PL than in NL, might explain that FA in PL respond more clearly to the diets.

ABBREVIATIONS USED

FA – fatty acid(s)

NL – neutral intramuscular lipids

PL – polar intramuscular lipids or phosphholipids

Cistus ladanifer – C. landanifer

PUFA – polyunsaturated fatty acids

SFA – saturated fatty acids

CLA – conjugated linoleic acid

18:1 *cis*-9 – oleic acid

16:0 – palmitic acid

18:0 – stearic acid

18:2*n*-6 – linoleic acid

$\delta^{13}C$ values – stable isotope ratios of carbon

GC-C-IRMS – gas chromatography-combustion-isotope ratio mass spectrometry

C0 – control diet

C6 – diet supplemented with oil

CL0 – diet with *Cistus ladanifer*

CL6 – diet supplemented with both oil and *Cistus ladanifer*

DM – dry matter

me-15:0 – Methyl pentadecanoate

me-16:0 – methyl palmitate

me-17:0 – methyl heptadecanoate

me-18:0 – methyl stearate

me-18:1 *cis*-9 – methyl oleate

me-18:3*n*-3 – methyl linolenate

me-18:2*n*-6 – methyl linoleate

me-18:1 *cis*-11 – methyl *cis*-11-octadecenoate

EA – elemental analysis

EA-IRMS – elemental analyser-isotope ratio mass spectrometer

FAME – fatty acid methyl ester(s)

VPDB – Vienna Pee Dee Belemnite

‰ – “per mil”

15:0 – pentadecanoic acid

17:0 – heptadecanoic acid

18:1 *cis*-11 – *cis*-11-octadecenoic acid

18:3*n*-3 – α-linolenic acid

SD – standard deviation

SI – supporting information

C – carbon

UN – unstructured covariance

Proc MIXED – MIXED procedure

SEM – Standard error of the means

Acknowledgments

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Table 1 – Fatty acid content (g/kg dry matter) and $\delta^{13}C$ values (‰) of the experimental diets [5].

	Diets[1]			
	C0	C6	CL0	CL6
Fatty acid content				
16:0	2.27	5.15	3.20	6.04
18:0	0.23	1.85	0.51	2.20
18:1	2.27	12.6	4.29	14.6
18:2*n*-6	4.20	20.5	8.98	25.7
18:3*n*-3	1.56	19.7	2.07	23.1
Total FA	14.2	66.0	24.4	78.4
$\delta^{13}C$				
16:0	-34.8	-32.8	-31.6	-32.4
18:0	-35.8	-32.7	-30.9	-32.8
18:1	-31.7	-30.0	-28.8	-29.8
18:2*n*-6	-29.3	-29.6	-28.9	-28.4
18:3*n*-3	-30.9	-30.7	-31.3	-29.1
Whole diet	-31.3	-29.8	-29.0	-29.3

[1] Diets: C0 – 0% oil and 0% *C. ladanifer*; C6 – 6% of oil and 0% *C. ladanifer*; CL0 – 0% oil and 25% *C. ladanifer*; CL6 – 6% of oil and 25% *C. ladanifer*

Table 2 – Effect of inclusion of oil (0 and 6%) and of *C. ladanifer* (0 and 25%) on content (mg/g muscle dry matter) [5] and $\delta^{13}C$ (‰) of fatty acids present in the neutral (NL) and polar (PL) lipid fraction of lamb meat.

Diets[1]	C0		C6		CL0		CL6		
Lipid fractions	PL	NL	PL	NL	PL	NL	PL	NL	SEM
Content, mg/g DM									
16:0 $^{o\times cl\times f}$	2.5^{c}	16.6^{ab}	1.6^{d}	12.1^{b}	2.6^{cd}	16.3^{ab}	2.0^{cd}	19.0^{a}	1.20
18:0 $^{o\times cl,\ f}$	2.0	11.6	1.7	10.2	1.9	9.6	2.2	13.2	1.08
18:1 *cis*-9 $^{o\times cl\times f}$	4.7^{c}	23.2^{ab}	1.9^{d}	18.1^{b}	4.3^{cd}	20.0^{ab}	2.5^{cd}	25.3^{a}	1.58
18:2*n*-6 $^{o,cl,f.}$	2.9	1.7	3.4	2.6	3.0	1.7	4.3	3.0	0.25
Total FA $^{o\times cl\times f}$	19.5^{c}	66.0^{b}	14.8^{d}	59.6^{b}	19.0^{cd}	60.4^{b}	19.7^{cd}	85.9^{a}	4.95
Profile, % FA									
16:0 $^{o\times f,\ cl\times f}$	12.9	25.2	11.0	20.6	13.4	27.0	10.1	22.0	0.57
18:0 $^{o\times f}$	10.1	17.6	11.7	16.6	9.9	15.9	11.1	15.2	0.67
18:1 *cis*-9 $^{o\times f}$	24.1	35.2	12.4	30.5	22.4	33.1	12.6	29.6	0.96
18:2*n*-6 $^{o\times f}$	14.8	2.4	23.1	4.2	15.8	2.9	21.8	3.5	0.38
$\delta^{13}C$ (‰)									
16:0 $^{o,cl,f.}$	-29.95	-29.33	-31.10	-29.82	-28.53	-27.58	-28.83	-27.83	0.377
18:0 $^{o\times f,\ cl\times f}$	-29.52	-28.58	-30.70	-29.10	-27.67	-27.57	-28.67	-27.82	0.249
18:1$^{cl,f.}$	-30.13	-29.25	-29.33	-29.68	-28.50	-27.83	-29.17	-28.40	0.317
18:2*n*-6 ^{o}	-30.90	-	-31.93	-	-30.80	-	-32.43	-	0.382

[1] Diets: C0 – 0% oil and 0% *C. ladanifer*; C6 – 6% of oil and 0% *C. ladanifer*; CL0 – 0% oil and 25% *C. ladanifer*; CL6 – 6% of oil and 25% *C. ladanifer*

^{a,b,c,d} Within a row, least squares means that do not have a common superscript letter differ, $P < 0.05$.

^{o} Significant effect ($P < 0.05$) of oil inclusion in the diet

^{c} Significant effect ($P < 0.05$) of *C. ladanifer* inclusion in the diet

^{f} Significant effect ($P < 0.05$) of lipid fraction

$^{o\times cl}$ Signficant interaction ($P < 0.05$) between oil and *C. ladanifer* effects

$^{o\times f}$ Signficant interaction ($P < 0.05$) between oil and lipid fraction effects

$^{c\times f}$ Signficant interaction ($P < 0.05$) between *C. ladanifer* and lipid fraction effects

$^{o\times cl\ \times f}$ Signficant interaction ($P < 0.05$) among oil, *C. ladanifer* and lipid fraction effects

Figure 1 – $\delta^{13}C$ (‰) of 16:0 and 18:1 present on muscle neutral (NL) and polar lipids (PL) of lambs.

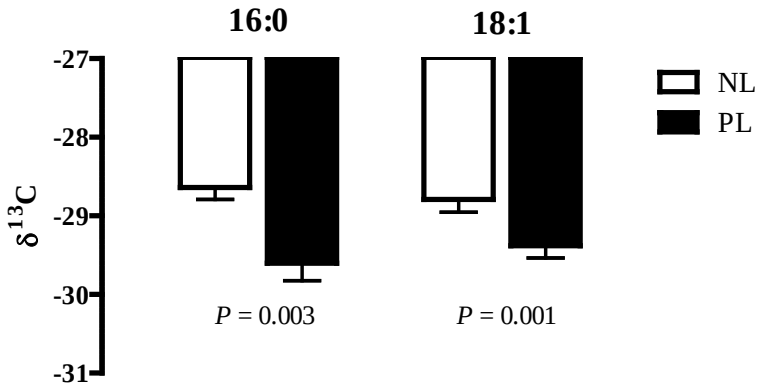


Figure 2 – Effects of dietary oil supplementation (A) and of inclusion of *C. ladanifer* (B) on $\delta^{13}C$ (‰) of 18:0 present on neutral (NL) or polar lipids (PL) of lamb meat. In each panel, columns that do not share subscript letters differ, $P < 0.05$.

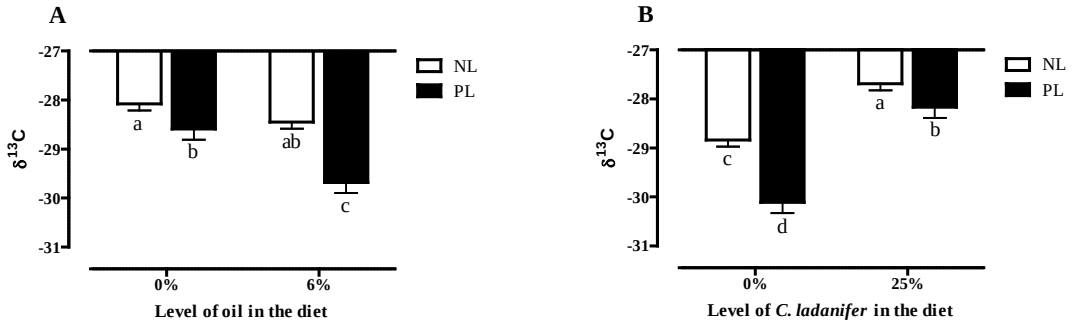


Figure 3 – Effect of inclusion of oil (A) and of *C. Ladanifer* (B) on the diet on the $\delta^{13}C$ (‰) of 16:0, 18:1 and 18:2*n*-6 present on muscle lipids of lambs.

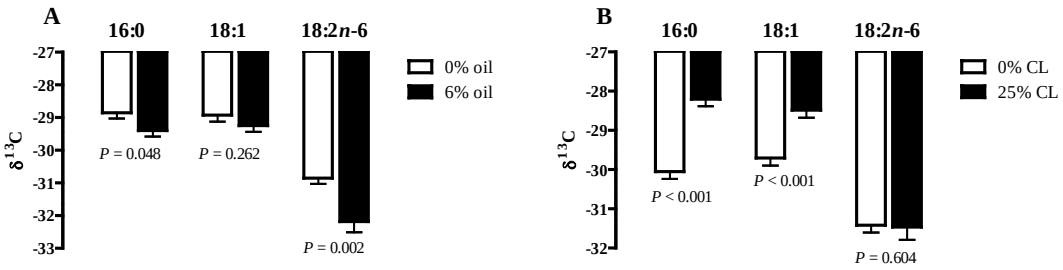
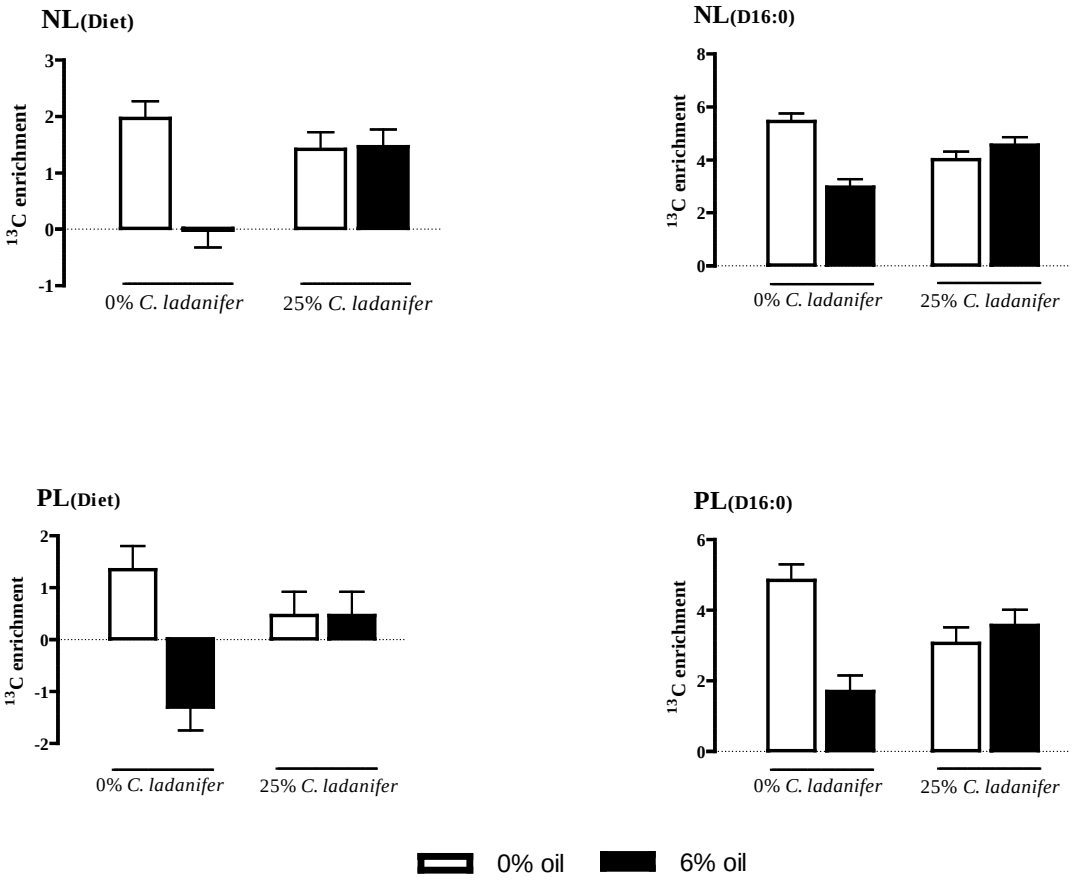
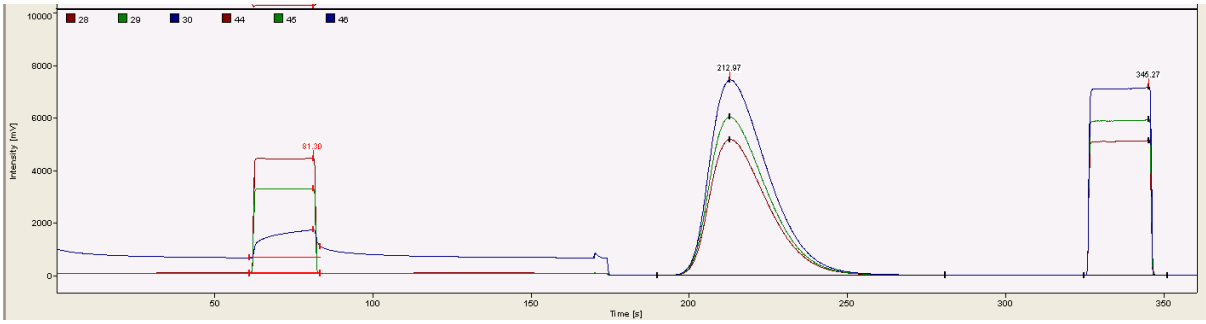


Figure 4 – Effect of inclusion of oil and of *C. ladanifer* on the diet on the ^{13}C enrichment (‰) of 16:0 present on NL (upper level) and PL (lower level) computed as the difference of $\delta^{13}C$ values of 16:0 in each lipid fraction minus $\delta^{13}C$ value of bulk diet (Diet) or $\delta^{13}C$ value of dietary 16:0 (D16:0). *P* value < 0.01 for the interaction between oil and *C. ladanifer*.

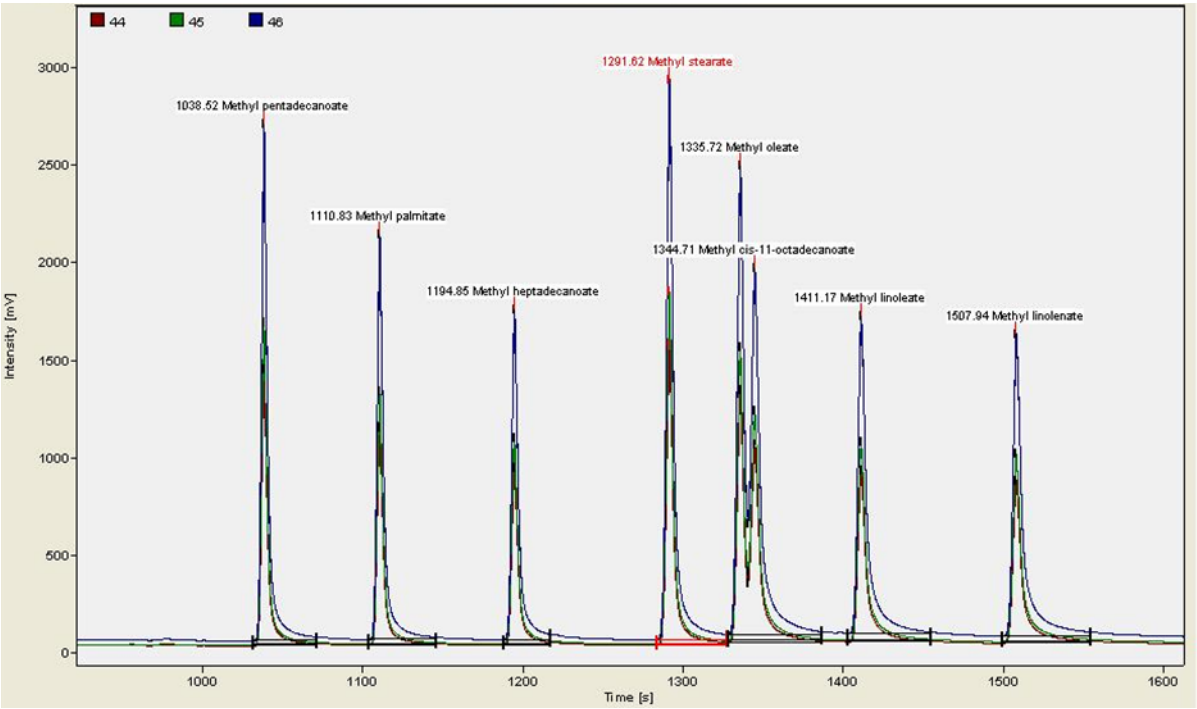


Supplementary Information (SI)

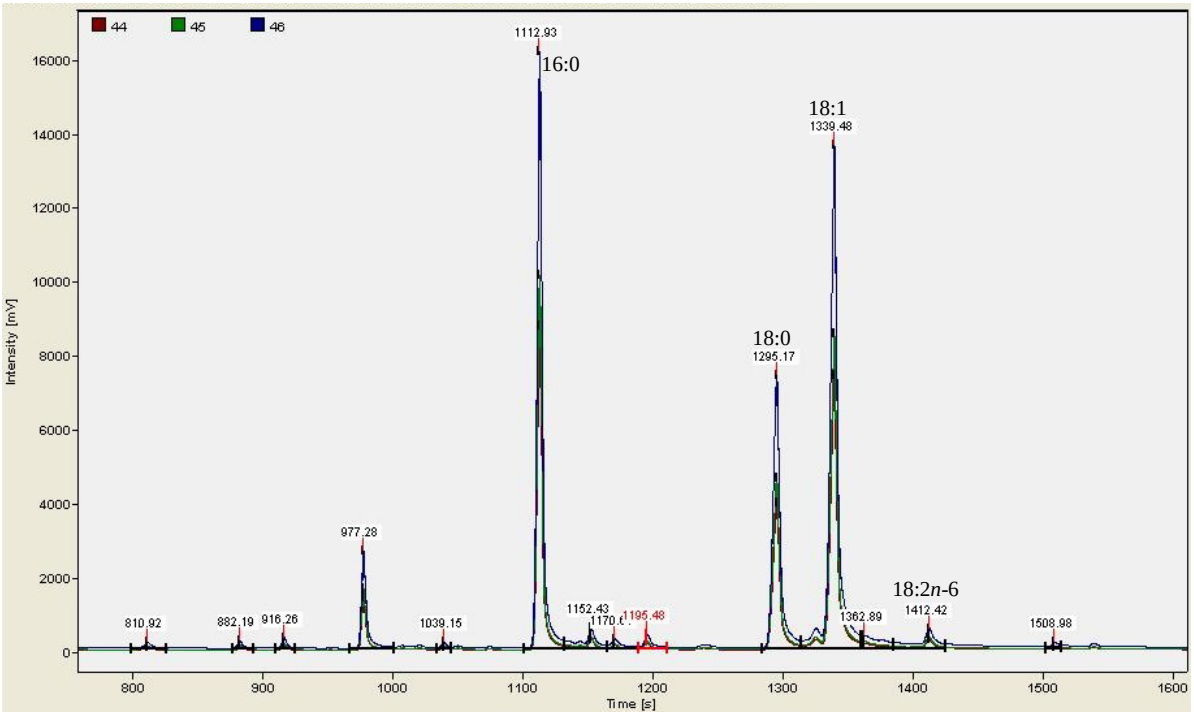
SI 1. EA-IRMS chromatogram of a fatty acid methyl ester (FAME) standard for carbon. The red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



