IMPACT OF ENVIRONMENTAL AND PRODUCTION FACTORS ON THE ISOTOPIC AND MOLECULAR COMPOSITION OF FOOD

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ABSTRACT

The globalization of the food market and increasing consumer awareness of food quality with respect to organic produce and virgin olive oils created the need for robust analytical approaches for their authentication. Stable isotope methodology can be used in food studies to investigate various environmental, chemical and biological processes that are linked with food origin and production techniques.

In the present work the effects of different organic and synthetic fertilisation strategies on the isotopic composition of greenhouse, field and retail vegetables were studied using bulk nitrogen isotope composition and, for the first time, nitrogen and oxygen isotope compositions of plant-derived nitrate. The second part of this work concerned the impacts of geographical location and production techniques on the molecular and, for the first time, carbon and hydrogen isotope compositions of *n*-alkanes in Mediterranean olive oils. The distribution and carbon isotope composition of *n*-alkanes in olives and leaves from different varieties were also examined.

The use of animal manures resulted in significantly higher bulk nitrogen isotope values of organic lettuces, potatoes, cabbages and onions compared to those cultivated with synthetic nitrogen fertilisers. Oxygen isotope values of plant-derived nitrate were significantly lower in organic compared to conventional potatoes and tomatoes. Intra-plant isotopic variation and a strong impact of the fertiliser application rate on the isotopic compositions of vegetables were observed.

The distribution and concentrations of *n*-alkanes in the Mediterranean extra virgin olive oils exhibited variation depending on the production country. Hydrogen and carbon isotope values of *n*-alkanes were significantly higher in olive oils from Southern compared to Northern Mediterranean countries. Significant difference in the *n*-alkane distribution of olives compared to olive leaves was observed throughout the growing season, which allowed differentiating between olive oils produced with mechanical and manual harvesting techniques.

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ACRONYMS AND ABBREVIATIONS

ACL	Average chain length
ANOVA	Analysis of variance
CAM	Crassulacean acid metabolism
CDA	Canonical discriminant analysis
DNA	Deoxyribonucleic acid
EA-IRMS	Elemental analysis - isotope ratio mass spectrometry
EC	European Commission
EU	European Union
Fera	Food and Environment Research Agency
FID	Flame ionisation detector
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-IRMS	Gas chromatography - isotope ratio mass spectrometry
GC-MS	Gas chromatography - mass spectrometry
GI	Geographical Indication
GIS	Geographic Information System
HPLC	High-performance liquid chromatography
IAEA	International Atomic Energy Agency
ICP-MS	Inductively coupled plasma mass spectrometry
IFOAM	International Federation of Organic Agriculture Movements
IPCC	Intergovernmental Panel on Climate Change
IRMS	Isotope ratio mass spectrometry
LC	Liquid chromatography
LC-IRMS	Liquid chromatography - isotope ratio mass spectrometry
LC-MS	Liquid chromatography - mass spectrometry
MIR	Mid-infrared
MS	Mass spectrometry
NIR	Near-infrared
NMR	Nuclear magnetic resonance
NPK	Nitrogen-phosphorus-potassium fertiliser
PCR	Polymerase chain reaction
PDO	Protected Designation of Origin

PEP	Phosphoenolpyruvate carboxylase
PGI	Protected Geographical Indication
ppb	Parts per billion
ppm	Part per million
PTV	Programmable temperature vaporisation
RNA	Ribonucleic acid
rpm	Revolutions per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SIRA	Stable isotope ratio analysis
TN	Total nitrogen
TSG	Traditional Speciality Guaranteed
VCDT	Vienna Canyon Diablo Troilite
VPDB	Vienna Pee Dee Belemnite
VSMOW	Vienna Standard Mean Ocean Water
WUE	Water use efficiency

CHAPTER 1

INTRODUCTION

1.1. Food authenticity

An authentic product, whether a raw material or a processed product on the supermarket shelf, is the one which strictly complies with the declaration given by the producer in terms of ingredients, production technology, absence of extraneous substances, geographical and varietal origin, production year and genetic identity (Kamm et al. 2001).