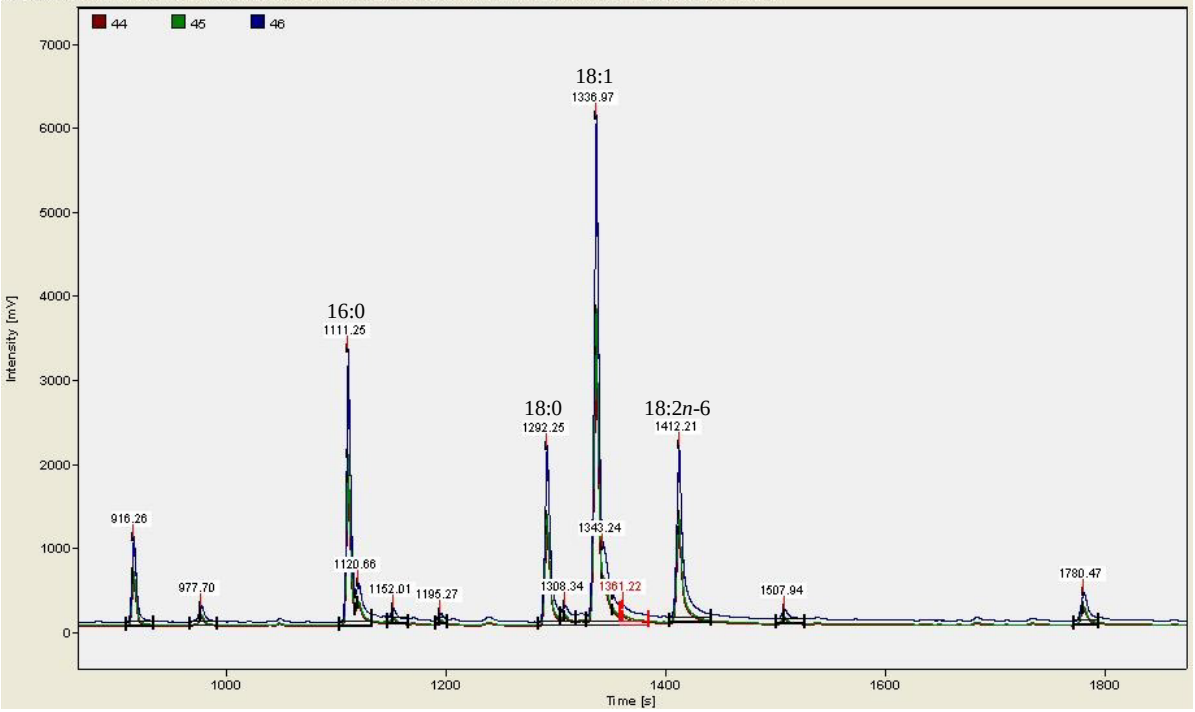
SI 2. GC-C-IRMS chromatogram of the fatty acid methyl ester (FAME) standard mix for carbon. The red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



SI 3. GC-C-IRMS chromatogram of a sample for carbon. FAME in muscle neutral lipids (NL) for feed group C. The red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



SI 4. GC-C-IRMS chromatogram of a sample for carbon. FAME in muscle polar lipids (PL) for feed group C. The red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



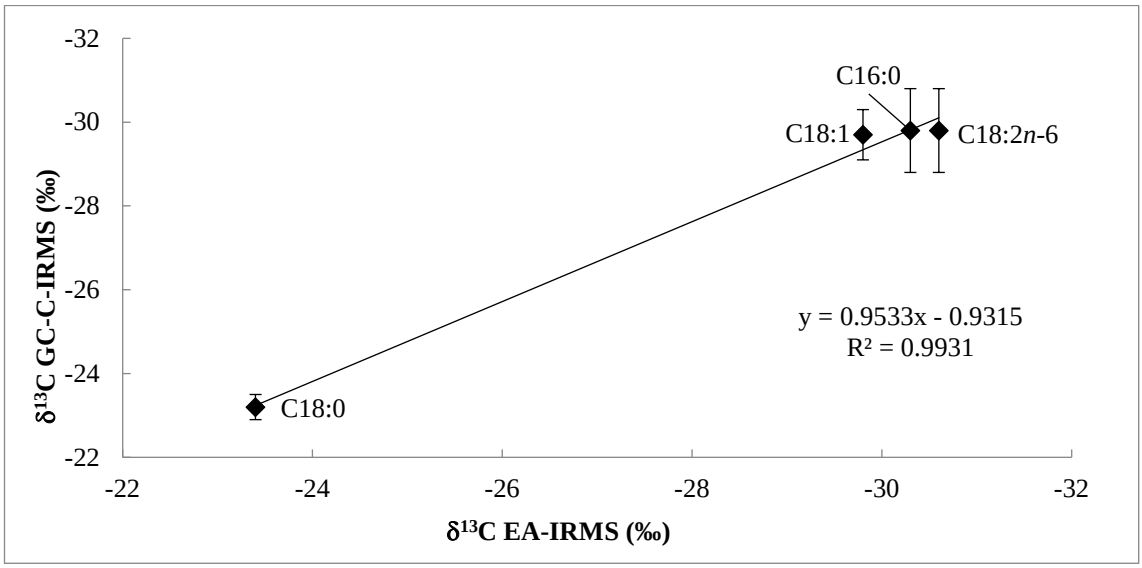
SI 5. FAME $\delta^{13}C$ values of the standard. Values are the mean ± standard deviations (SD) over all the runs.

FAME	**$\delta^{13}C$ (‰)**		
	value	SD	EA value
C16:0	-29.8	1.0	-30.4
C18:0	-23.2	0.6	-23.4
C18:1	-29.7	0.3	-29.8
C18:2*n*-6	-29.8	1.0	-30.6

SI 6. FAME $\delta^{13}C$ values of the standard. Values are the mean ± standard deviations (SD) of 10 repeated measurements.

FAME	**$\delta^{13}C$ (‰)**		
	value	SD	EA value
C16:0	-29.5	0.6	-30.4
C18:0	-23.2	0.3	-23.4
C18:1	-29.9	0.4	-29.8
C18:2*n*-6	-30.6	1.5	-30.6

SI 7. Linear correlation of isotopic measurements of FAMEs for GC-C-IRMS plotted against EA-IRMS measurements for carbon. Error bars represent the standard deviation (±SD) of repeated measurements.



SI 8. FAME $\delta^{13}C$ values of a sample. Values are the mean ± standard deviations (SD) of 9 repeated measurements.

	$\delta^{13}C$ (‰)	
FAME	**value**	**SD**
C16:0	-28.2	0.8
C18:0	-28.4	0.8
C18:1	-28.4	0.7

SI 9. The fatty acid methyl esters (FAME) standards, $\delta^{13}C$ EA values, the calculations to determine the corrected value for the fatty acid due to the added methyl group during derivatisation and the difference (‰) between the corrected FA $\delta^{13}C$ value and the FAME $\delta^{13}C$ EA value.

Compound		FAME $\delta^{13}C$ EA	Number of carbons (C)	$\delta^{13}C_{FA}$ =	$(((n+1)\delta^{13}C_{FAME})-\delta^{13}C_{MeOH})/n$	Corrected FA value (‰)	$\delta^{13}C$ Difference between Corrected FA & FAME EA value (‰)
Methyl Pentadecanoate	C15:0	-33.3	15	$\delta^{13}C_{FA\ C15:0}$ =	(((15+1)*-33,3)--38,3)/15 =	-33.0	0.3
Methyl Palmitate	C16:0	-30.3	16	$\delta^{13}C_{FA\ C16:0}$ =	(((16+1)*-30,3)--38,3)/16 =	-29.8	0.5
Methyl Heptadecanoate	C17:0	-29.8	17	$\delta^{13}C_{FA\ C17:0}$ =	(((17+1)*-29,8)--38,3)/17 =	-29.3	0.5
Methyl Stearate	C18:0	-23.4	18	$\delta^{13}C_{FA\ C18:0}$ =	(((18+1)*-23,4)--38,3)/18 =	-22.6	0.8
Methyl Oleate	C18:1 *cis*-9	-29.8	18	$\delta^{13}C_{FA\ C18:1\ cis\text{-}9}$ =	(((18+1)*-29,8)--38,3)/18 =	-29.3	0.5
Methyl Cis-11-Octadecenoate	C18:1 *cis*-11	-30.2	18	$\delta^{13}C_{FA\ C18:1\ cis\text{-}11}$ =	(((18+1)*-30,2)--38,3)/18 =	-29.8	0.4
Methyl Linoeate (reference)	C18:2*n*-6	-30.6	18	$\delta^{13}C_{FA\ C18:2n\text{-}6}$ =	(((18+1)*-30,6)--38,3)/18 =	-30.2	0.4
Methyl Linolenate (reference)	C18:3*n*-3	-30.8	18	$\delta^{13}C_{FA\ C18:3n\text{-}3}$ =	(((18+1)*-30,8)--38,3)/18 =	-30.4	0.4
Methoxide in methanol (MeOH)	CH_3	-38.3	1				

CHAPTER FIVE

INTRODUCTORY COMMENTS

REFERENCES

RESEARCH PAPER

Paper 4 van Leeuwen, K. A., Paolini, M., Laursen, K. H., Micheloni, C., Prenzler, P. D., Ryan, D., & Camin, F. (2017). Bulk H, C, N, O and S stable isotope ratios and $\delta^{15}N$ and $\delta^{13}C$ of amino acids for possible differentiation between organic and conventional tomatoes.

In the format of the journal: Journal of Agricultural Food Chemistry

To be submitted.

EXPERIMENTAL REPORT

Report 1 Tomato fruit extraction of free amino acids

INTRODUCTORY COMMENTS

Nowadays there is increasing international pressure to develop sustainable horticultural practices. Organic management is often depicted as a viable sustainable approach, and products produced under these protocols commonly attract premium prices. The appeal, then for tomatoes grown conventionally to be mislabelled as organic, is high.

To protect the producer and the consumer from this type of fraud, distinguishing tomatoes grown under different farming regimes is imperative. Evidence from the literature has suggested that bulk analysis using N isotopic ratios could be used to assist in the differentiation of organic and conventional growing regimes but the analysis could not unequivocally distinguish between the systems (Bateman, Kelly, & Woolfe, 2007). The combined bulk analysis of isotopic values for multiple elements (C, N, O, H and S) could possibly achieve this since the isotopic values in plants are dependent upon a variety of factors, such as the fertilizer used, soil moisture, nitrogen in the atmosphere, drought, soil types, photosynthetic pathway, temperature and light intensity (Masclaux-Daubresse et al., 2010; Styring, Fraser, Bogaard, & Evershed, 2014).

Amino acids are vital for plants growth, protection, etc (Molero, Aranjuelo, Teixidor, Araus, & Nogués, 2011). Investigating the metabolic paths of plant amino acids could prove to be a useful tool to differentiate between the two farming systems.

In this chapter, bulk analysis of C, N, S, H and O isotopic ratios of tomatoes grown under different farming systems was undertaken to distinguish between the farming regimes As well as bulk analysis, compound analysis of chemically varied compounds, i.e. amino acids, in tomatoes for two elements, N and C, was achieved to differentiate between the systems on a molecular level.

Note: This chapter is in two parts. Part 1 presents a manuscript based on the study using a method developed and data collected by another researcher. Part 2 describes attempts by the candidate to develop a suitable method for free amino acid isotope ratio analysis.

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Bulk H, C, N, O and S stable isotope ratios and $\delta^{15}N$ and $\delta^{13}C$ of amino acids for possible differentiation between organic and conventional tomatoes

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Abstract

In this study the analysis of the stable isotope ratios of H, C, N, O and S of bulk tomatoes and of C and N in the amino acids Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and Phe were considered for distinguishing organic from conventional tomatoes.

21 samples of tomatoes grown in two Italian regions (Emilia Romagna and Basilicata), over two years (2012 & 2013) on controlled commercial organic and conventional farms were collected. Bulk analysis was performed using IRMS interfaced with an Elemental Analyser and a Pyroliser, whereas GC-C-IRMS was implemented to measure isotopic ratios in amino acids after separation and derivatisation.

Of the bulk isotope ratios, $\delta^{15}N$ was confirmed to be the most significant parameter. In some cases, the other isotopic ratios were influenced by the farming system, but year and regions also had a strong impact.

By combining $\delta^{13}C$ and $\delta^{15}N$ of the different amino acids separation between organic and conventional tomatoes was achieved, regardless of the considered years and regions.

Keywords

Amino acids; authentication; compound-specific; GC-C-IRMS; tomatoes

Chemical compounds http://www.ncbi.nlm.nih.gov/pccompound.

Chemical compounds studied in this article: L-Alanine (PubChem CID:5950); Glycine (PubChem CID:750); L-Valine (PubChem CID:6287); L-Isoleucine (PubChem CID:6306); L-Leucine (PubChem CID:6106); L-Proline (PubChem CID:145742); L-Aspartic acid (PubChem CID:5960); L-Glutamic acid (PubChem CID:33032); L-Glutamine (PubChem CID:5961); L-Asparagine (PubChem CID:6267); L-Phenylalanine (PubChem CID:6140); L-Threonine (PubChem CID:6288)

1. Introduction

Tomatoes are a significant agricultural product and are produced under conventional or organic farming systems. Organically grown tomatoes demand high market prices due to the cost of production whereas conventional tomatoes retail at much lower prices. Because of this significant price difference producers may be tempted to sell their conventionally grown tomatoes as organic. Mislabelling like this is rife and detection of such fraudulent activity is challenging.

The choice of fertilizer and other farming conditions are critical in growing tomatoes and will differ depending on whether the tomatoes are grown organically or conventionally, as in organic farming system synthetic fertilizers are not permitted and the use of organic fertilizer is compulsory (European Community Council Regulation, EC No 834/2007 and Commission Regulation, EC No 889/2008 (www.ec.europa.eu) and National Organic Program (NOP) in the USA (www.ams.usda.gov)). These conditions will impact significantly on the nitrogen content of the soil and plant including $\delta^{15}N$, the ratio of $^{15}N/^{14}N$ (Masclaux-Daubresse et al., 2010; Styring, Fraser, Bogaard, & Evershed, 2014a). Indeed synthetic fertilizers have $\delta^{15}N$ values (from -6 to +6‰) lower than those of organic fertilizers, ranging between +0.6 and +36.7‰ (manure between +10 and +25‰) (Bateman & Kelly, 2007) and therefore plants grown conventionally have lower $\delta^{15}N$ values than those grown organically (Laursen, Schjoerring, Kelly, & Husted, 2014; Nakano, Uehara, & Yamauchi, 2003).

The roots of non N_2-fixing plants uptake N generally in the form of ammonium (NH_4^+) or nitrate (NO_3^-) from the soil and can have a different $\delta^{15}N$ value depending on the type of fertilizer used, whether synthetic or organic (Bateman & Kelly, 2007). Conversely, N_2-fixing plants acquire N from the atmosphere and hence have a $\delta^{15}N$ value around 0‰ (Bateman, Kelly, & Jickells, 2005). Assimilation of NO_3^- and NH_4^+ in plants occurs

differently; the root takes up NH_4^+ immediately whereas NO_3^- can be taken up by the root and shoots of a plant (Evans, Bloom, Sukrapanna, & Ehleringer, 1996). NO_3^- is then reduced to NH_3, which is converted by glutamine synthetase to glutamine, whereas NH_4^+ is directly integrated into glutamine such that, in the absence of isotopic fractionation, the $\delta^{15}N$ will be the same as for ammonium (Styring et al., 2014a). As NO_3^- assimilation and relocation transpires through several processes, fractionation consequently occurs. Glutamate synthase forms glutamate from glutamine. Glutamate is the source of N for all amino acids, which could have different $\delta^{15}N$ values than for glutamate due to fractionation occurring during the metabolic processes within the plant (Styring et al., 2014a; Werner & Schmidt, 2002).

Several papers in the literature have showed that analysis of stable isotope ratios of N, sometimes in combination with those of C, O, H and S, bulk (whole plant) and compound-specific has the potential to differentiate vegetable and fruits grown under different farming systems (Longobardi, Casiello, Centonze, Catucci, & Agostiano, 2017; Paolini, Ziller, Laursen, Husted, & Camin, 2015; Styring, Fraser, Bogaard, & Evershed, 2014b). Most analytical methods implemented to determine if a product is organic or derived conventionally are based on bulk isotopic analysis of the product, i.e. whole plant. Research has shown however, that compound specific analysis is more powerful (van Leeuwen, Prenzler, Ryan, & Camin, 2014). Amino acids in plants are involved in protein synthesis, plant defense, cell component synthesis, secondary metabolism and osmotic movements (Molero, Aranjuelo, Teixidor, Araus, & Nogués, 2011) and are therefore ideal to analyse for isotopic changes within the plant. Analysis of amino acids, for $\delta^{15}N$ and $\delta^{13}C$, can be undertaken implementing gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

In this study, the analysis of the isotopic ratios of H, C, N, O and S of bulk tomatoes was combined with those of C and N for amino acids to distinguish between organic and conventional growing regimes for tomatoes.

2. Materials and methods

2.1 Standards and reagents

The standards L-Alanine (≥ 98%); Glycine (≥ 99%); L-Valine (≥ 98%); L-Isoleucine (≥ 98%); L-Leucine (≥ 98%); L-nor Leucine (≥ 98%); L-Proline (≥ 99%); L-Aspartic acid (≥ 98%); L-Glutamic acid (≥ 99%); L-Glutamine (≥ 99%); L-Asparagine (≥ 98%); L-Phenylalanine (≥ 98%); L-Threonine (≥ 98%) and the analytical grade Amberlite® IR120 cation-exchange resin (hydrogen form) were purchased from Sigma-Aldrich (Milan, Italy). Triethylamine (≥ 99.5%) and acetic anhydride (≥ 99%) reagents were purchased from Sigma-Aldrich (Milan, Italy). Analytical grade acetone, isopropanol, diethyl ether, dichloromethane and ethyl acetate were purchased from VWR (Milan, Italy) and Sigma-Aldrich (Milan, Italy). Petroleum ether 40-60% was purchased from VWR Chemicals (Milan, Italy).

2.2 Growth conditions and sampling of tomato samples

Twenty-one samples of tomatoes (*Solanum lycopersicum*, 2 varieties, round (1) and long (2), for each region and each farming system) were grown at two different geographical locations, about 600 km apart, in Italy, Emilia Romagna (ER) and Basilicata (BAS), on controlled commercial farms and were collected over two years (2012 & 2013). There were 3 replicates for each plot. The tomatoes were grown under two different systems: organic (Org) and conventional (Conv). The organic fields were prepared and crops grown according to the European Community Council Regulation (EC 834/2007) for growing organic crops (at least for 10 years). Nutrients for the organic crops derived from

the previous crops grown in those fields, addition of organic fertilizers (commercial, pelleted) for some crops and also from cover crops. The conventional fields implemented inorganic fertilizers and pesticides as per normal practice for conventional crops (Table 1). Irrigation was implemented on both the organic and conventional fields. The tomatoes were harvested from mid-August through to early September and at the maturity stage which, for the different regions, was on different days. Due to weather conditions in 2012 the fruit available was limited compared to the fruit available in 2013, also due to the extreme drought in 2012 a plot in Emilia Romagna was lost.

Table 1. Tomato sample information: variety, geographic location, farming system and fertilizer application. Org (organic), Conv (conventional), BAS (Basilicata), ER (Emilia-Romana).

Variety	Year	Geographic location	Farming system	Fertilizer application
1	2012	BAS	Org	Guanito, 400kg/ha (NP org. fertilizer with 6%N, 15%P_2O_5, 2%MgO, 10% Ca)
2	2012	BAS	Org	Guanito, 400kg/ha (NP org. fertilizer with 6%N, 15%P_2O_5, 2%MgO, 10% Ca)
1	2013	BAS	Org	Guanito, 400kg/ha (NP org. fertilizer with 6%N, 15%P_2O_5, 2%MgO, 10% Ca)
2	2013	BAS	Org	Guanito, 400kg/ha (NP org. fertilizer with 6%N, 15%P_2O_5, 2%MgO, 10% Ca)
1	2012	ER	Org	farmyard manure
2	2012	ER	Org	dehydrated chicken manure
1	2013	ER	Org	farmyard manure
2	2013	ER	Org	dehydrated chicken manure
1	2012	BAS	Conv	synthetic fertilizer: 3%P 22%K, Auxines sprayed on leaves
2	2012	BAS	Conv	synthetic fertilizer: 3%P 22%K, Auxines sprayed on leaves
1	2013	BAS	Conv	synthetic fertilizer: 3%P 22%K, Auxines sprayed on leaves
2	2013	BAS	Conv	synthetic fertilizer: 3%P 22%K, Auxines sprayed on leaves
1	2012	ER	Conv	mineral N by fertigation
2	2012	ER	Conv	mineral N by fertigation
1	2013	ER	Conv	mineral N by fertigation
2	2013	ER	Conv	mineral N by fertigation

2.3 Tomato sample preparation

Tomatoes were weighed, washed with milliQ water and frozen at -20 °C. The whole tomatoes were then freeze-dried in an open plastic bag for approximately 48 hrs. The tomatoes were homogenized and then milled using a retch centrifugal mill (ZM 200) with titanium instrumentation. The tomato powder was then stored at room temperature.

2.4 EA analysis of standards and tomato samples

An elemental analyser (Flash EA 1112, Thermo Scientific, Bremen, Germany) furnished with an autosampler (Finnigan AS 200, Thermo Scientific) and coupled to a DELTA V isotope ratio mass spectrometer (Thermo Scientific) through a ConFlo dilutor (Thermo Finnigan, Bremen, Germany) was implemented to determine the $\delta^{15}N$ and $\delta^{13}C$ values for the single amino acid standards including the internal standard before derivatisation and for the bulk tomato samples (SI Figure 1). The temperature of the combustion reactor was 910 °C and for the reduction reactor the temperature was 680 °C, the post reactor GC-column temperature was 45 °C. The He carrier gas had a flow rate of 120 mL/min, the reference gas flow rate was 150 mL/min and the oxygen flow rate was 250 mL/min. The cycle (run time) was 320 s, with a sampling delay of 15 s and an oxygen injection end of 5 s. Each amino acid was weighed (0.8 mg) in tin capsules (SÄNTIS analytical AG, Teufen, Switzerland), in duplicate. A blank sample was run first in the sequence and then the working standard (x2), the samples in replicate, the control (x2) and at the end of the sequence, the working standard (x2). Daily calibration checks were undertaken using the calibrated working casein standard (MRI 64) and controlled with the other casein standard (MRI 63). The isotopic ratios were calculated against two in-house standards and calibrated against the international reference materials: L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), mineral oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA). The values were expressed in δ‰ against the international standard (Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$). The uncertainty of measurements (2σ) was ±0.3‰.

Tomato samples were measured in bulk for isotopes C, N, S, H and O. The bulk tomato sample (0.5 mg), in tin capsules, was combusted in an elemental analyser (VARIO CUBE, Elementar Analysensysteme GmbH, Germany) coupled to an isotope ratio mass

spectrometer (IRMS, Isoprime, Isoprime Ltd., UK) to measure the $^{13}C/^{12}C$ ($\delta^{13}C$), $^{15}N/^{14}N$ ($\delta^{15}N$) and $^{34}S/^{32}S$ ($\delta^{34}S$) isotopic ratios in one run. The oxidation reactor operated at 1180 °C, the reduction reactor was 630 °C and the Helium carrier flow was 220 mL/min. A pyrolyser (Finnigan TC/EA, high temperature conversion elemental analyser, Thermo Scientific) coupled to an IRMS (Finnigan DELTA XP, Thermo Scientific) was implemented to measure the $^{2}H/^{1}H$ ($\delta^{2}H$) and $^{18}O/^{16}O$ ($\delta^{18}O$) isotopic ratios in one run for the bulk tomato sample (0.2 mg) in silver capsules. The pyrolyser reactor operated at a temperature of 1450 °C, the helium carrier flow was 100 ml/min and the GC column (1.2 m, ¼ inch OD, 5.4 mm ID with a molecular sieve of 5 Å and 80/100 mesh) was at 110 °C. Samples were measured in duplicate. The isotope ratios were expressed in δ ‰ against Vienna Standard Mean Ocean Water for $\delta^{18}O$ and $\delta^{2}H$, Vienna-Pee Dee Belemnite for $\delta^{13}C$, air for $\delta^{15}N$ and Vienna Canyon Diablo Troilite for $\delta^{34}S$ according to the following equation:

$$\delta = (R_{sample} - R_{standard}) / R_{standard} \quad (1)$$

where R_{sample} is the isotope ratio measured for the sample and $R_{standard}$ is the international standard isotope ratio. The isotopic values for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ were calculated against working in-house standards which were themselves calibrated against international reference materials: potassium nitrate IAEA-NO_3 (IAEA-International Atomic Energy Agency, Vienna, Austria) for $\delta^{15}N$ L-glutamic acid USGS 40 (U.S. Geological Survey, Reston, VA, USA) for $^{13}C/^{12}C$and $^{15}N/^{14}N$ fuel oil NBS-22, IAEA-CH-6 for $^{13}C/^{12}C$, barium sulphates IAEASO-5, NBS 127 (IAEA) and USGS 42 and USGS 43 for $\delta^{34}S$. Through the development of a linear equation the values were calculated against two working standards. The isotopic values for $\delta^{2}H$ and $\delta^{18}O$ were calculated against KHS (Kudu Horn Standard, $\delta^{2}H$ =-54±1‰ and $\delta^{18}O$ =+20.3 ±0.3‰) and CBS (Caribou Hoof Standard $\delta^{2}H$ =-197±2‰ and $\delta^{18}O$ =+3.8±0.3‰) through the development of a linear

equation and implementing the comparative equilibration procedure (Carter, Barwick, & (Eds), 2011). These keratinous standards were exploited because of the absence of any international organic reference material with a matric similar to our tomato samples. For the $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and $\delta^{2}H$ the uncertainty of measurement (2σ) was 0.1‰, 0.2‰, 0.3‰, 0.3‰ and 1‰, respectively. Isotopic data for the bulk tomato samples is in the supplementary information (SI Table 1).

2.5 Extraction, hydrolysis, purification and derivatisation of amino acids

Petroleum ether/diethyl ether (2:1, v/v, 30 mL) was added to defat powdered tomato samples (5 g) which were then homogenized (Ultraturrax, model X-620, Staufen, Germany; 3 min at 11500 rpm) and centrifuged (ALC PK 131IR, Thermo Electron Corporation, Germany; 6 min at 4100 rpm). The residues (defatted powdered tomato samples) were then left to dry and were stored at room temperature until required.

The defatted powdered tomato samples (250 mg) in Pyrex vials with a PTFE-cap were hydrolysed for 24 h at 110 °C with 6 M HCl (2 mL) to obtain individual amino acids. The hydrolysed solution was cooled, filtered through glass wool, dried under N_2 and then redissolved in 0.1 M HCl (2 mL). An internal standard, norleucine (8 mg/mL in 0.1 M HCl), was added and the sample was stored at -18 °C.

To purify the amino acids the samples were run through a cation-exchange resin (Amberlite® IR120) with all exchange sites previously saturated with H^+. To saturate the exchange sites the resin was soaked in 3 M NaOH overnight, washed in distilled water and then soaked again overnight in 6 M HCl followed by washing in distilled water. The glass pipette was prepared with a quartz wool plug and then the resin (saturated H^+) was filled above the quartz wool to approximately 3 cm before loading the hydrolysed sample (0.5 mL). Salts were washed out with distilled water. The amino acids were eluted with NH_4OH (10 wt%) and subsequently dried under N_2. The utilisation of resin (with the

above-mentioned conditions) for sample purification does not incur isotopic fractionation (Styring et al., 2014a; Takano, Kashiyama, Ogawa, Chikaraishi, & Ohkouchi, 2010).

The samples were then derivatised using an *N*-acetylisopropyl method described by Corr (Corr, Berstan, & Evershed, 2007). Esterification of the samples was undertaken by the addition of the sample to acidified isopropanol (1 mL; 1:4 acetyl chloride/isopropanol) for 1 hr at 100 °C. The remaining isopropanol was removed under a gentle stream of N_2 at 40 °C and then, to wash and remove excess water and isopropanol from the sample, dichloromethane (0.25 mL x 2) was added, followed by evaporation under N_2 at 40 °C. A solution of acetic anhydride/triethylamine/acetone (1:2:5 v/v/v, 1 mL, 10 mins at 60 °C) was added to acetylate the amino acid esters which were subsequently dried under a stream of N_2 at room temperature. The derivatised amino acids were then dissolved in ethyl acetate (1 mL). Saturated NaCl solution (1 mL) was added and the mixture was vortexed and allowed to separate into phases. The organic layer containing the amino acids was removed and dried under N_2 at room temperature. Any remaining water or reagent was removed with the addition of dichloromethane (0.25 mL x 2) which was subsequently evaporated under a steady stream of N_2. Finally, the amino acid samples were dissolved in ethyl acetate (0.2 mL) and stored at -18 °C prior to analysis.

2.6 GC-C-IRMS analysis of amino acids for $\delta^{13}C$ and $\delta^{15}N$

GC-C-IRMS analysis determined the isotopic ratios and retention times for derivatised individual amino acids, alanine (Ala), glutamate (Glx), glycine (Gly), isoleucine (Ileu), leucine (Leu), phenylalanine (Phe), proline (Pro), threonine (Thr), and valine (Val). The amino acid glutamine was converted to glutamic acid, due to acid hydrolysis. Accordingly, the isotopic values of $\delta^{13}C$ and $\delta^{15}N$ for Glx reflect the isotopic values for carbon and nitrogen for both glutamate + glutamine, correspondingly (SI Table 2 and SI Table 3, SI Figure 2 and SI Figure 3) (Styring, 2012, Paolini 2015).

The amino acid reference standards were analysed separately to determine retention time with a Trace GC Ultra (GC Isolink + ConFlo IV, Thermo Scientific) furnished with an autosampler (Triplus, Thermo Scientific) and for $\delta^{15}N$ analysis a HP-INNOWAX column (60 m x 0.32 mm i.d. x 0.25 μm film thickness, Agilent) was installed. The injector temperature was 250 °C throughout the run with the injector mode set on splitless, and the amount of sample injected was 0.8–1.0 μL. The initial oven temperature was 40 °C and maintained for 2 min, then ramped to 140 °C at a rate of 40 °C/min, then increased to 180 °C at a rate of 2.5 °C/min, then ramped again to 220 °C at a rate of 6 °C/min, then increased to 250 °C at a rate of 40 °C/min and maintained for 15 min. The carrier gas was He (Rivoira, purity: 99.999 %) with a constant flow of 1.4 mL/min. For analysis of $\delta^{13}C$ a more polar column was installed (ZB-FFAP; 30 m x 0.25 mm i.d. x 0.25 μm film thickness, Phenomenex) and the injector was set on split mode (1:30), with an injection volume of 1.0 μL. The initial oven temperature was 40 °C and maintained for 1 min, then ramped to 120 °C at a rate of 15 °C/min, then increased to 190 °C at a rate of 3 °C/min, then ramped again to 250 °C at a rate of 5 °C/min and maintained for 7 min. The same conditions were used for the tomato samples. After separation in the GC the sample then flowed through an oxidation reactor which consisted of three braided wires of 0.125 mm diameter, centred inside a 320 mm alumina tube (1 x nickel oxide, 1 x copper oxide, 1 x platinum), kept at 1030 °C and housed in the GC whereby it was quantitatively combusted to N_2, CO_2 and H_2O (removed via a Nafion® membrane). The GC-C was coupled via an open split interface to an isotope ratio mass spectrometer (IRMS Delta V). The CO_2 was removed from the analyte for the analysis of $\delta^{15}N$ isotopic ratio via a liquid nitrogen trap situated after the oxidation combustion reactor.

Instrumental performance was monitored using a standard mixture of derivatised L-amino acids with the addition of an internal standard, L-norleucine (Nleu), and their isotopic values for $\delta^{13}C$ and $\delta^{15}N$, were measured during each analytical run. Because of its

absence in tomatoes, Nleu was chosen as the internal standard for the samples and standard mix and its isotopic value was measured in each sample run. The L-amino acid EA-IRMS isotopic values determined for Nleu were -27.6‰ for $\delta^{13}C$ and 14.0‰ for $\delta^{15}N$. The internal standard, Nleu, was used for quality control of the method, it was added to every amino acid sample and underwent the same processes as the sample, such as hydrolysis and derivatisation. The $\delta^{15}N$ and $\delta^{13}C$ values obtained for the amino acid mix (which underwent the same rigorous process as the samples) from the GC-C-IRMS were the same as that obtained by EA-IRMS which also shows the validity of the method. Repetition of the same sample by analysis also confirmed the quality of the process.

When the difference between the values determined by EA-IRMS and GC-C-IRMS for $\delta^{13}C$ and $\delta^{15}N$ were no more than 1.5‰ and 1.0‰, respectively, the analytical run was accepted.

Data Analysis and Corrections

The reference gases, CO_2 and N_2, of known carbon and nitrogen isotopic composition, respectively, were directly introduced at the beginning and end of each run and the $\delta^{13}C$ and $\delta^{15}N$ values reported are initially relative to these gases. Samples were measured in triplicate. The isotopic ratios were expressed in δ ‰ against Vienna-Pee Dee Belemnite for $\delta^{13}C$ and air for $\delta^{15}N$ according to equation (1) mentioned above. The derivatised amino acid $\delta^{13}C$ values are a composite of the initial starting amino acid carbon and of the reagents implemented in the derivatisation reaction. Due to the addition of these extra carbons, an empirical correction was applied to determine the $\delta^{13}C$ value of the amino acid (Docherty 2001):

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad (2)$$

where *n* is the number of moles of carbon, and the subscripts c, d, and cd represent the compounds of interest, the derivative group, and the derivatised compound, respectively. Measurement errors as well as the total analytical error, which arises from the different derivatisation steps, were considered when calculating the $\delta^{13}C$ measurement of uncertainty:

$$\sigma_c^2 = \sigma_s^2 \left(\frac{n_s}{n_c}\right)^2 + \sigma_{sd}^2 \left(\frac{n_s + n_d}{n_c}\right)^2 + \sigma_{cd}^2 \left(\frac{n_c + n_d}{n_c}\right)^2 \quad (3)$$

where *n* is the number of moles of carbon, and the subscripts c, s, d, cd and sd represent the underivatised compound, the underivatised standard, the derivatising agent, the derivatised compound and the derivatised standard, respectively.

Accuracy and Precision of GC-C-IRMS

EA-IRMS isotopic values of $\delta^{13}C$ and $\delta^{15}N$ for the single non-derivatised amino acids were the average of two measurements, however for GC-C-IRMS the determined $\delta^{13}C$ and $\delta^{15}N$ isotopic values for the derivatised amino acids were the mean of 3 runs. A comparison between the isotopic values for $\delta^{13}C$ and $\delta^{15}N$ of single non-derivatised amino acids by EA-IRMS and their respective derivatised forms in a standard mix by GC-C-IRMS was used to test the accuracy of the determined isotopic values for the amino acids measured (SI Table 4). The isotopic values for the amino acids determined by EA-IRMS and by GC-C-IRMS were linearly correlated against each other, separately, for $\delta^{13}C$ and $\delta^{15}N$. The difference after the empirical calculation for $\delta^{13}C$ and $\delta^{15}N$ was not more than ±1.6‰ and ±0.5‰, respectively, for the measured values by EA-IRMS and compared with GC-C-IRMS (SI Figure 4 & SI Figure 5). Precision was evaluated by derivatising 6 replicates of the amino acid standard mixture, and analysing each one in triplicate by GC-C-IRMS and a standard deviation (1σ) of ±0.8‰ for $\delta^{13}C$ and ±0.4‰ for $\delta^{15}N$ was obtained.

2.7 Statistical analysis

The data were statistically analysed utilising the R program (R Core Team, 2017) and with the factoextra (Kassambara & Mundt, 2017) and ggplot2 (Wickham, 2009) packages.

3. Results and discussion

3.1 BULK tomato samples

The tomato samples were analysed in bulk for C, N, S O and H stable isotope ratios. In Table 2, the means of the $\delta^{2}H_{bulk,}$ $\delta^{18}O_{bulk,}$ $\delta^{13}C_{bulk,}$ $\delta^{15}N_{bulk}$ and $\delta^{34}S_{bulk}$ values for the different farming systems, regions and years as well as the results of a one-way analysis of variance (ANOVA) without interaction effects are reported. The $\delta^{15}N_{bulk}$ values of the tomatoes ranged between 4.0‰ to 14.6‰ for the organic systems and 1.6‰ to 8.2‰ for the synthetic systems (SI Table 1) and these values depict the nature of the fertilization system or farming system implemented. This is further reflected by the means of each system as shown in Table 2, with the mean of the $\delta^{15}N_{bulk}$ values for the organic system significantly higher ($p < 0.05$) than the mean of the $\delta^{15}N_{bulk}$ values for the conventional system. Synthetic fertilizers have isotopic $\delta^{15}N$ values between -6‰ and +6‰ whereas organic fertilizers have an isotopic $\delta^{15}N$ range between +0.6‰ to 36.7‰ (Bateman & Kelly, 2007). Besides farming system, there was a significant difference also for year and region for $\delta^{15}N_{bulk}$ ($p<0.05$).

The $\delta^{13}C_{bulk}$ values of the tomatoes (-29.9‰ to -26.0‰, SI Table 1) were representative of C3 plants (-24‰ to -34‰) (Krueger & Reesman, 1982). The $\delta^{13}C$ value of a plant (whole plant i.e. bulk) is influenced mainly by the plant's photosynthetic pathway; however, fertilizers, soil types and CO_2 pools, nitrogen and water availability can also affect the plant $\delta^{13}C$ values (Longobardi et al., 2017). Moreover these values relate to the

uptake from CO_2 from the atmosphere and also to factors which have an impact on stomatal aperture and photosynthesis such as temperature, salinity and light intensity (O'Leary, 1981). The photosynthetic rate of the plant increases with N supply and thereby influences the efficiency of water use (Klaus et al., 2013). The use of different farming systems, organic and conventional, could lead to different $\delta^{13}C$ values as under conventional conditions the availability of N is high and leads to lower $\delta^{13}C$ values whereas lower N availability leads to higher $\delta^{13}C$ values, which could help differentiate between the systems (Paolini et al., 2015). According to Table 2 the $\delta^{13}C_{bulk}$ means for the organic system and conventional system are different ($p < 0.1$) suggesting that the farming system does affect the $\delta^{13}C_{bulk}$ value. Results in Table 2 also show that the year influenced the $\delta^{13}C_{bulk}$ value ($p < 0.1$), but there was no difference between the regions. The $\delta^{13}C_{bulk}$ value for the organic system is higher than for the conventional system and this is due to lower N availability and therefore a decrease in the rate of photosynthesis which in turn has a higher discrimination of the enzyme RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) against $^{13}CO_2$ (Högberg, Johannisson, & Hällgren, 1993; Rapisarda et al., 2010). Photosynthetic rates are reduced when stomatal conductance is reduced due to decreased CO_2 assimilation under drought conditions (Reddy, Chaitanya, & Vivekanandan, 2004).

The $\delta^{2}H_{bulk}$ values of the tomatoes (-74.4‰ to -23.0‰, SI Table 1) and the $\delta^{18}O_{bulk}$ values (29.2‰ to 23.9‰, SI Table 1) are influenced by a number of factors such as temperature, precipitation and humidity, geographic zone, longitude, latitude, and altitude. There was no significant difference between means for both the conventional system and organic system for $\delta^{2}H_{bulk}$ and $\delta^{18}O_{bulk}$, which means that the farming system (organic and conventional) does not influence the isotopic values (Table 2). There were however significant differences ($p < 0.05$) for the year and region for $\delta^{2}H_{bulk}$ and $\delta^{18}O_{bulk}$ values suggesting that the environment/water was different for both regions and years (Table 2).

There was a drought for the ER region in 2012, which would affect plant growth rates and plant density, which influences evapotranspiration, and this in turn affects the $\delta^{18}O_{bulk}$ and $\delta^{2}H_{bulk}$ values of the plant (Barbour, Walcroft, & Farquhar, 2002; Camin, Perini, Colombari, Bontempo, & Versini, 2008).

The $\delta^{34}S_{bulk}$ values of the tomatoes were between -6.6‰ and +2.1‰ (SI Table 1). According to Table 2 conventional and organic farming conditions cannot be differentiated based on ^{34}S. The year also did not have an effect on the $\delta^{34}S_{bulk}$ values; however, there was a significant difference between the BAS and ER regions for $\delta^{34}S_{bulk}$ values, which are due to closeness to the sea and also to soil conditions (Schmidt, Robins, & Werner, 2015).

Table 2. Isotopic mean values for tomato bulk samples.