Over the recent years consumer demand for authentic, high quality foods has been increasing. To be able to make informed choices about their diets consumers need clear and accurate information about the products they purchase. Consumer choices may reflect lifestyle and religious concerns (e.g. vegetarianism, preference for organic products, absence of pork for Muslims and Jews) or health concerns (e.g. absence of lactose, nuts, gluten for individuals with particular allergies) (Woolfe and Primrose 2004, Montealegre et al. 2010, Primrose et al. 2010).

There is currently a growing enthusiasm among consumers for purchasing high quality sustainable food products, such as organic and locally produced foods (e.g. Cowell and Parkinson 2003, Weatherell et al. 2003, Pretty et al. 2005, Mirosa and Lawson 2012). The major reasons for buying local products include i) specific culinary, organoleptic qualities, or purported health benefits associated with regional products; ii) patriotism; iii) a decreased confidence in the quality and safety of foods produced outside their local region or country; iv) concerns about environmental impact of food production and “food miles” (Kelly et al. 2005). Increased consumer interest in purchasing organic food is mainly prompted by the demand for healthier and higher quality food with an absolute minimum of additives, and willingness to contribute to the environmental protection, animal welfare and more ethical and sustainable living (Magnusson et al. 2003, Mondelaers et al. 2009, Herrmann 2010, van Doorn and Verhoef 2011, Shepherd 2011).

Some food products are popular with consumers because of their geographical and/or ethnic origin. There are a great number of branded goods declaring provincial high quality characteristics. These products have an identifiable geographical origin and in the European Union they are protected using certification systems known as

Geographical Indications (GIs). Three main GIs are Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG). The PDO label is used to describe foodstuffs with a strong regional identity that are produced, processed and prepared in a specific geographical area using prescribed techniques that may be unique to that region. Foods with PGI status must have a geographical link in at least one of the stages of production, processing or preparation. TSG does not refer to a specific geographical origin, but defines products that are traditional or have customary names and have a set of features which distinguish them from other similar products (Council Regulation (EC) No 510/2006). There are currently more than 10,000 protected GIs in the world with an estimated trade value of more than US$ 50 billion (Giovannucci et al. 2009). Many of these are well-known names, such as Bordeaux wine (France), Grana Padano and Parmigiano-Reggiano cheese (Italy), table olives Noires de Nyons (France), Roquefort cheese (France), Noord-Hollandse Gouda cheese (the Netherlands), Opperdoezer Ronde potatoes (the Netherlands) and many others (Luykx and van Ruth 2008).

Thus, various kinds of high quality and speciality food products with certified geographical or production origin are currently available on the market. Typically, these products are much more expensive compared to their uncertified counterparts (Brown and Sperow 2005, Oberholtzer et al. 2005, Winter and Davis 2006, Lin et al. 2008, Giovannucci et al. 2009). This price difference creates an incentive for fraudulent activities when genuine products can be substituted with inferior ones for financial gain (Kelly et al. 2005). Therefore, consumers paying a premium price for the certified speciality foods need to be sure that they are purchasing high quality and authentic products, which would satisfy their dietary preferences. The description and labelling of food must be honest and accurate, particularly in the case of processed foods (Woolfe and Primrose 2004, Montealegre et al. 2010, Primrose et al. 2010).

Food authentication is the process by which a food product is verified as complying with its label description, i.e. it is authentic. Food labelling legislation aims to ensure that food is properly described. It seeks to protect the consumer from being sold an inferior product with a false description (Dennis 1998, Kamm et al. 2001, Reida et al. 2006, Montealegre et al. 2010, Shears 2010). The authenticity of food is an important issue not only for consumers but also for food producers due to

the legal compliance, economic reasons (right goods for the right prices) and the assurance of a consistent well-defined quality. Genuine food producers do not wish to be subjected to unfair competition from unscrupulous processors who could gain an economic advantage from the misrepresentation of the food they are selling (Kamm et al. 2001, Reida et al. 2006, Montealegre et al. 2010).