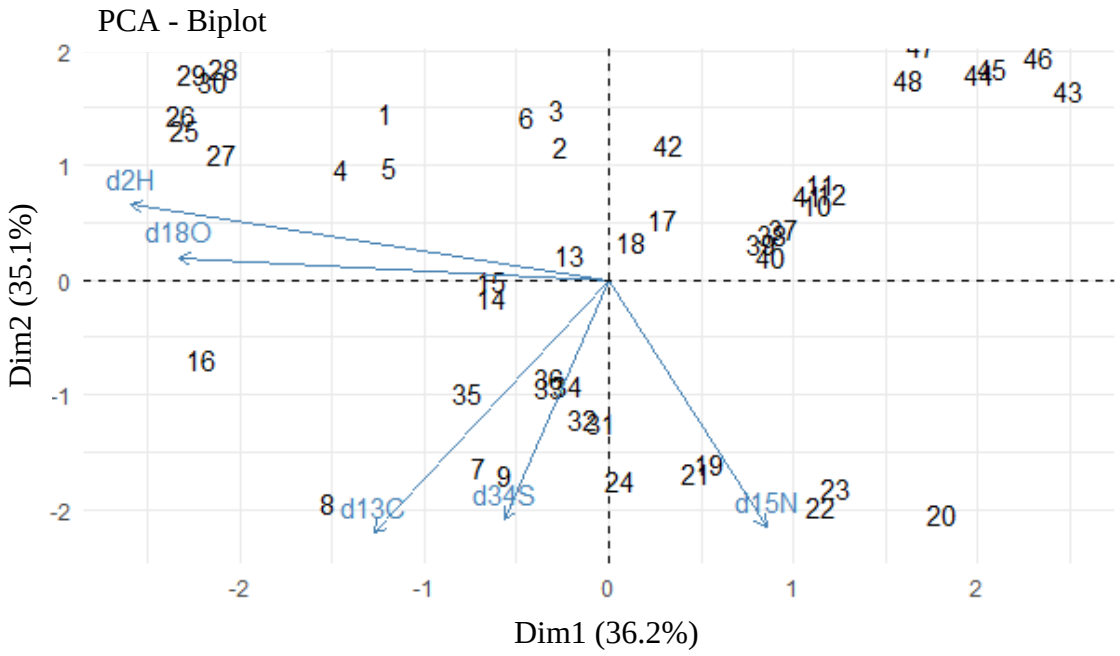
		tomato bulk values (‰)				
system		$\delta^{2}H$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S_{corr}$
Org	mean	-48.8	27.3	-27.5c	9.3a	-1.7
	SD	12.0	1.3	0.7	3.4	3.0
Conv	mean	-52.8	26.5	-28.0d	4.5b	-1.7
	SD	15.6	2.1	1.2	2.1	2.3
region						
BAS	mean	-44.8a	28.2a	-27.8	5.5a	-3.0a
	SD	16.0	1.0	0.6	2.3	1.5
ER	mean	-56.9b	25.6b	-27.7	8.2b	-0.4b
	SD	9.0	1.3	1.2	4.3	2.9
year						
2012	mean	-43.1a	27.4a	-27.5c	6.1a	-1.5
	SD	9.2	1.8	0.8	3.0	3.0
2013	mean	-58.6b	26.5b	-28.0d	7.7b	-1.9
	SD	15.9	1.5	1.1	4.1	2.3

Significantly different mean values (one-way ANOVA, $p<0.05$) between groups are indicated with “a” and “b”. Significantly different mean values (one-way ANOVA, $p<0.1$) between groups are indicated with “c” and “d”. Org, organic, Conv, conventional, SD, standard deviation, corr (corrected).

The bulk tomato samples were also analysed by ANOVA for interaction effects between the groupings. There was significant interaction effects between system and region for $\delta^{2}H_{bulk}$ (p<0.05), $\delta^{18}O_{bulk}$ (p<0.001), $\delta^{13}C_{bulk}$ (p<0.05), $\delta^{15}N_{bulk}$ (p < 0.001), between system and year for $\delta^{2}H_{bulk}$ (p<0.001), $\delta^{18}O_{bulk}$ (p<0.01), $\delta^{13}C_{bulk}$ (p<0.01) and $\delta^{34}S_{bulk}$ (p<0.001) and between region and year for $\delta^{13}C_{bulk}$ (p<0.001) and $\delta^{18}O_{bulk}$ (p<0.05).

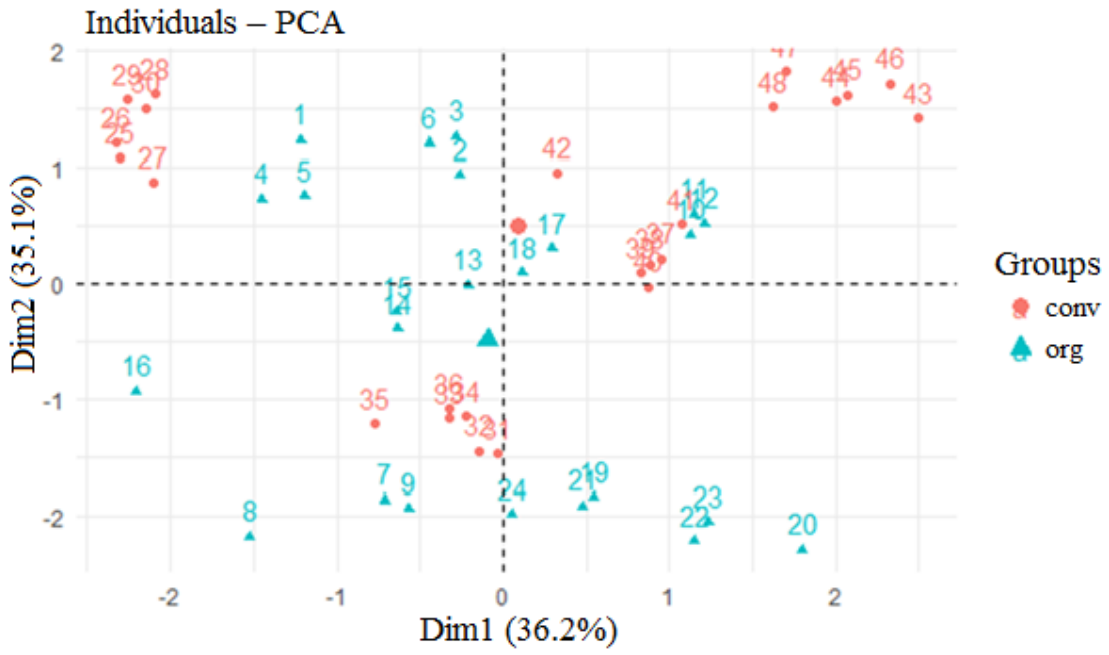
Principal component analysis (PCA) was implemented for the bulk data for $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. The majority of the data variability is explained with the first three PCs with 36% variance for the first PC, 35% variance for the second PC and 17% variance for the third PC. To visualise which variables were influencing the first two PCs a biplot was used (see Figure 1). The first PC has two major contributors, $\delta^{2}H$ and $\delta^{18}O$, which appear to be highly correlated whereas $\delta^{13}C$, $\delta^{34}S$ and $\delta^{15}N$ mainly contribute to the second PC.

Figure 1. A biplot of PC1 and PC2 for bulk $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values. The numbers on the biplot refer to the sample numbers. The label d2H refers to $\delta^{2}H$, d18O refers to $\delta^{18}O$, d13C refers to $\delta^{13}C$, d15N refers to $\delta^{15}N$ and d34S refers to $\delta^{34}S$.



Analysing the PCA by farming system, it can be seen (Figure 2) that when plotting the first two PCs there is no clear separation between the groups. There are some organic and conventional samples associated with higher levels of $\delta^{2}H$ and $\delta^{18}O$, some organic and conventional samples associated with higher levels of $\delta^{13}C$, $\delta^{34}S$ and $\delta^{15}N$ and there are some organic and conventional samples that appear to be associated with lower levels of all measured isotopes ($\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{34}S$ and $\delta^{15}N$).

Figure 2. PCA plot for bulk $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{34}S$ and $\delta^{15}N$ for farming system. The numbers on the PCA plot refer to the sample numbers.



Having analysed isotope ratios for bulk samples of organic and conventional tomatoes, a clear distinction between farming system (independent of region) was not established. Therefore, it was necessary to investigate isotope ratios of individual amino acids to gain a better understanding of the influence of farming system.

3.2 GC-C-IRMS

Amino acids, Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and Phe, in tomato samples were analysed for $\delta^{13}C$ and $\delta^{15}N$. In Table 3, the means of the $\delta^{13}C_{aa}$ and $\delta^{15}N_{aa}$ values for the different farming systems, regions and years as well as the results of a one-way analysis of variance (ANOVA) without interaction effects are reported. There were strong significant differences (p<0.05) between the organic and conventional farming systems for all amino acids for $\delta^{15}N$. For $\delta^{13}C$ there was only a significant difference (p<0.01) between systems for Glx. For amino acids Ala, Val, Ileu, Leu, Gly, Pro and Phe there

were significant differences (p<0.05) between the regions BAS and ER. For $\delta^{13}C$ there was only a significant difference (p<0.01) between regions for Val and Leu. There are no significant effects for $\delta^{15}N$ or $\delta^{13}C$ for all amino acids between the different years, 2012 and 2013.

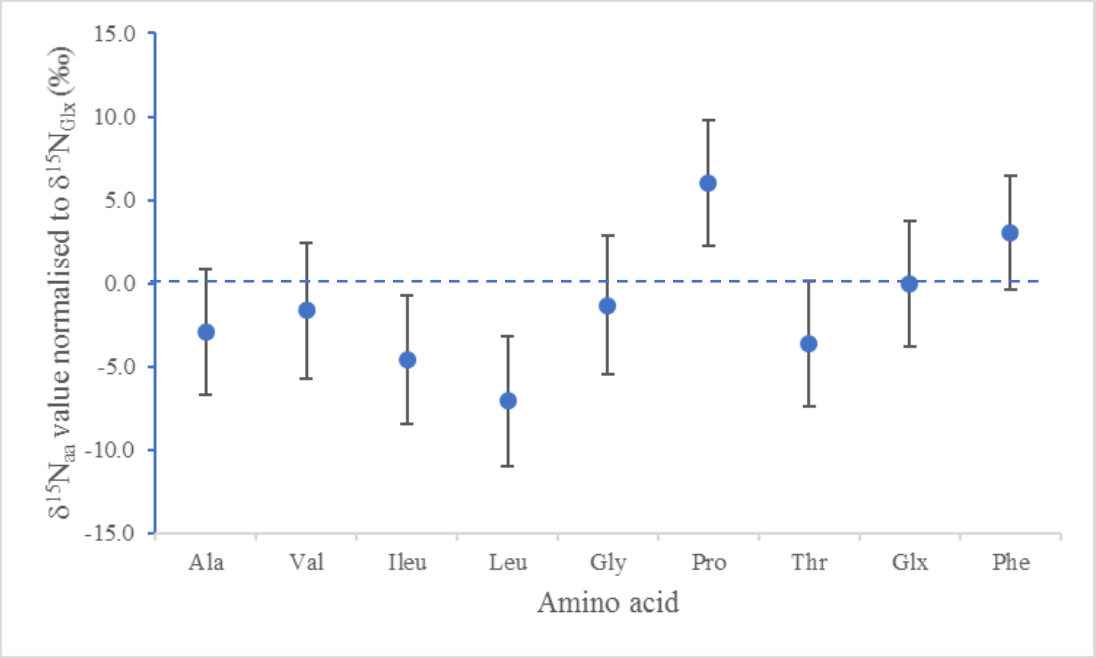
Table 3. Amino acid $\delta^{15}N_{aa}$ and $\delta^{13}C_{aa}$ values of tomato samples and ANOVA analysis

system		Tomato Amino Acid $\delta^{15}N$ and $\delta^{13}C$ values (‰)																	
		Ala		Val		Ileu		Leu		Gly		Pro		Thr		Glx		Phe	
		N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C
Org	mean	6.9a	-27.3	8.5a	-33.8	5.6a	-25.5	2.9a	-36.5	8.3a	-38.9	15.6a	-25.8	6.3a	-22.7	10.1a	-26.9a	12.9a	-29.2
	SD	3.3	0.8	3.5	0.8	3.0	1.2	3.3	0.9	3.7	1.7	3.4	2.2	3.3	2.0	2.6	0.6	2.8	0.7
Conv	mean	1.9b	-27.8	2.8b	-34.0	-0.2b	-25.6	-2.5b	-36.9	3.7b	-38.8	10.9b	-26.2	1.0b	-23.7	4.4b	-28.4b	7.8b	-29.4
	SD	2.5	1.2	2.1	0.7	2.0	1.3	2.3	0.9	3.5	2.3	2.4	2.5	2.0	2.6	2.2	0.5	1.6	0.9
region																			
BAS	mean	2.8a	-27.7	4.2a	-34.2c	1.1a	-25.8	-1.3a	-37.1c	4.4a	-38.4	11.9c	-26.0	2.7	-23.6	6.1c	-27.8	9.1a	-29.8
	SD	3.3	0.8	2.1	0.7	2.3	0.6	2.1	0.5	3.6	1.2	2.4	1.8	1.8	1.7	3.1	0.9	2.6	0.6
ER	mean	5.9b	-27.4	7.1b	-33.6d	4.3b	-25.3	1.7b	-36.3d	7.6b	-39.3	14.7d	-26.0	4.6	-22.7	8.4d	-27.6	11.5b	-28.8
	SD	3.8	1.2	5.1	0.6	4.5	1.6	4.9	1.1	4.3	2.5	4.5	2.8	5.0	2.8	4.2	1.0	3.9	0.6
year																			
2012	mean	3.4	-27.3	5.7	-33.6	2.9	-25.2	0.3	-36.6	4.7	-38.2	12.4	-25.1	3.5	-22.4	6.6	-27.5	10.5	-29.1
	SD	3.5	0.9	3.6	0.6	3.7	1.1	3.6	0.8	3.9	1.4	3.3	2.0	3.3	1.7	3.5	0.9	3.5	0.8
2013	mean	5.3	-27.8	5.6	-34.2	2.5	-25.9	0.1	-36.8	7.2	-39.6	14.2	-26.9	3.9	-23.9	8.0	-27.9	10.1	-29.5
	SD	4.1	1.1	4.7	0.7	4.3	1.2	4.5	1.1	4.4	2.3	4.2	2.3	4.4	2.6	4.1	0.9	3.6	0.7

Significantly different mean values (one-way ANOVA, p<0.05) between groups are indicated with “a” and “b”. Significantly different mean values (one-way ANOVA, p<0.1) between groups are indicated with “c” and “d”. Org, organic, Conv, conventional, SD, standard deviation.

As mentioned earlier, glutamine and glutamic acid $\delta^{15}N$ value is central to all the $\delta^{15}N$ amino acid values as the amino acids are derived from these amino acids, therefore the $\delta^{15}N$ amino acid values were normalised against the $\delta^{15}N$ Glx values to cancel out the differences due to peripheral sources of nitrogen such as locality and fertilizer applications (see Figure 4)(Paolini et al., 2015; Styring et al., 2014a).

Figure 4. Tomato amino acid $\delta^{15}N$ values normalised to Glx for organic and conventional farming systems. Dots represent mean values, error bars represent ± standard deviation (n=16).

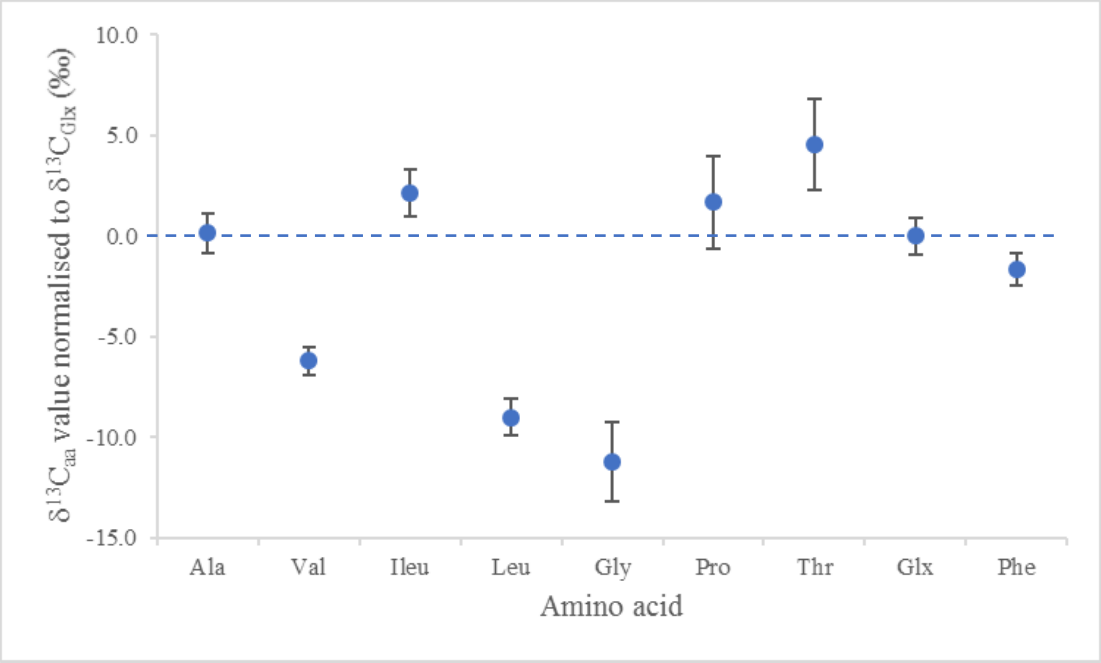


The normalised $\delta^{15}N$ values for both farming systems of Pro (6.0‰ ± 3.8‰) and Phe (3.0‰ ± 3.4‰) are higher when compared to the $\delta^{15}N$ values for Glx. Phe is enriched in $\delta^{15}N$ compared to Glx due to a kinetic isotope effect in the synthesis of phenylpropanoids (for structural and protection purposes in plants) where it is deaminated as an intermediate (Styring et al., 2014a; Werner & Schmidt, 2002). Pro is also enriched in $\delta^{15}N$ compared to Glx and this is due to a kinetic isotope effect associated with its catabolism via enzymatic reactions (Hermes, Weiss, & Cleland, 1985; Styring et al., 2014a). The $\delta^{15}N$ values for Ala (-2.9‰ ± 3.8‰), Val (-1.6‰ ± 4.1‰) Ileu (-4.6‰ ± 3.9‰), Leu (-7.1‰ ± 3.9‰) Gly (-1.3‰ ± 4.2‰) and Thr (-3.6‰ ± 3.8‰) are lower when compared to the $\delta^{15}N$ values for Glx. Val, Ileu and Leu are branch-chain amino acids (BCAA) and are precursors to synthesising proteins (Werner & Schmidt, 2002). Val is synthesised directly from pyruvate which is derived from photosynthesis (Bryan, 1980) and Leu is synthesised

from the Val metabolic pathway via a succession of reactions branching off from it (Kochevenko & Fernie, 2011; Pátek, 2007) and these successive reactions could be the cause for the greater discrimination for $\delta^{15}N$ in Leu than for Val (Bol, Ostle, & Petzke, 2002; Hofmann, Jung, Segschneider, Gehre, & Schüürmann, 1995). Ileu is synthesized from Thr, which is deaminated, and during this metabolic process, there is a slight depletion in $\delta^{15}N$ for Ileu compared to Thr. Gly is involved in the photorespiratory nitrogen cycle and is derived from the Glu, Ala or Asp amino group (Styring et al., 2014a; Werner & Schmidt, 2002). Gly $\delta^{15}N$ is enriched compared with Ala and is slightly depleted to Glx. Ala is also slightly depleted compared to Glx.

In addition, for $\delta^{13}C$, to remove the external factors that could be affecting the isotopic value such as the atmosphere and the fertilizer regime, the amino acid $\delta^{13}C$ values were normalised to Glx $\delta^{13}C$ value (see Figure 5).

Figure 5. Tomato amino acid $\delta^{13}C$ corrected value means normalised to Glx for organic and conventional farming systems and ER and BAS regions. Dots represent mean values, error bars represent ± standard deviation (n=16).

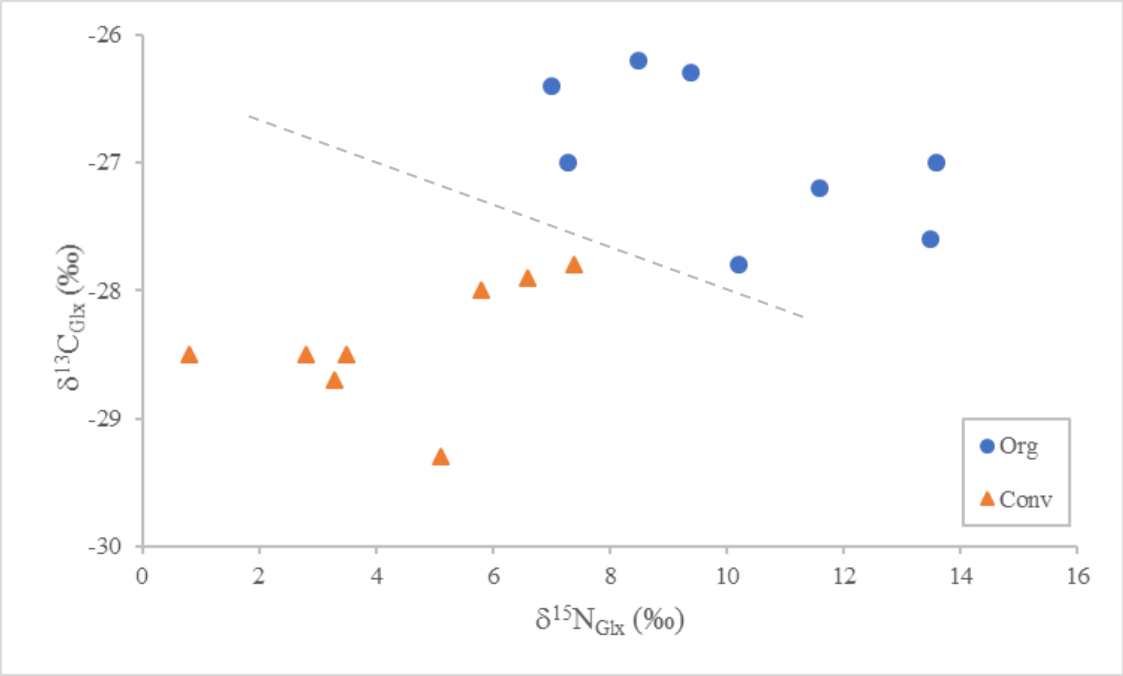


The $\delta^{13}C$ values for both farming systems and regions of Pro (1.7‰ ± 2.3‰), Ileu (2.1‰ ± 1.2‰) and Thr (4.5‰ ± 2.3‰) are higher when compared to the $\delta^{13}C$ values for Glx. The $\delta^{13}C$ values for Ala (0.1‰ ± 1.0‰) are the same as for Glx and the $\delta^{13}C$ values for Val (-6.2‰ ± 0.7‰), Leu (-9.0‰ ± 0.9‰), Gly (-11.2‰ ± 2.0‰) and Phe (-1.6‰ ± 0.8‰) are lower when compared to the $\delta^{13}C$ values for Glx.

The fixation of CO_2 in plants by the enzyme RuBisCO has a kinetic isotope effect. RuBisCO generates glucose and aids in the photorespiration of the plant, these affect the amino acids involved in these systems, such as glycine in photorespiration (Paolini et al., 2015; Styring et al., 2014a; Werner & Schmidt, 2002). As previously mentioned, Val is derived from photosynthesis as is Leu, by subsequent branching reactions from the Val metabolic pathway, Gly is derived from photosynthesis and this can explain the lower $\delta^{13}C$ values for these 3 amino acids: Val (-34.2‰ to -33.6‰), Leu (-37.1‰ to -36.3‰) and Gly (-39.6‰ to -38.2‰) are very low (Table 3).

As can be seen in Figure 6 there is separation between the farming systems, organic and conventional, of the amino acids when the $\delta^{13}C_{Glx}$ values are plotted against $\delta^{15}N_{Glx}$ values.

Figure 6. Tomato $\delta^{15}N_{Glx}$ samples plotted against $\delta^{13}C_{Glx}$ samples. Org (organic), Conv (conventional), dashed line is for visual purposes and does not represent statistical analysis.

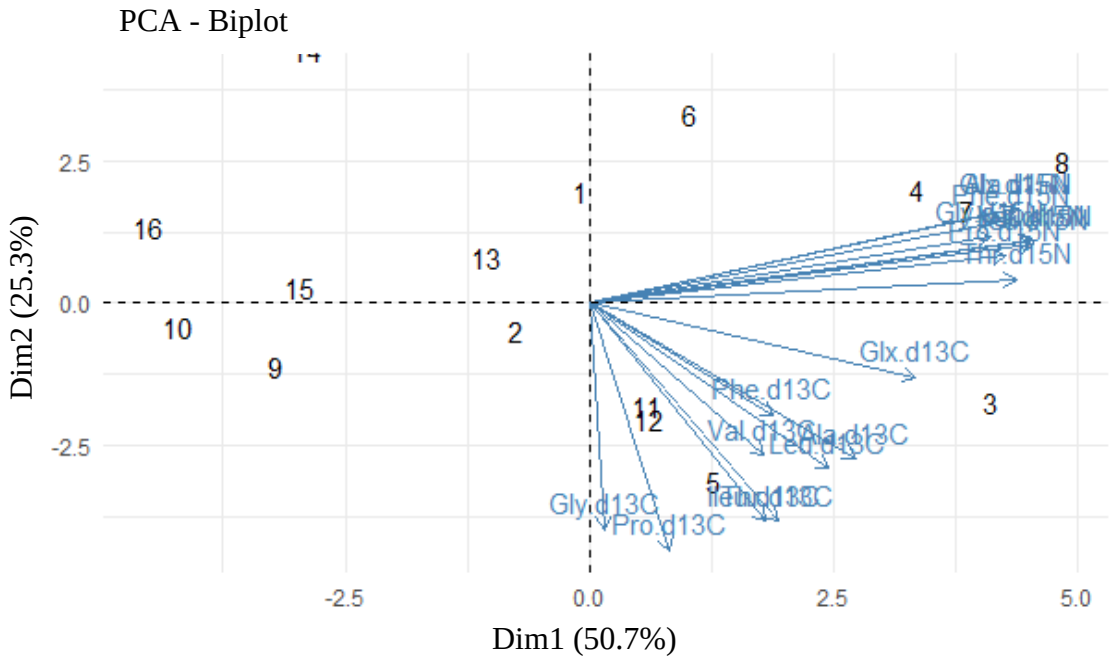


The amino acids were also analysed by ANOVA for interaction effects between the groupings. For $\delta^{15}N$ there was significant interaction effects between system and region for $\delta^{15}N$ for the amino acids Val (p<0.01), Ileu (p<0.05), Leu (p<0.05), Pro (p< 0.1), Thr (p<0.01) and Phe (p<0.001) and between region and year for $\delta^{15}N$ for the amino acids Ala (p<0.01) and Leu (p<0.05). For $\delta^{13}C$, there was significant interaction effects between system and year for $\delta^{13}C$ for the amino acid Thr (p<0.05). There was also a significant interaction effects between region and year for $\delta^{13}C$ for the amino acid Ala (p<0.05).

Principal component analysis (PCA) was implemented for the single amino acids for $\delta^{13}C$ and $\delta^{15}N$ values. The majority of the data variability is explained with the first three PCs with 51% variance for the first PC, 25% variance for the second PC and 9% variance for the third PC. To visualise which variables were influencing the first two PCs a biplot was

implemented (Figure 7). The first PC has a number of amino acid contributors: Leu, Ileu, Val, Ala, Phe, Glx, Thr, Pro, and Gly for $\delta^{15}N$ with a few which are highly correlated. Another contributor to the first PC is Glx for $\delta^{13}C$. The amino acids which contribute to PC 2 are Pro, Gly, Thr, Ileu, Leu, Val and Ala for $\delta^{13}C$, with Phe and Ala showing a good degree of degree of correlation, Thr and Ileu highly correlated.

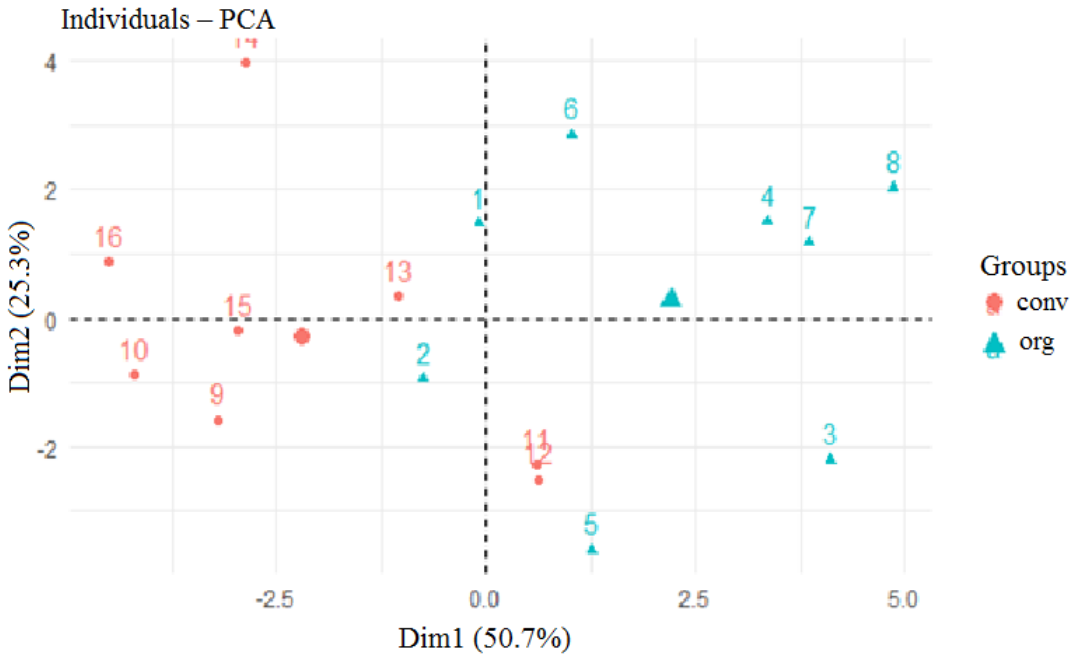
Figure 7. A biplot of PC1 and PC2 for amino acid $\delta^{13}C$ and $\delta^{15}N$ values. The numbers on the biplot refer to the sample numbers. The label d13C refers to $\delta^{13}C$ and d15N refers to $\delta^{15}N$.



Analysing the PCA for farming system for amino acid $\delta^{15}N$ and $\delta^{13}C$, it can be seen (Figure 8) that when plotting the first two PCs there is much better separation between the organic and conventional systems than for the bulk tomato samples. Half of the organic samples are associated with Leu, Ileu, Val, Ala, Phe, Glx, Thr, Pro, and Gly for higher $\delta^{15}N$ values. A few of the organic and conventional samples are associated with

Pro, Gly Thr, Ileu, Leu, Val and Ala for $\delta^{13}C$ values whereas the majority of conventional samples appear to be associated with lower levels of all measured amino acids for $\delta^{15}N$ and $\delta^{13}C$.

Figure 8. PCA plot for $\delta^{13}C$ and $\delta^{15}N$ of the amino acids for farming system. The numbers on the PCA plot refer to the sample numbers.



4. Conclusions

By analysing multi-element stable isotope ratios in bulk samples and in amino acids of Italian organic and conventional tomatoes, it was confirmed that the most significant parameter for characterising organic production is $\delta^{15}N$; however, year and region also have an impact.

By implementing the analysis of $\delta^{15}N$ and $\delta^{13}C$ of amino acids Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and Phe, a better separation between the 2 farming systems was achieved, regardless of the considered year and region. This confirms that compound specific isotopic analysis is a more powerful technique compared to the bulk analysis.

Future research should encompass a larger number of samples from different regions to confirm and test the reliability of the method.

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Supporting Information

SI Table 1. Bulk $\delta^{2}H$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S_{corr}$ values of tomato samples. BAS (Basilicata), ER (Emilia-Romagna), org (organic), conv (conventional), corr (corrected).

Variables					Tomato bulk values (‰)				
system	region	year	variety	replicate	$\delta^{2}H$	$\delta^{18}O$	$\delta^{13}X$	$\delta^{15}N$	$\delta^{34}S_{corr}$
org	BAS	2012	1	1	-43.1	29.9	-28.3	6.5	-4.8
org	BAS	2012	1	2	-48.0	28.2	-28.2	8.1	-5.4
org	BAS	2012	1	3	-48.6	28.3	-28.6	6.1	-4.4
org	BAS	2012	2	1	-35.6	28.7	-28.1	5.6	-2.0
org	BAS	2012	2	2	-37.6	28.4	-28.1	5.9	-2.5
org	BAS	2012	2	3	-41.7	27.7	-28.9	6.0	-2.8
org	ER	2012	1	1	-48.1	27.4	-26.7	10.8	1.6
org	ER	2012	1	2	-41.3	28.0	-26.1	10.7	2.0
org	ER	2012	1	3	-51.0	27.7	-26.8	11.6	1.5
org	ER	2012	2	1	-50.8	24.7	-27.7	10.1	-6.6
org	ER	2012	2	2	-56.4	25.2	-27.8	8.6	-6.3
org	ER	2012	2	3	-53.7	24.9	-27.8	9.5	-6.5
org	BAS	2013	1	1	-34.1	27.4	-27.4	6.9	-2.7
org	BAS	2013	1	2	-28.7	26.9	-26.8	6.4	-1.9
org	BAS	2013	1	3	-29.2	27.0	-26.9	5.0	-1.4
org	BAS	2013	2	1	-22.9	29.2	-26.0	4.0	0.8
org	BAS	2013	2	2	-34.8	27.7	-28.1	9.7	-4.4
org	BAS	2013	2	3	-38.8	27.7	-27.5	7.3	-3.6
org	ER	2013	1	1	-60.5	27.4	-27.7	14.0	1.1
org	ER	2013	1	2	-74.4	25.7	-27.5	14.6	1.1
org	ER	2013	1	3	-60.1	27.3	-27.6	13.9	1.3
org	ER	2013	2	1	-68.1	26.4	-27.4	14.0	1.4
org	ER	2013	2	2	-67.5	26.1	-27.5	13.2	1.5
org	ER	2013	2	3	-56.0	27.6	-27.4	13.6	1.6
conv	BAS	2012	1	1	-32.5	29.8	-27.9	2.8	-1.7
conv	BAS	2012	1	2	-32.4	29.8	-27.9	2.2	-1.9
conv	BAS	2012	1	3	-35.5	29.6	-27.7	2.7	-1.5
conv	BAS	2012	2	1	-26.5	28.6	-28.2	1.6	-2.4
conv	BAS	2012	2	2	-27.4	29.0	-28.1	1.6	-2.4
conv	BAS	2012	2	3	-27.7	29.0	-28.3	1.9	-1.8
conv	ER	2012	1	1	-55.0	25.4	-26.5	5.9	2.0
conv	ER	2012	1	2	-52.7	25.4	-26.5	5.9	2.1
conv	ER	2012	1	3	-47.7	25.4	-26.8	5.7	2.1
conv	ER	2012	2	1	-49.6	25.4	-26.7	5.7	1.6
conv	ER	2012	2	2	-41.7	25.4	-26.4	5.5	1.7
conv	ER	2012	2	3	-47.2	25.1	-26.7	5.2	1.8
conv	BAS	2013	1	1	-67.7	27.4	-28.1	8.0	-3.6
conv	BAS	2013	1	2	-70.9	27.4	-27.4	7.1	-4.7
conv	BAS	2013	1	3	-71.7	26.9	-27.4	7.3	-4.4
conv	BAS	2013	2	1	-64.8	26.7	-27.5	8.2	-4.2
conv	BAS	2013	2	2	-66.7	26.6	-27.9	6.6	-4.6
conv	BAS	2013	2	3	-66.0	28.7	-28.2	5.4	-4.6
conv	ER	2013	1	1	-68.6	24.1	-29.6	3.4	-2.3
conv	ER	2013	1	2	-61.6	23.9	-29.6	3.5	-2.5
conv	ER	2013	1	3	-65.8	24.4	-29.8	3.1	-2.1
conv	ER	2013	2	1	-70.1	24.3	-29.8	2.8	-2.6
conv	ER	2013	2	2	-56.7	24.2	-29.9	3.1	-2.2
conv	ER	2013	2	3	-58.5	24.1	-29.5	3.0	-1.9

SI Table 2. Amino acid $\delta^{13}C$ corrected values for tomato samples. Values are the mean of three analytical replicates. Org (organic), Conv (conventional), BAS (Basilicata), ER (Emilia-Romagna).

Variables				Tomato amino acid $\delta^{13}C$ values (‰)									
system	region	year	variety	Ala	Val	Ileu	Leu	Gly	Nleu	Pro	Thr	Glx	Phe
Org	BAS	2012	1	-27.9	-34.1	-26.4	-36.7	-40.3	-27.9	-28.2	-25.8	-26.2	-29.4
Org	BAS	2012	2	-27.3	-33.5	-26.2	-37.1	-37.6	-27.0	-24.0	-23.0	-27.0	-29.4
Org	ER	2012	1	-26.8	-32.7	-23.9	-35.8	-36.9	-27.5	-23.1	-20.4	-26.3	-28.7
Org	ER	2012	2	-27.5	-33.6	-25.9	-36.3	-40.2	-28.1	-28.1	-23.5	-27.2	-28.5
Org	BAS	2013	1	-27.9	-33.4	-26.1	-36.4	-39.6	-27.2	-25.4	-22.0	-27.6	-29.0
Org	BAS	2013	2	-26.0	-35.0	-24.7	-36.8	-38.3	-27.6	-27.3	-23.9	-27.0	-30.6
Org	ER	2013	1	-28.3	-34.7	-26.8	-38.0	-41.4	-27.5	-27.4	-23.0	-27.8	-29.6
Org	ER	2013	2	-26.6	-33.4	-23.9	-34.8	-37.0	-26.8	-23.0	-19.6	-26.4	-28.3
Conv	BAS	2012	1	-28.2	-34.0	-25.4	-37.1	-37.3	-27.2	-24.0	-21.9	-28.5	-29.5
Conv	BAS	2012	2	-28.4	-34.6	-25.5	-37.9	-36.7	-27.1	-24.5	-22.2	-28.5	-30.7
Conv	ER	2012	1	-26.1	-33.3	-23.7	-36.4	-38.1	-27.1	-25.1	-21.4	-27.9	-28.0
Conv	ER	2012	2	-26.1	-33.2	-24.3	-35.6	-38.1	-27.4	-23.8	-20.8	-28.0	-28.6
Conv	BAS	2013	1	-27.3	-33.7	-26.5	-37.2	-39.4	-27.7	-26.6	-23.3	-28.7	-29.5
Conv	BAS	2013	2	-28.2	-35.3	-25.5	-37.4	-38.1	-27.5	-28.1	-26.5	-28.5	-30.4
Conv	ER	2013	2	-28.7	-33.7	-26.1	-35.7	-38.8	-26.8	-26.3	-25.4	-27.8	-29.6
Conv	ER	2013	1	-29.2	-34.0	-27.9	-37.7	-44.2	-27.4	-31.2	-27.7	-29.3	-29.1

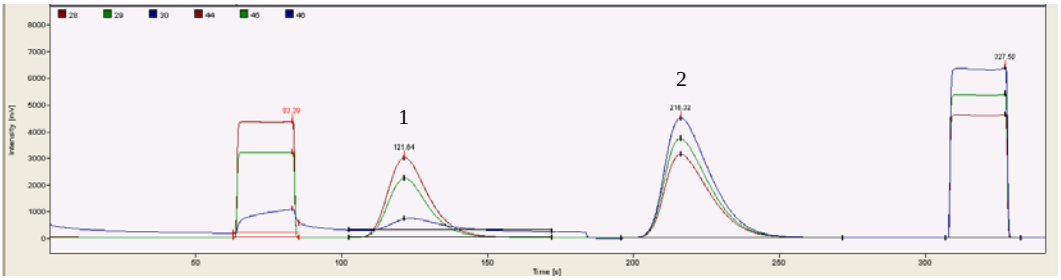
SI Table 3. Amino acid $\delta^{15}N$ values for tomato samples. Values are the mean of three analytical replicates. Org (organic), Conv (conventional), BAS (Basilicata), ER (Emilia-Romagna).