1.2. Food fraud

Food fraud is a collective term used to encompass the deliberate and intentional substitution, addition, tampering or the misrepresentation of food ingredients or food packaging, or false statements made about a product for economic gain (Spink and Moyer 2011). Food fraud techniques can be very sophisticated and at least as laborious as those needed to detect the falsification. Some examples of how food fraud can happen include:

i) product/ingredient substitution by a similar but cheaper one (e.g. labelling cheaper varieties of vegetables or fruits as well known and more expensive varieties);

ii) food adulteration with a cheaper or base material (e.g. adding cheaper vegetable oils to named higher value vegetable oils, mixing long-grain rice with more expensive Basmati rice, mixing cow's milk with buffalo milk before production of buffalo mozzarella cheese, adding common wheat to durum wheat pasta labelled 100% durum wheat, adding water, sugar, acids and colouring to fruit juices);

iii) addition of undeclared ingredients;

iv) false claims about the food processing/storage (e.g. labelling meat as fresh even though it has been previously frozen, failure to declare that food has been irradiated, failure to declare that juice has been prepared from concentrate);

v) misleading information about the amount of a quantitative ingredient;

vi) false claims regarding geographical or production origin (e.g. labelling conventionally produced food as organic, claiming that extra virgin olive oil is from a particular geographical region, labelling South American beef as British beef, declaring farmed fish as 'wild').

Currently one of the biggest food fraud issues in the EU is the adulteration of extra virgin olive oil. Various cases of selling lower grade olive oils as extra virgin

olive oil or mislabelling country of origin have been detected in recent years in Italy and throughout the Mediterranean (Shears 2010).

With the growth of the organic food market the mislabelling of conventional food products as organic has become another great fraud problem in Europe and worldwide (Beck 2012, Huber 2012, Piva 2012, van den Idsert 2012). For example, a major fraud case in the organic food sector took place in Europe from 2007 to 2011. Tons of conventional products, mainly of plant origin, had false organic certificates and were sold at premium prices throughout the EU during five consequent years (Piva 2012, Ferrante 2012).

Another example of a serious food fraud in Europe is the so called 'circular trade' of butter. When butter produced in the EU is exported to a 'third' (non-EU) country it attracts a subsidy payment due to the lower market prices outside the EU. To gain profit the same consignment of butter is then re-labelled as 'produce of the third country' and re-imported back to the EU. The profit is made because the import tax is much lower than the original subsidy paid on the export of butter. Therefore, by simply re-labelling the geographic origin of butter, a dishonest trader can make a significant financial gain. Widespread transportation of animals and foodstuffs also presents the opportunity for unscrupulous traders to misdescribe the origin of meat and other products of animal origin (Kelly et al. 2005).

The motivation for food fraud is mainly financial benefit, however this practice can have a significant effect on consumer health and safety. The adulteration or misbranding of genuine food products can sometimes be harmful to consumers' health and even fatal. For example, the well known Chinese melamine scandal is an example of such food scam. It took place in 2008 in China when melamine was added to milk powder to raise its nitrogen content and resulted in thousands of hospitalizations and several baby deaths (Shears 2010, Pei et al. 2011). Melamine was added also to pet food and animal feed in China between 2006 and 2008. Pet food was exported to USA and resulted in a large number of deaths among animals (Shears 2010).

The prospect of high profits associated with a low risks of detection and subsequent penalties has resulted in various food fraud activities in Europe and worldwide. Consequently, there is a growing demand from the enforcement agencies to have access to reliable and robust analytical tools that can verify origin labels on food products.