Variables				Tomato amino acid $\delta^{15}N$ values (‰)									
system	region	year	variety	Ala	Val	Ileu	Leu	Gly	Nleu	Pro	Thr	Glx	Phe
Org	BAS	2012	1	4.7	5.6	3.8	0.8	2.5	13.3	12.4	0.9	8.5	13.1
Org	BAS	2012	2	1.6	3.8	1.6	-2.1	5.6	13.4	10.5	3.7	7.3	9.6
Org	ER	2012	1	6.7	11.0	8.1	4.2	7.4	13.5	17.6	9.7	9.4	13.9
Org	ER	2012	2	8.0	10.9	7.7	6.2	8.8	13.8	16.5	6.9	11.6	15.8
Org	BAS	2013	1	3.9	4.4	1.9	-0.2	6.1	12.9	14.5	4.3	7.0	8.0
Org	BAS	2013	2	8.6	8.5	4.2	2.1	9.8	14.2	15.1	5.7	10.2	12.4
Org	ER	2013	1	9.9	11.6	7.7	4.6	12.7	14.0	16.4	9.7	13.5	14.7
Org	ER	2013	2	11.4	12.3	9.4	7.7	13.3	13.3	21.9	9.6	13.6	15.6
Conv	BAS	2012	1	-0.6	2.4	-1.2	-3.5	-0.8	14.0	9.1	1.7	0.8	8.1
Conv	BAS	2012	2	-2.1	1.5	-2.0	-4.0	-0.2	12.9	8.8	0.5	2.8	5.1
Conv	ER	2012	1	4.9	3.6	2.7	0.7	5.5	14.0	13.1	3.6	6.6	8.8
Conv	ER	2012	2	4.3	6.6	2.4	0.3	9.1	13.3	10.8	0.7	5.8	9.7
Conv	BAS	2013	1	3.5	3.4	-1.3	-2.1	6.9	12.6	13.9	3.0	7.4	9.2
Conv	BAS	2013	2	2.9	3.7	1.4	-1.2	4.9	12.8	10.8	1.7	5.1	7.5
Conv	ER	2013	2	0.8	0.0	-1.7	-5.1	2.4	13.3	7.4	-2.8	3.5	6.0
Conv	ER	2013	1	1.2	0.8	-1.7	-5.0	1.6	12.1	13.6	-0.4	3.3	7.6

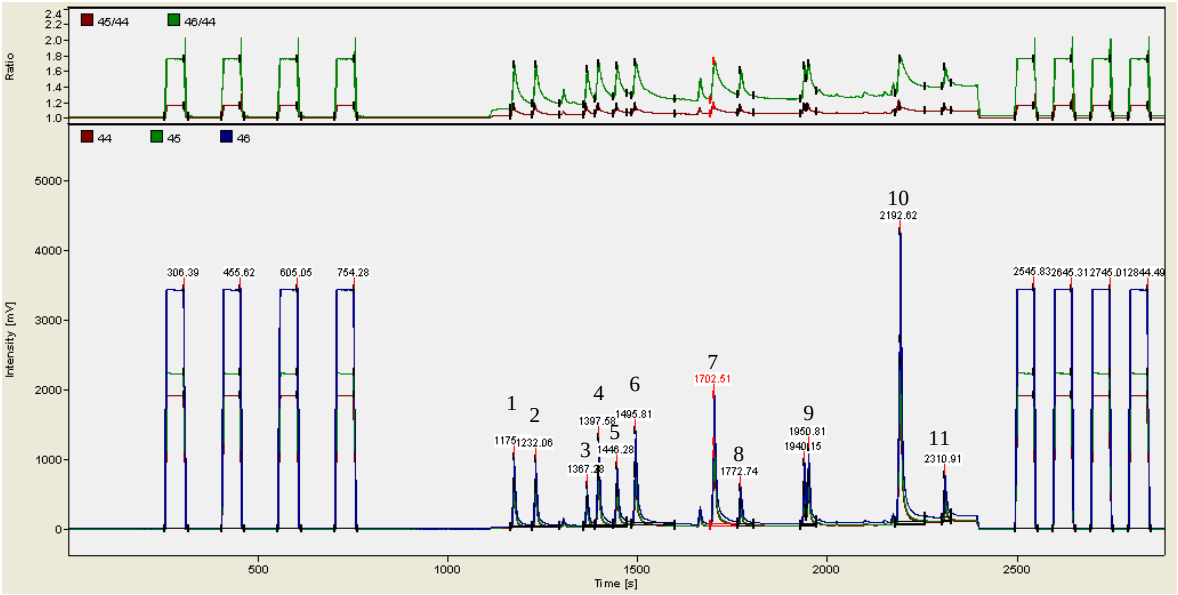
SI Table 4. Amino acid $\delta^{13}C$ values of the standard mixture amino acids, their respective *N*-acetylisopropyl esters (in brackets standard deviations of 3 repeated measurements) and after empirical correction.

Amino acids	$\delta^{13}C$ (‰)		
	underivatized amino acid	amino acid *N*-acetylisopropyl esters	corrected amino acid
Ala	-19.7	-26.0 (0.2)	-19.4
Val	-11.9	-21.5 (0.1)	-11.5
Ileu	-13.5	-23.1 (0.1)	-13.6
Leu	-13.5	-21.7 (0.0)	-13.3
Gly	-41.6	-28.5 (0.1)	-42.8
Nleu	-27.6	-29.4 (0.2)	-27.1
Pro	-11.2	-21.2 (0.1)	-11.1
Thr	-10.5	-24.6 (0.3)	-11.0
Glx	-13.7	-22.5 (0.1)	-14.0
Phe	-11.1	-19.4 (0.1)	-10.6

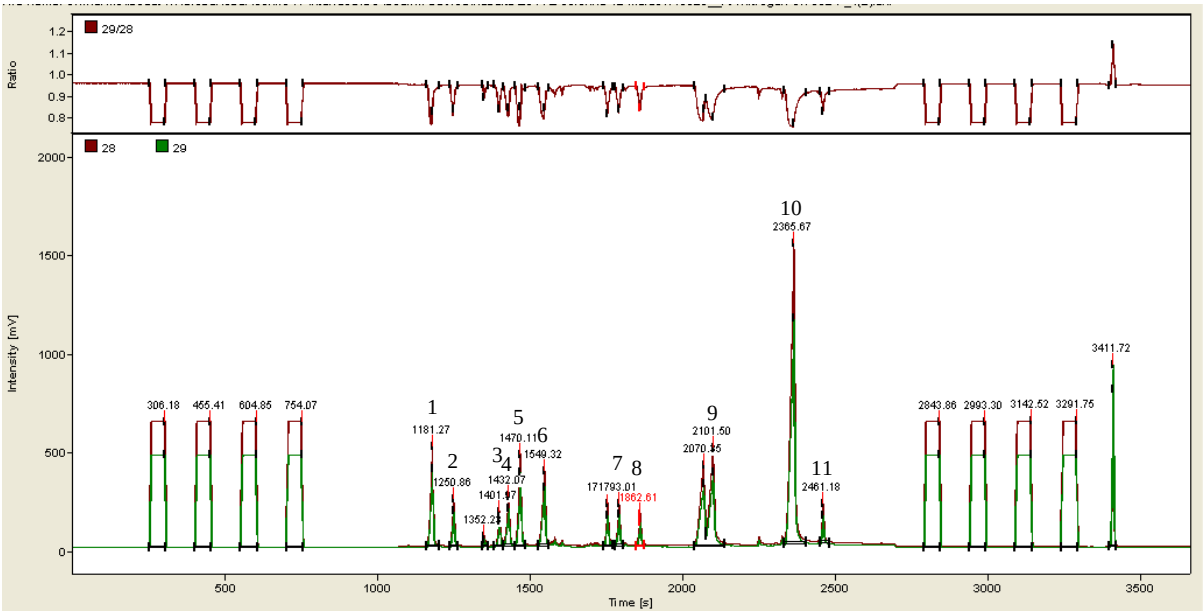
SI Figure 1. EA-IRMS chromatogram of an amino acid for nitrogen (peak 1) and carbon (peak 2). For the nitrogen peak the red trace refers to isotope 28, the green trace refers to isotope 29 and the blue trace refers to isotope 30. For the carbon peak the red trace refers to isotope 44, the green trace refers to isotope 45 and the blue trace refers to isotope 46.



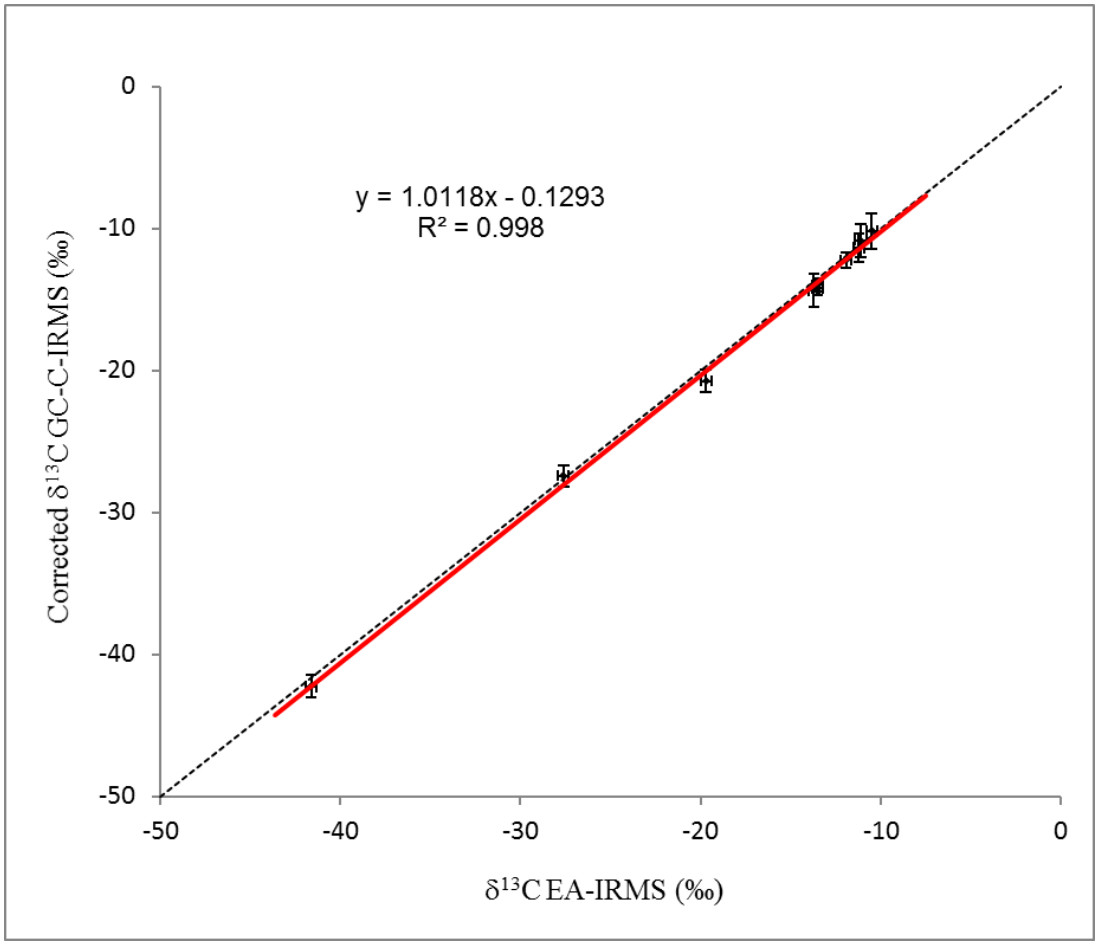
SI Figure 2. GC-C-IRMS chromatogram of *N*-acetyl-isopropyl derivatives of amino acids in a tomato sample for carbon. Peaks: 1, Ala; 2, Val; 3, Ile; 4, Leu; 5, Gly; 6, Nleu (internal standard); 7, Pro; 8, Thr; 9, Asx; 10, Glx; 11, Phe. The first and last four peaks are reference gas signals. The ratio of the isotopes is represented by the top chromatogram, the green trace refers to the ratio of isotope 46 to isotope 44 and the red trace refers to the ratio of isotope 45 to isotope 44. The intensity of the isotopes are reflected in the bottom chromatogram where the red trace refers to carbon isotope 44, the green trace refers to carbon isotope 45 and the blue trace refers to carbon isotope 46.



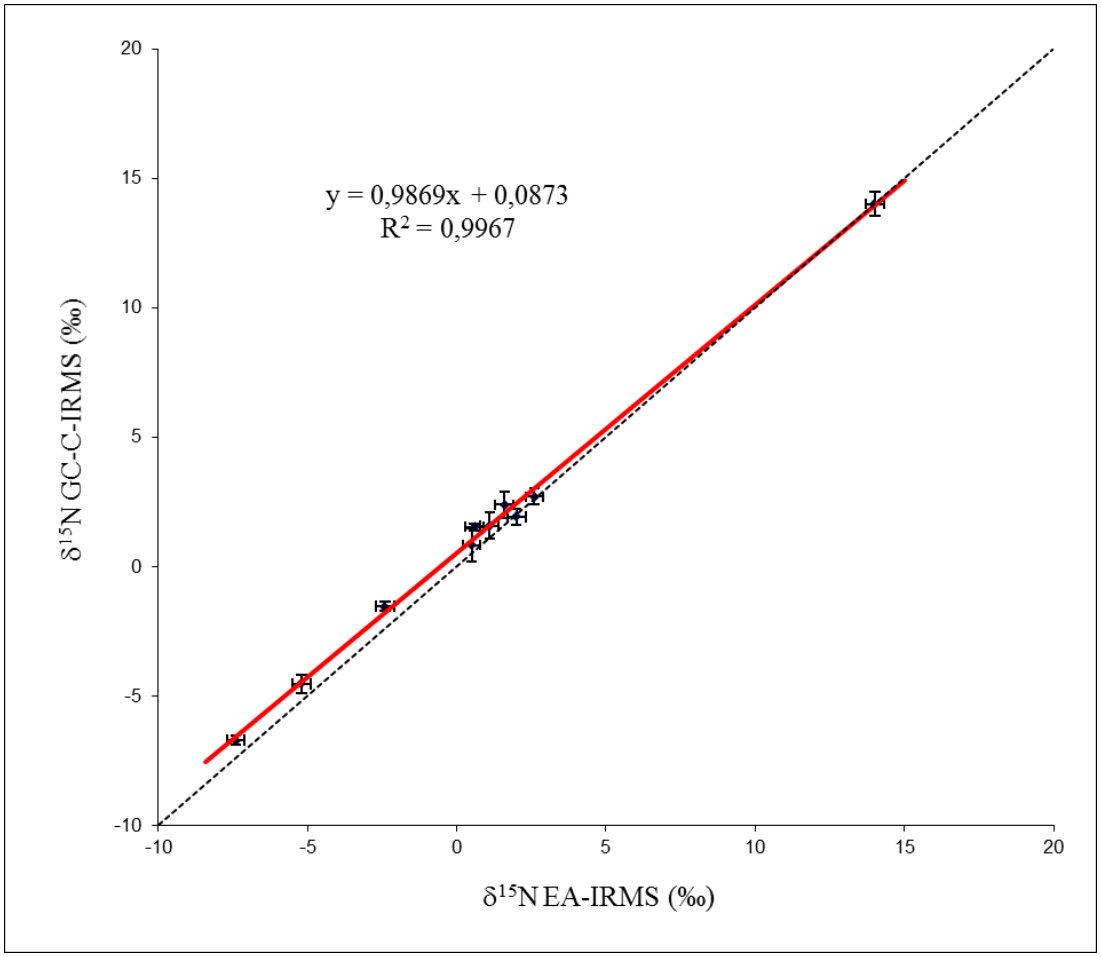
SI Figure 3. GC-C-IRMS chromatogram of *N*-acetyl-isopropyl derivatives of amino acids in a tomato sample for nitrogen. Peaks: 1, Ala; 2, Val; 3, Ile; 4, Leu; 5, Gly; 6, Nleu (internal standard); 7, Pro; 8, Thr; 9, Asx; 10, Glx; 11, Phe. The first and last four peaks are reference gas signals. The ratio of the isotopes is represented by the top chromatogram, the red trace refers to the ratio of isotope 29 to isotope 28. The intensity of the isotopes are reflected in the bottom chromatogram where the red trace refers to nitrogen isotope 28 and the green trace refers to nitrogen isotope 29.



SI Figure 4. Isotopic $\delta^{13}C$ measurements from GC-C-IRMS (n=3) plotted against EA-IRMS (n=2) measurements for carbon. Error bars represent the standard deviation (±1σ) of repeated measurements. The dashed line is the y=x line. The red line is the regression line.



SI Figure 5. Isotopic $\delta^{15}N$ measurements from GC-C-IRMS (n=3) plotted against EA-IRMS (n=2) measurements for nitrogen. Error bars represent the standard deviation (±1σ) of repeated measurements. The dashed line is the y=x line. The red line is the regression line.



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Experimental Report

Tomato fruit extraction of free amino acids

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Experimental Report

Tomato fruit extraction of free amino acids

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1. Introduction

In the preceding research paper, the implementation of GC-C-IRMS to differentiate tomatoes grown organically from tomatoes grown conventionally was investigated and the results reported. The extraction and derivatisation methods for amino acids in tomatoes was developed by another researcher and I collected, analysed and discussed the isotope ratio data. The method reported in a refereed publication is usually the final developed method implemented in the particular study. However, for a thesis a substantial amount of time and knowledge goes into method development, which is a significant part of research training for a PhD candidate that cannot be encapsulated in a manuscript prepared for peer-reviewed publication. Important methodologies relevant to my study which emphasise my proficiency in problem solving and critical thinking in relation to analytical chemistry are therefore conveyed in this experimental report.

The following method development was not continued due to time constraints and prevailing problems with pyridine and the inability to remove it from the sample prior to analysis. The effect of the pyridine on the oxidation column in the GC-C-IRMS was paramount. The drawbacks to the method developed in the previous research paper was that the derivatisation and extraction methods were time-consuming which creates the inability to analyse samples in a short timeframe. The method I was developing would overcome this as the ethyl chloroformate reaction is very quick and would be appropriate to use for high throughput sample analysis.

Amino acids are non-volatile compounds, hence derivatisation is required for analysis by GC-C-IRMS (Corr, Berstan, & Evershed, 2007). Methods for the extraction, derivatisation and analysis of amino acids were explored to establish optimum conditions.

2. Experimental Methods and Discussion

Throughout this research, when required, various GC conditions were explored to adjust for peak optimisation and separation such that over 100 GC methods were trialled via changes in GC columns, GC oven temperature, temperature programs and flow rates. For the purposes of this experimental report only the GC methods relevant to the communicated results are described. In this report amino acids not included in the previous research paper were analysed and therefore their EA-IRMS values were required, and an extensive list of these values for $\delta^{15}N$ and $\delta^{13}C$ can be found in the Supplementary Information (SI) Table 1.

2.1 Tomato sample preparation

Tomato fruits (oblong) used for method development purposes were purchased locally. The tomato fruits were weighed, washed, dried and separated into four glass beakers and put in the freezer (-20 °C) for 24 hr before being transferred to the freeze-dryer (72 hr). The samples were removed from the freeze-drier and reweighed. There was a problem with some of the tomatoes not being completely frozen, these were separated from the properly frozen tomatoes. The frozen tomatoes (6 tomatoes) were weighed separately and then 3 were pulverised with a mortar and pestle under liquid N_2. The remaining three were cut in half and those halves separated and combined so three halves of the tomatoes were combined and then were powdered with a mortar and pestle under liquid N_2 while the other 3 halves were separated into pulp, seeds and skins, weighed and then powdered under liquid N_2 with a mortar and pestle. The powdered samples (whole tomatoes, half

tomatoes, pulp, seed and skin) were put in the freezer (-20 °C) overnight and then to remove any excess moisture were transferred to the freeze-drier (72 hr). The tomato samples were then reweighed and then stored in the fridge (4 °C) (see SI. Figure 1).

2.2 Preparation of amino acids

2.2.1 Free amino acids in tomato fruit

For $\delta^{15}N$ analysis by GC-C-IRMS the amount of sample is important. It is easier to measure $\delta^{13}C$ as there are more C atoms in a compound but for amino acids there may be only one N atom or sometimes 2 N atoms and this affects the peaks heights to be measured. Therefore, it is necessary to determine which free amino acids can be feasibly measured. The free amino acid concentrations found in ripe tomato fruit are glutamine (Gln; 5490 nmol/g), glutamic acid (Glu; 2769 nmol/g), γ-aminobutyric acid (GABA; 2060 nmol/g), aspartate (Asp; 1159 nmol/g), asparagine (Asn; 998 nmol/g), serine (Ser; 773 nmol/g), phenylalanine (Phe; 676 nmol/g), isoleucine (Ileu; 451 nmol/g), alanine (Ala; 209 nmol/g), leucine (Leu; 161 nmol/g), valine (Val; 145 nmol/g), proline (Pro; 129 nmol/g) and glycine (Gly; 97 nmol/g) (Boggio, Palatnik, Heldt, & Valle, 2000).

2.2.2 Derivatisation Experimentation

2.2.2.1 Derivatisation by ethyl chloroformate (method 1)

Derivatisation by ethyl chloroformate (ECF) was based on the method of Husek (Hušek, 1991). Standard solutions (2.5 M in 0.1 M HCl) were made for the single amino acids Gly and Ala and citric acid (2.5 M in 0.1 M HCl) was used as an internal standard (iSTD). To a 5 mL test tube, 15 μL of standard solution was added (i.e. Gly; Ala; citric acid; Gly + citric acid; Ala + citric acid and octadecane) to which 45 μL of deionised water was added, to make the total volume 60 μL. The solution was then vortexed. To the sample

ethanol-pyridine solution (EtOH:pyr; 40 μL; 4:1, v/v) was added and then vortexed, then 5 μL ethylchloroformate (ECF) was added, vortexed (CO_2 released). Then chloroform (1% ECF, v/v; 100 μL) solution was added and the sample vortexed. The layers were allowed to separate and the chloroform layer was removed and analysed by GC-MS. The amino acids were derivatised as evidenced by their molecular ions in the GC-MS trace and by identification match in the NIST database. N(*O*,*S*)-ethoxycarbonyl (EOC) Gly ethyl ester eluted at 9.712 min, and N(*O*,*S*)-EOC D-Ala ethyl ester eluted at 9.043 min, N(*O*,*S*)-EOC L-Ala ethyl ester at 9.313 min and N(*O*,*S*)-EOC citric acid ethyl ester at 21.730 min. Octadecane was used as a non-derivatised iSTD and it eluted at 21.197 min.

The preferred method to derivatise the free amino acids found in tomato fruits was from Husek (Hušek, 1991)(method 1) as it was quick, easy and effective. Two other methods, trifluoroacetylation and acetylmethylation (Corr et al., 2007) were attempted, but were found to be ineffective and time consuming. Method 1 was further refined as detailed below.

2.2.2.2 Refining the derivatisation method using amino acid standards

A 5 mg/mL in 0.1 M HCl solution for each amino acid (Gly (×2), Ala, Phe, Ileu, Pro, GABA, and Val) was made. Each amino acid was then derivatised using derivatisation method 1 and analysed via GC-MS, to check for retention time, detector response (peak height and shape) and completeness of derivatisation. All amino acid derivatives gave acceptable detector responses. However when analysed by GC-C-IRMS for $\delta^{15}N$, unacceptable detector responses were observed (e.g. apparent overloading, low response, peak broadening etc.). To improve the chromatography in the GC-C-IRMS system, the oven temperatures, ramp rates, hold times and flow rates were changed and tested with a derivatised amino acid mix.

The chromatograms in the GC-C-IRMS showed evidence of an impurity that affected the baseline, and peaks for the amino acids derivatives. The m/z value for this impurity was 79, corresponding to the molecular ion of pyridine. In addition, a pyridine peak (compared with NIST database) eluted in the spectrum before the solvent delay was adjusted appropriately. Over time, it also affected the combustion column of the GC-C-IRMS. Many steps were taken to attempt to remove the pyridine from the sample before analysis without removing or affecting the amino acids. Initially, to remove pyridine, saturated (sat.) $CuSO_4$ was added to the sample with the result that pyridine was removed but so too were the amino acids. Different washes for the chloroform layer (final step of the derivatisation) were also tested, such as washing the chloroform layer with 0.025 M HCL, 10% $CuSO_4$, 0.025 M HCl + 10% $CuSO_4$, 0.5 M $NaHCO_3$, sat. $CuSO_4$ + 0.0125 M HCl, 0.0125 M HCl (pH 1.9), sat. $NaHCO_3$ & sat. NaCl, 0.00625 M HCl (pH 2.2), 0.003125 M HCl (pH 2.5), 0.0015625 M HCl (pH 2.8), 0.00078125 M HCl (pH 3.1) and 0.0005 M HCl (pH 3.42). The result was that washing the chloroform layer with 0.0005 M HCl (pH 3.4) removed the pyridine and the amino acid peaks were not affected. The other concentrations of HCl were too acidic, which affected the peaks.

The final derivatisation method that was accepted was the Husek (Hušek, 1991) method with some refinement (method 2): Water (45 μL) was added to a standard mix or sample (15 μL), vortexed, then 40 μL ethanol:pyridine solution (EtOH:pyr; 4:1) was added, vortexed , then 5 μL ethyl chloroformate (ECF) was added, vortexed (CO_2 released) then finally 100 μL chloroform (1% ECF) was added and vortexed. The chloroform layer was removed, washed with HCl solution (pH 3.4; 3 × 30 μL), vortexed, and then transferred to a GC-MS insert and vial for analysis.

The possibility that the wash step might cause fractionation of carbon was checked and found to not occur, except for Glu, for which the average $\delta^{13}C$ of the washes was 1.7 (‰) ± 0.7 (‰) lower than when the sample was not washed. The results (Table 1) also

confirmed the repeatability of the method, except for Glu which has fractionation probably because when it is derivatised it is not only converted to its respective ethyl ester, but it also forms pyro-glutamic acid (Hušek, 1991).

Table 1. Amino acid mix, before and after wash (5 replicates) with HCl (pH 3.4) for $\delta^{13}C$.

Mix	**before wash**	**after wash with HCl solution pH 3.4**						
	sample	**rep 1**	**rep 2**	**rep 3**	**rep 4**	**rep 5**	**ave**	**SD**
amino acid	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{13}C$ (‰)**
Ala	-29.7	-30.1	-29.7	-29.8	-29.6	-29.9	-29.8	0.2
Gly	-37.4	-38.0	-37.8	-37.6	-37.3	-37.4	-37.6	0.3
Val	-24.8	-24.9	-24.7	-24.8	-24.6	-25.0	-24.8	0.2
Pro	-24.4	-24.6	-25.0	-24.3	-24.8	-26.2	-25.0	0.7
Ileu	-25.0	-25.3	-25.3	-25.1	-25.2	-25.6	-25.3	0.2
Leu	-24.2	-24.1	-24.3	-24.0	-24.1	-24.5	-24.2	0.2
Asp	-30.3	-30.4	-30.8	-31.4	-30.7	-31.5	-31.0	0.5
Phe	-21.8	-21.7	-22.0	-21.8	-22.5	-22.3	-22.1	0.3
Glu	-25.6	-27.0	-26.2	-27.7	-27.9	-27.5	-27.3	0.7

The derivatised amino acids were each run separately to determine their retention time and their respective ions prior to the preparation and analysis of the combined standard mixture.

2.2.2.3 Optimisation of derivatisation agents on free amino acids

A mix of compounds and single amino acids were derivatised using various derivatisation methods summarised in (Table 2) this was done to optimise the derivatisation conditions.

Table 2. Different reagent volumes for derivatisation of a standard mix

Sample/reagent	Vol. added (μL)	Vol. added (μL)	Vol. added (μL)	Vol. added (μL) *	Vol. added (μL)
Mix 1a	60	60	60	60	60
water	240	360	480	600	720
EtOH:pyr (4:1)	220	340	460	580	700
ECF	30	45	60	75	90
chloroform	100	100	100	100	100
Na_2SO_4	~ 0.1 mg	~ 0.1 mg	~ 0.1 mg	~ 0.1 mg	~ 0.1 mg

Single amino acids were derivatised as per * in Table 2. Table 3 shows the chromatographic and isotopic results for $\delta^{15}N$ for the single amino acids and for the mix with ECF (90 μL).

Table 3. Results for derivatisation selected for similar abundance heights in GC-MS with an INNOWAX column installed, a comparison between the mix and the single amino acids

Amino acid	Mix: 90 μL ECF			Single: 75 μL ECF			EA
	GC-MS Peak height (Abundance)	GC-C-IRMS peak height (mV)	GC-C-IRMS δ^{15} N value (‰)	GC-MS Peak height (Abundance)	GC-C-IRMS peak height (mV)	GC-C-IRMS δ^{15} N value (‰)	δ^{15} N value (‰)
Ala	2.31E9	212	-8.0	2.26E9	266	-8.3	-7.4
Val	4.14E9	352	-1.2	4.70E9	387	0.7	0.5
Ileu	3.81E9	230	-4.0	4.86E9	424	-2.9	-2.4

Leu	3.53E9	237	-0.2	4.42E9	358	1.5	1.6
Nleu	3.07E9	189	12.0	4.22E9	380	13.8	14.5
Asp	3.09E9	139	-3.9	4.27E9	339	-4.0	-4.2
Met	2.49E9	968	-2.7	4.29E9	187	-0.3	-1.1
Asn	1.11E9	85	-0.1	8.09E8	56	-1.2	1.3
Glu	9.66E8	11	-19.8	3.36E8 (2 peaks ^)	-	-	-2.3
Phe	3.81E9	177	1.9	4.72E9	255	2.6	2.6

^ the other peak is pyro-glutamic acid

As can be seen in Table 3 the $\delta^{15}N$ values are very close to that of EA for most of the compounds measured which is a very promising result. When those same amino acids are combined in a mix the $\delta^{15}N$ GC values when compared to the EA $\delta^{15}N$ values, are not as close and this is due to the peak heights of the compounds in the mix being smaller than for the single compounds. The peak heights are smaller for the compounds in the mix as there is more matrix for the instrument to scan than for a single compound, it is less sensitive.

Other experiments were performed to check the derivatision method and these were to keep the volume of EtOH:pyr (4:1, 700 μL), water (720 μL), chloroform (100 μL) and standard mix (60 μL) constant while changing the ECF volume (105 μL, 120 μL, 135 μL, 150 μL, 165 μL, 180 μL and 240 μL), and to keep the ECF (90 μL) and chloroform (200 μL) volumes the same while changing the EtOH:pyr (4:1; 820 μL, 940 μL, 1060 μL, 1180 μL and 1300 μL) and water (840 μL, 960 μL, 1080 μL, 1200 μL and 1320 μL) volume. The results showed that addition of 105 μL of ECF to the derivatisation reaction was better than all the other volumes tested in this reaction but it needs to be compared with the addition of 90 μL of ECF (see Table 2). The chromatographic peak shapes and heights were terrible when the ECF volume remained the same and the EtOH:pyr (4:1) addition

changed so it is not worthwhile. The best result achieved for analysis of amino acids for $\delta^{15}N$ was for single amino acids (see Table 3) was with the HP-INNOWAX GC column (60 m) and when 75 μL of ECF was added to the derivatisation reaction. When all these reactions are compared the best result was when ECF (90 μL) and EtOH:pyr (4:1; 700 μL) was added to the derivatisation reaction for the mix.

The $\delta^{15}N$ value is very similar or even the same, as that for the EA, for the single compounds, which is required as it shows there is no fractionation with the method. However, the $\delta^{15}N$ values for those same compounds in the mix show slight fractionation and this is due to the peak heights not being high enough for an accurate measurement. To improve the response of the sample peaks the volume of sample injected in the GC-C-IRMS was increased from 1 μL to 2 μL which resulted in less separation between the amino acids in the mix. Therefore, the analytical method is good, method development is required to increase the peak height of the amino acids in the mixes to avoid fractionation.

2.2.3 Extraction and Concentration Experimentation

2.2.3.1 Optimisation of tomato extraction conditions

Various solvents were compared (0.1 M HCl, 0.1 M NaOH, water and HCl (pH 3.4)) to determine which solution is best for the extraction of free amino acids from dried tomato samples. The samples were prepared in duplicate and one half implemented the wash stage after derivatisation (method 2) and the other half did not (method 1). The result of this experiment determined that the samples extracted with 0.1 M NaOH and derivatised with method 2 (wash) had peaks of good symmetry and with good height (9000 mV, ion 46) but some peaks were missing. The extractions with all solvents were then derivatised with method 1 (no wash). The derivatisations without the wash had peaks of good

symmetry and therefore the extraction solvent 0.1 M HCl was chosen for extraction as it was the same solution for the amino acid standards.

The tomato extraction experiment was a 3 × 3 × 3 design. The tomato powder was weighed at 100 mg (×3), 250 mg (×3) and 500 mg (×3), in centrifuge tubes, with an extraction solution (0.1 M HCl) volume of 3 mL, 6 mL or 9 mL. The extraction time was 20 mins, 1 hr or 2 hrs, and each sample was extracted 3 times, so in total there were 81 samples (see SI. Figure 2). The samples were then derivatised following method 1, and then analysed by GC-MS. The areas under the peak for each amino acid was measured for all 81 samples.

The results from the 3x3x3 factorial design experiment were analysed using R statistics (R Core Team, 2017). The results of this analysis show that:

1) longer extraction time leads to less amino acids, except in the case for Leu and Val where the effect is not significant;

2) higher amounts sample lead to the recovery of greater amounts of amino acids, always significant;

3) higher extraction volumes lead to lesser amounts of the amino acids, always significant;

4) the 2^{nd} and 3^{rd} extractions are useful for 7 of the amino acids measured but are insignificant for 4.

Therefore, considering these results, the optimised extraction method for amino acids in tomatoes was to have a short extraction time (20 min), greater amount of sample (500 mg) and less extraction volume (3 mL).

For clean up purposes, the tomato extracts (from the above experiment) in 0.1 M HCl were put through a column of resin (Amberlite® IR 120 hydrogen form, Fluka, Milan, Italy) which was prepared by pipetting a slurry of water and resin into a glass pipette plugged with glass wool to the height of approximately 1 cm. The resin was then washed with 5 loads of methanol (MeOH; 100 μL) and then with water (5 × 100 μL). The sample extract (1 mL) was added to the pipette. The extract was then washed with MeOH (80%, 3 × 100 μL), then eluted with NH_4OH (30%):MeOH (1:1; 5 × 25 μL) (method 3). The eluent was evaporated to dryness, under a steady stream of N_2, and a residue remained. These samples were then derivatised as per the derivatisation method for residues ie addition of water (60 μL), mixed, then EtOH:pyr (4:1, 40 μL), vortexed, ECF (5 μL), vortex 30 s, stand for 10 min, then 100 μL (1% ECF) chloroform was added (method 4) and an aliquot take for analysis by GC-C-IRMS.

2.2.3.2 Clean up and derivatisation of tomato extraction

The measurement of free amino acids by GC-C-IRMS for $\delta^{15}N$ requires a lot of sample, so to try to concentrate the sample to increase the peak heights in the chromatograms, different extraction solvents were used, the solvents in the wash step of the derivatisation method were changed and then the samples were purified and concentrated through a resin step.

In a centrifuge tube tomato powder (0.2 g × 5; 0.0 g, ×1 - blank) was weighed, then a different solvent (5 mL) was added to each tube (1 – 0.1 M HCl, 2 – 0.1 M NaOH, 3 – H_2O, 4 – 50% EtOH, 5 – 80% EtOH, 6 – H_2O). Each tube was spiked with iSTD Nleu (10 μL, ~10 mg/mL), capped, hand shaken and then centrifuged for 5 min at 3400 rpm. The pH of the supernatant after centrifugation was ≤1, ≥11, 6, 6, 6, 6 for samples 1, 2, 3, 4, 5 and 6, respectively. The samples (1, 2, 3, 4, 5 & 6; 15 μL) were then derivatised as per method 1. Another derivatisation was completed as per method 1 but with a change

in the water step, so instead of water a different solvent was added, so there were 6 samples with an additional 4 solvents to try, resulting in 24 samples. To samples (15 μL sample 1, 2, 3, 4, 5 & 6) 45 μL of solvent was added including aq. HCl (pH 4), 0.1 M HCl, 0.1 M NaOH, and 1M NaOH. All samples were then derivatised as per method 1.

Next, 12 resin filled pipettes were prepared using a glass wool plug with resin (1 cm in height from the plug; Amberlite IR120 H^+ strongly acidic). Each pipette was washed with deionised water until the pH was not too acidic (~ pH 6 -7). From each sample (1, 2, 3, 4, 5 & 6) 2 mL of supernatant was removed to be put separately through the resin. The resin was washed with 2 (pipette full) loads of H_2O, then washed with EtOH (80%, 1 mL). The sample (2 mL; 1, 2, 3, 4 ,5 & 6) was loaded gradually, then washed with EtOH (80%; 3 × double the height of the resin) and then with water (2 × double the height of the resin). The sample was eluted with 30% ammonia solution (2 mL, 2 × 1mL) into a clean tube before being concentrated (from between 0.5 and 1 mL) under a steady stream of N_2 (method 5). The samples were derivatised as per method 1. To the remaining supernatant extract for all samples (1, 2, 3, 4, 5 & 6), sodium tungstate (10%, 1 mL; Sodium tungstate dihydrate, greater than or equal to 99.0% (AT), 500 g, Fluka, Milan, Italy) was added to remove proteins, the solution was shaken and then centrifuged for 5 min at 3400 rpm. The samples were then put through the resin-filled pipettes with the above-mentioned method without the concentration step. The samples (1 to 5) when loaded to the pipette became gelatinous and stuck, the ethanol-based samples were a cloudy yellow precipitate; these eventually flowed through with the washes. Sample 6 was fine as it was a blank and contained no tomato sample. The elution with 30% ammonia was good. The samples were then derivatised as per method 1. The samples were run on the GC-MS and GC-C-IRMS, on a HP-1 column.

The results of this experiment were:

- There were good heights in the GC-C-IRMS for samples extracted with 50% EtOH and 0.1M NaOH
- Good separation of peaks in the GC-MS but the peak shape was not good for tomato samples extracted with water and with the solvent being 0.1M NaOH
- Good separation and symmetry of important peaks in GC-MS and GC-C-IRMS for tomato samples extracted with 0.1 M HCl and purified with resin
- With tomato samples extracted with 0.1 M HCl and purified with resin, ethyl citrate was removed but it was not removed for the tomato samples extracted with water and with the solvent being 0.1 M NaOH as evidenced by a large co-eluting chromatographic peak.

Overall, it was determined that the tomato extracts should be purified and concentrated with resin after being extracted with 0.1 M HCl, as there was good separation and symmetry of amino acid peaks and the ethyl citrate peak was removed.

2.3 Instrumental method development

2.3.1 GC-MS conditions

Initially, a chiral column was used for analysis such to separate the D and L amino acid isomers. The GC column was installed in the GC-MS (Trace GC Ultra; GC Isolink + ConFlo IV, Thermo Scientific) furnished with an autosampler (Triplus, Thermo Scientific) was a CP-Chirasil-L-Val (25 m × 0.25 mm × 0.12 μm, Agilent Technologies, Netherlands) with deactivated FSOT column (1.45 m × 0.25 mm I.D., Alltech Associates, Italy) attached as a guard column. The injector temperature was 190 °C. The initial oven temperature was 50 °C and maintained for 3 min, then ramped to 105 °C at a rate of 30 °C/min, then increased to 126 °C at a rate of 1 °C/min and then increased to 190 °C at a

rate of 2 °C/min and held for 10 min. The carrier gas was He (Rivoira, purity: 99.999%) with a constant flow of 2 mL/min. This column allowed for the separation between D- and L-amino acids but later in the study was not required as D-amino acids are not found in tomato fruits as they are not naturally occurring however the synthetic fertilizers used in the conventionally grown tomatoes could contain both D and L amino acid isomers. The column was designed specifically to analyse amino acids, therefore for analysis of $\delta^{15}N$ for derivatised amino acids by GC-C-IRMS should be more sensitive than for other columns and could provide good peak heights as required for N analysis.

The GC conditions that provided the best results for $\delta^{15}N$ with respect to peak separation and peak heights throughout this experimental phase was a HP-INNOWAX (60 m × 0.320 mm × 0.25 μm, Agilent Technologies) column used in conjunction with the following GC method. The initial oven temperature was 80 °C and maintained for 2 min, then ramped to 140 °C at a rate of 45 °C/min and maintained for 2 min, then increased to 250 °C at a rate of 5 °C/min and then increased to 320 °C at a rate of 45 °C/min and held for 5 min. The injector temperature was 250 °C. The carrier gas was He (Rivoira, purity: 99.999%) with a constant flow of 2.4 mL/min.

Standard mixes were prepared in 0.1 M HCl and tomato samples were extracted in 0.1 M HCl.

2.3.2 Repeatability checks

The repeatability of the GC method was checked by running 3 derivatised samples of Phe and 3 samples of the iSTD norvaline (Nval) (Table 5). As can be seen in Table 5 the repeatability of the method is good and the method is good for the iSTD, which shows that the $\delta^{15}N$ value is similar to the EA value, however there was fractionation later in the run as can be seen for Phe which has a difference of ~ 4‰ between $\delta^{15}N$ values for GC-

C-IRMS and EA-IRMS. More work therefore needs to be done on the GC method to reduce the fractionation towards the end of the run.

Table 5. Repeatability check for GC method

Amino acid	Replicate	Retention time GC-MS (min)	Retention time GC-C-IRMS (s)	GC-MS Peak height (abundance)	GC-C-IRMS Ampl 28 (mV)	GC-C-IRMS $^{15}N/^{14}N$ (‰)	EA-IRMS $^{15}N/^{14}N$ (‰)
Phe	1	31.53	1934.30	1.13E9	310	7.027	2.6
	2	31.51	1932.83	9.73E8	273	6.005	
	3	31.52	1934.09	1.05E9	336	6.882	
Nval (iSTD)	1	16.88	1044.58	7.38E8	292	15.960	15.4
	2	16.82	1041.03	6.09E8	240	16.115	
	3	16.88	1044.16	6.88E8	301	16.260	

2.3.3 Tomato sample comparison with tomato samples from CORE Organic II.

Powdered tomato samples (1 & 2) used in the previous sections (Sections 2.2.3.1, 2.2.3.2 and 2.2.3.3) for method development purposes were purchased commercially in Italy. However, powdered tomato samples (3 & 4) from the CORE Organic II project where tomato plants were grown under conventional and organic fertilizers were the subject samples for this research. It was therefore necessary to compare the extraction of tomato samples 1 and 2 to that from the CORE Organic II project (samples 3 and 4).

The tomato powder (0.2 g × samples 1, 2, 3 & 4) was weighed into a centrifuge tubes, 0.1 M HCl (2 mL, pH 4) was added, samples vortexed for 1 min, then centrifuged for 10 min at 4100 rpm. The supernatant was decanted in a clean tube. Samples 1 and 3 went through sample clean-up by elution and extraction from resin using method 5 but with NH_3 (10%,

10 × 100 μL) instead of 30% ammonia solution as the elution step. The pipette was prepared as previously mentioned (Section 2.2.3.3) but also with glass wool on top of the resin bed. The residue was then derivatised as per method 4 but with the volumes up scaled due to larger amounts of sample. Samples 2 & 4 were not cleaned up through the resin, they were directly derivatised (method 2) also with volumes up scaled due to greater sample amounts. The chromatograms for samples 1 & 3 yielded sharp symmetrical peaks that were well resolved; both samples had the same peaks though the peak height varied a little. With respect to baseline noise, chromatograms for samples 1 & 3 were superior to the chromatograms of samples 2 & 4.

These results showed that for derivatised amino acids in the tomato samples, purification with the resin provided enhanced chromatography than without purification. To provide improved chromatography for the analysis of free amino acids for $\delta^{15}N$ in tomatoes, purification of the sample would therefore be required.

2.4 Preparation of total amino acids

Based on the collective results presented in Section 2.2.3, analysis of free amino acids in tomatoes could not be optimised sufficiently to ensure the appropriate peak heights required for the analysis of $\delta^{15}N$ by GC-C-IRMS. As such, it was necessary to analyse for total amino acids rather than for free amino acids. This then required hydrolysis, purification and derivatisation of the sample.

2.4.1 Hydrolysis, purification and derivatisation of tomato samples for total amino acids

Tomato samples (0.25 g × 4) were weighed into a 3 mL reaction tube and iSTD (100 μL; 8 mg/mL Nleu) was added. HCl (6M, 2 mL) was added (reddish colour), the reaction vial was capped, vortexed and heated at ~ 105 °C for 24 hr. The tomato samples went black, and they were then filtered through a glass-wool plugged glass pipette. The filtered sample was a red/brown colour. The samples were then evaporated under a steady stream of N_2 and at 70 °C, which took 2 hr. The residue was redissolved in HCl (0.1 M, 1 mL). For purification, the sample residues were then put through resin (Amberlite® cation exchange, 2.5 cm in height) filled pipettes, with a glass wool plug at the bottom of the resin and a glass wool layer on the top of the bed of the resin (to prevent disturbance of the resin). The resin beds were wet with water (1 mL), then the samples were put through (5 × 100 μL), and washed with water (4 × 1 mL). Samples were then eluted with 10% NH_3 (2 × 1 mL) into a clean glass tube and evaporated to dryness under N_2 at 70 °C. For derivatisation, the samples were redissolved in 0.1 M HCl (60 μL). Water was (720 μL) added and the mixture was vortexed, then EtOH:pyr (4:1; 700 μL) was added and the mixture was again vortexed. ECF (90 μL) was added, the mixture was vortexed and rested for 5 min. Chloroform (100 μL) was then added, the mixture was vortexed and the emulsion was left to stand until the layers were separated. The chloroform layer was then removed into a clean vial, dried under Na_2SO_4 and put in a GC-MS insert before analysis. There were no peaks for these samples when it was run on the GC-MS and GC-C-IRMS, which means that something went wrong with the hydrolysis, purification or derivatisation step. Most likely it was the latter with too much pyridine remaining in the sample after derivatisation.

The derivatisation and purification of the total amino acids mentioned in the previous research paper was therefore implemented for the analysis of tomatoes grown under two

different farming regimes, since although it was time-consuming, it did not have problems with analysis.

3. Conclusion

The extraction of free amino acids in tomato fruits and subsequent derivatisation and analysis proved to be quite difficult. The derivatisation method (method 4) was derived from Husek (Hušek, 1991) and it was very effective for derivatising the amino acids; it was also simple and quick. However, there was a problem with the derivatisation method and this was that pyridine was present in the sample and was subsequently analysed. To address this problem the chloroform layer was washed with HCl (0.0005 M, pH 3.4) which removed the pyridine and the amino acid peaks were not affected which is important as it means that this step does not incur fractionation. Method 2 was the derivatisation method that was finally accepted (see SI. Figure 3). The best result achieved for analysis of amino acids for $\delta^{15}N$ was for single amino acids (see Table 2) was with the HP-INNOWAX GC column (60 m) and when 75 μL of ECF was added to the derivatisation reaction. For the mix, the best result was when 90 μL of ECF was added to the derivatisation reaction.

To extract free amino acids from powdered tomatoes it was established that higher starting amounts of the sample would yield higher amounts of amino acids whereas greater extraction volume leads to lower amounts of amino acids extracted due to dilution effects. It was also established that the longer the extraction times, the concentration of amino acids extracted were lower, except for leucine and valine, and that for a number of amino acids a second or third extraction was also beneficial for recovery.

Clean up of the tomato sample through the Amberlite® resin removed a peak, ethyl citrate, which was affecting the analysis of the tomato sample. The elution through the resin also cleaned up the sample producing a chromatogram with sharp symmetrical peaks

of appropriate height. With the derivatisation step for the tomato samples the wash step (to remove pyridine) was excluded as it appeared to remove all of the amino acid peaks.

There were many problems with the oxidation column of the instrument and this was probably due to the pyridine not being totally removed from the sample prior to analysis. The oxidation column deteriorated more and more towards the end of all my experimentation as the volumes of pyridine were increased quite substantially, especially from the beginning when the derivatisation method was first used. Therefore, to implement or to continue work on this method, the pyridine needs to be completely removed before analysing samples on the GC-C-IRMS.

4. References

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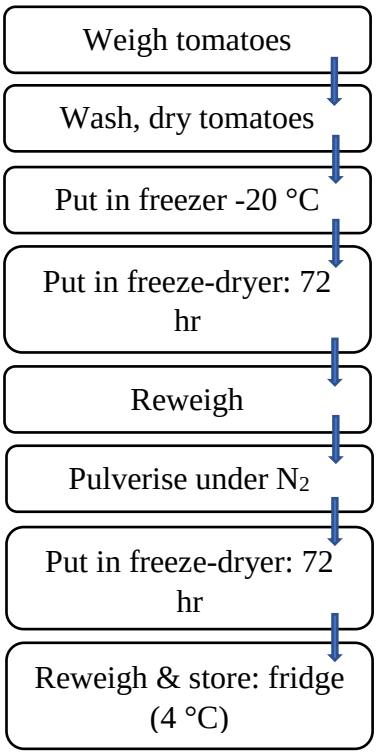
5. Supplementary Information

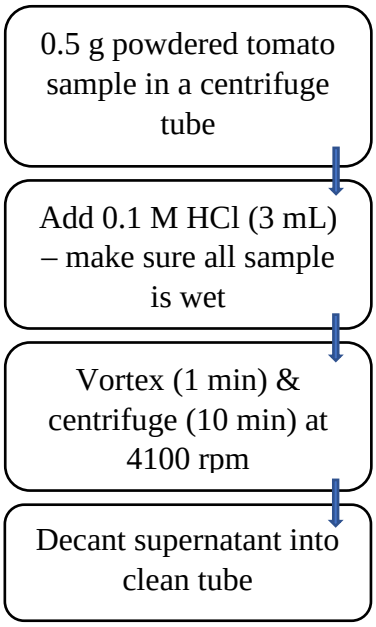
SI. Table 1. Isotopic ratios of $\delta^{15}N$ and $\delta^{13}C$ for amino acids and internal standards by EA-IRMS

Compound	$\delta^{15}N/^{14}N$ (‰)	$\delta^{13}C/^{12}C$ (‰)
L-Alanine	-7.4	-19.7
D-Alanine	-0.7	-33.4
L-Arginine	-2.2	-13.4
L-Asparagine	1.3	-23.4
L-Aspartic acid	-4.2	-22.2
L-Cysteine	-5.5	-30.1
L-Cystine	8.5	-15.2
L-Glutamic acid	-5.2	-13.7
D-Glutamic acid	-2.3	-13.7
L-Glutamine	-2.0	-11.7
Glycine	2.0	-41.6
L-Histidine	-8.6	-9.9
L-Isoleucine	-2.4	-13.5
L-Leucine	1.6	-13.5
D-Leucine	-2.1	-19.0
L-Lysine	1.1	-25.8
L-Methionine	-1.1	-31.9
D-Methionine	-0.4	-29.7
L-Phenylalanine	2.6	-11.1
D-Phenylalanine	3.6	-11.9
L-Proline	1.1	-11.2
L-Serine	-1.0	-6.9
D-Serine	1.9	-32.2
L-Threonine	0.6	-10.5
D-Threonine	7.1	-29.8
L-Tryptophan	-3.5	-10.4
L-Tyrosine	3.0	-25.8
L-Valine	0.5	-11.9
D-Valine	6.2	-25.4
γ-Aminobutyric acid	-5.1	-30.9
nonadecane	na	-26.8
L-Norleucine	14.5	-27.6
L-Norvaline	15.4	-28.6
trans -hydroxy-L-proline	-8.7	-12.3

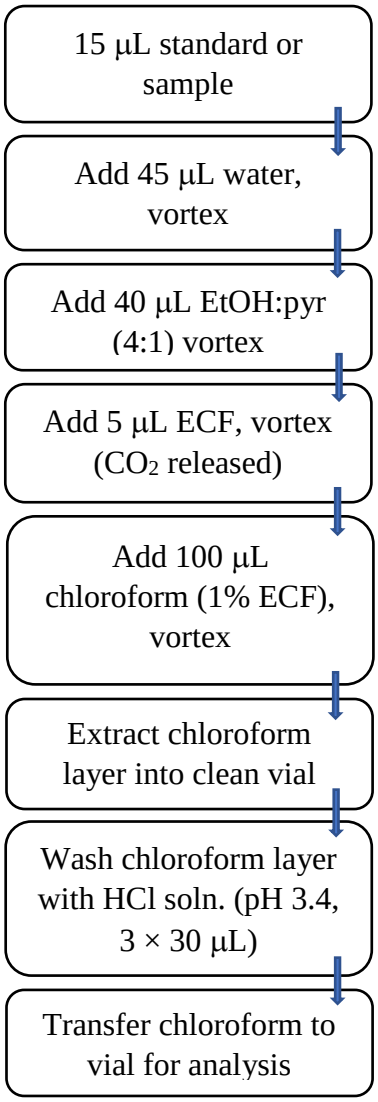
SI. Figure 1. Flow Chart of tomato sample preparation



SI. Figure 2. Flow Chart of tomato extraction for free amino acids



SI. Figure 3. Flow Chart of the derivatisation of free amino acids in a tomato sample



CHAPTER SIX

CONCLUSIONS

REFERENCES

CONCLUSIONS

The overall research objective for this project was to implement GC-C-IRMS for compound specific analysis, for $\delta^{13}C$ and $\delta^{15}N$, for the authenticity and traceability of foods and beverages. The importance of being able to trace and authenticate foods and beverages is paramount to the respective industries to protect the consumer and the producer. Adulterations and frauds occur through practices such as the addition of non-declared additives to increase product mass, mislabelling and misrepresentation, and the addition of flavours/aromas to enhance a product at a lower cost. Tracing a food or beverage means to be able to follow its production from beginning to the end thereby allowing the consumer to have faith in the product.

Three systems were investigated of increasing analytical complexity, by which the advantages and disadvantages of the GC-C-IRMS methodology could be evaluated. In the first study, a single isotope ratio ($\delta^{13}C$), from a single compound (vanillin) was studied to investigate adulteration of distillates. The second study involved measuring $\delta^{13}C$ of fatty acid methyl esters in intramuscular fat from lambs, to understand more about fatty acid metabolism from an animal nutrition viewpoint. This system was more complex due to the fact that isotope ratios of multiple fatty acids were measured, and there was a derivatisation step to convert fatty acids to the methyl esters. The final study involved more compounds with different chemistries (amino acids) and more isotope ratios (C, N, H, O and S for bulk samples, and $\delta^{13}C$ and $\delta^{15}N$ for individual amino acids). In this system, the extraction and derivatisation steps proved particularly challenging, and multiple methods were trialled before one that was compatible with GC-C-IRMS was found.

Vanillin is a marker for quality in distillates hence to adulterate lesser quality distillates by the addition of vanillin would prove tempting. An analytical method was developed to determine the authenticity of distillates, with vanillin as the target compound for analysis. The isotopic $\delta^{13}C$ values vanillin for the 48 samples derived from different sources including synthetic, ex-lignin and natural, were consistent with those reported in the literature. Synthetic vanillin in commercial flavourings were able to be distinguished from natural vanillin with the developed method. A $\delta^{13}C$ range for tannin-derived vanillin was determined and was consistent with the $\delta^{13}C$ values found in the literature. Measurement of a distillate aged in a barrel showed that there was no fractionation of vanillin over time, which is important to be able to measure the authenticity of a distillate (if there was fractionation, determination of authenticity of vanillin in a distillate would not be feasible). To apply the method for analysis of distillates the level of determination of adulteration by the addition of synthetic vanillin was investigated and it was determined that adulteration could be detected when the total amount of vanillin present in the distillate comprised of more than 60% of synthetic vanillin. For a producer looking to adulterate their product without detection by this method they would therefore need to know the initial concentration of vanillin in their distillate. The analysis of vanillin for $\delta^{13}C$ was undertaken for 32 distillates, including whisky, cognac, bourbon, rum, grappa and brandy. A rum sample was shown to be adulterated with its $\delta^{13}C$ value found to be within the $\delta^{13}C$ range for synthetic vanillin. Problems with some of the distillates, such as in rums, were with co-elution of 5-HMF with vanillin. Also, the analysis is only appropriate for discerning synthetic vanillin addition, therefore the addition of bio-synthetic vanillin to the distillate would not be detected as the method cannot distinguish between bio-synthetic vanillin and the vanillin derived from wood.

Future work in the area could entail the implementation of a multidimensional GC coupled to a C-IRMS (Tobias, Zhang, Auchus, & Brenna, 2011) to aid in separation of

the compounds, 5-HMF from vanillin. The method could be implemented to analyse for other quality markers for distillates such as cis-whisky lactone, syringaldehyde and furfural (Alcarde, Souza, & Bortoletto, 2014; Franitza, Granvogl, & Schieberle, 2016). Another recommendation for future work could be to conduct further ageing studies of distillates in barrels and monitor the effect time has on $\delta^{13}C$ values for vanillin. Other future work could look at the analysis of $\delta^{13}C$ of vanillin and related compounds for the authenticity of wine stored in barrels or on oak. As wine is different to distillates, the method would need to be adjusted. The reduction in ethanol content would certainly need to be taken into account. Head-space solid-phase microextraction (HS-SPME) could be a viable alternative to be investigated: as SPME fibres are negatively impacted by high ethanol concentrations sample dilution would be required. Given that HS-SPME relies on the intrinsic volatility of the different aroma compounds, isotopic fractionation could occur during sampling as reported by Schipilliti, et al. (Schipilliti, Bonaccorsi, & Mondello, 2017).

The tracking of an animal feed to the animal is called traceability as defined by the European Union Regulation(EC) Nr 178/2002 as “the ability to trace and follow a food, feed, food producing animal, or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution.” It is usually applied to prevent unfair trading of fake products as well as to determine contamination source and to track the origin of the ingredients.

From a nutritional aspect, to be able to trace the metabolic pathway, from the animal to its diet, in order to understand how to improve meat quality is of great importance. Therefore a method was to developed to measure the $\delta^{13}C$ of the main FA present in neutral (NL) and polar (PL) intramuscular lipids of meat samples from lambs fed with 4 different dietary regimes namely a control diet, a diet supplemented with oil, a diet supplemented with *Cistus ladanifer* and a diet supplemented with *Cistus ladanifer* and

oil. The research objective was then to determine whether the increase in NL of intramuscular FA for lambs fed a diet supplemented with both oil and *C. ladanifer* could be explained mostly by the incorporation of diet preformed FA or by increased *de novo* FA synthesis (question derived from previous research, (Jerónimo et al., 2010)).

A conclusion of this research was that PL $\delta^{13}C$ values of FA were lower than those in NL, which could indicate that the incorporation of exogenous FA is greater for PL than for NL and this was likely to be due to their functions in the cells of animals with PL having a higher turnover than NL for carbon (Harrison et al., 2010). PL are cell membrane components and NL are generally found in adipocytes. The results also showed that, with respect to 16:0, the increase of intramuscular FA was due to continual *de novo* FA synthesis for lambs fed a diet supplemented with oil and *C. ladanifer*. The results also showed that for lambs fed diets supplemented with oil, *de novo* FA synthesis was inhibited. Therefore, the oil effect was repressed with the inclusion of *C. ladanifer* to the diet.

Future work would be to determine the mechanism of the *C. ladanifer* effect for *de novo* FA synthesis to enable implementation of more efficient nutritional approaches to increase the intramuscular fat in lamb meat.

Tracing the metabolic pathway, with isotopic ratios, from soil to fruit or vegetable could also be important for the authentication of organic food from conventional produce. Bulk isotopic ratio analysis for C, N, H, O and S was undertaken to differentiate between tomatoes grown organically or conventionally, as these isotopes can be affected from a number of factors such as the surrounding environment, soil and fertilizer conditions and photosynthetic rates. The result was that there was overlap with the conventional and organic tomato isotopic values, so the separation between systems was not established. Therefore, compound specific isotope ratio analysis was conducted for total amino acids

found in tomatoes and measured for $\delta^{15}N$ and $\delta^{13}C$ values. As amino acids are involved in many metabolic pathways in the plant, tracing the N uptake from the fertilizer to the plant and subsequent fruit would be possible. The results of the analysis showed that there is greater separation between the systems when plotting the first 2 PCs from the PCA analysis undertaken. When the external factors were removed, by using Glx to normalise the amino acid $\delta^{15}N$ and $\delta^{13}C$ values and when these values are then plotted against each other, then there is separation between the organic and conventional farming systems. Therefore, the analysis of the amino acids Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and Phe for $\delta^{15}N$ and $\delta^{13}C$ with particular focus on Glx for $\delta^{13}C$, provides separation between tomatoes grown organically and tomatoes grown conventionally. Future work in this area to enable this method to be used for authenticity purposes would be to test its validity on a greater set of samples and covering more locations and not only for tomatoes but for other organic produce.

For the method development work for the report on free amino acids it was determined that to extract the most amino acids from tomatoes, a larger amount of sample, less volume of extractant solvent and a short extraction time with repeated extractions, was required. The wash to remove pyridine worked well on small concentrations of sample mixes but when the volumes became larger due to larger sample mass the pyridine was difficult to remove; also, it seemed to remove the amino acid peaks in the tomato samples. The clean-up of the tomato sample was effective for purification of the sample and it also removed the ethyl citrate peak which was required. The analysis of the single amino acids for $\delta^{15}N$ proved that the GC-C-IRMS method was free of fractionation. The method derived from the Husek derivatisation method (Hušek, 1991) warrants further study as it is an inexpensive and quick method compared to method implemented for this study, which would be beneficial for high volumes of samples.

Overall, the application of GC-C-IRMS to diverse matrices of differing complexities was found to be a promising tool for the purposes of addressing adulterations and traceability of foods and beverages.

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