1.3. Analytical techniques for food authentication

The development of analytical methodologies for food authentication is a challenging task. Primrose et al. (2010) in their review on food forensics discuss three main challenges in the development of food authentication methodology. Firstly, the issue under investigation is generally linked to a legal requirement or standard. The interpretation of the results has to be made in the light of analytical uncertainty, natural variation and any tolerance permitted by the requirements defining a particular food product, whereas the conclusion made must be beyond reasonable doubt. The second difficulty is finding a marker (or markers) that characterises this food product, one of its ingredients, the adulterants in question, or its processing, production or geographic origin. The marker has to be specific, its natural variation must be limited and well characterised, and be measured accurately if necessary. Another potential problem is obtaining authentic samples for the development and the evaluation of the method. There is less difficulty if these can be obtained or prepared nationally, but it is often more difficult when the samples have to be obtained from other countries.

Despite these analytical and conceptual challenges, substantial progress has been made in a number of research areas and in some cases reliable analytical methodologies have been developed and are currently applied for controlling food authenticity at national and international levels (Primrose et al. 2010). Some of the most widely used approaches include: separation techniques, spectroscopic techniques, DNA-based techniques and mass spectrometry techniques.

Separation techniques, which include high performance liquid chromatography (HPLC) and gas chromatography (GC) analyses, have been widely applied for the characterization and quantification of various major and minor food components. HPLC has been used in food analysis for measuring numerous compounds, e.g. carbohydrates, vitamins, amino acids, proteins, tryglycerides in fats and oils, lipids, pigments and mycotoxins (Luykx and van Ruth 2008). HPLC analyses have been applied to determine the authenticity of dairy products, honey and nuts, the varietal and geographical origin of olive oils, wines and coffee (e.g. Tomas-Barberan et al. 1993, Romero et al. 2002, Ferreira and Cacote 2003, Chen e t al. 2004, Gómez-Ariza et al. 2006, Aparicio-Ruiz et al. 2009). GC is one of the most universal separation

techniques used in food analysis, mainly in volatile and semi-volatile composition studies (Luykx and van Ruth 2008). GC analyses have been successfully applied to determine the varietal and geographical origin of olive oils, wines, coffee, fruit juices and other foodstuffs (e.g. Casal et al. 2003, Reid et al. 2004, Alves et al. 2005, Etièvant et al. 2006, Lopez Oritz et al. 2006, Temime et al. 2008).

Spectroscopic techniques that are widely used for food authentication include nuclear magnetic resonance (NMR) spectroscopy, Raman spectroscopy and infrared (NIR, MIR, FTIR) spectroscopy. NMR is based upon the measurement of absorption of radiofrequency radiation by atomic nuclei with non-zero spins in a strong magnetic field and can provide detailed information about the molecular structure of a food sample (Ibañez and Cifuentes 2001). ^{1}H, ^{13}C and ^{31}P NMR spectroscopy has been the most widely applied in food authentication studies. Selected examples of the application of NMR include detection of olive oil adulteration with lower grade olive, seed and nut oils, tracing the geographical origin of olive oils, wines, and production techniques of juices and coffee (e.g. Le Gall et al. 2001, Charlton et al. 2002, Brescia et al. 2003, Fragaki et al. 2005, D'Imperio et al. 2007, Alonso-Salces et al. 2010, Mannina et al. 2011).

Raman spectroscopy is the measurement of the wavelength and the intensity of inelastically scattered light (Raman scatter) from molecules. A particular molecule and its environment will determine what Raman signals will be observed. Raman spectroscopy has high sensitivity to C=C, C≡C and C≡N bonds and low sensitivity to water (Reid et al. 2006). This technique has been used to determine the varietal and geographical authenticity of olive oils, honey and other commodities (e.g. Baeten et al. 2001, Lopez-Diez et al. 2001, Goodacre et al. 2002, Paradkar and Irudayaraj 2002).

IR spectroscopy is a non-invasive and non-destructive technique that analyses the wavelength and intensity of the absorption of infrared light by measuring the absorption frequencies of the molecules (Karoui et al. 2004, Reid et al. 2006, Luykx and van Ruth 2008). Each functional group of a molecule has a unique vibrational frequency that can be used to determine which functional groups are present in a sample. This creates a unique molecular “fingerprint” that can be used to confirm the identity of a sample. IR spectroscopy has been used for the geographical classification of olive oils, wines and grapes, rice, fruit juices and honey (e.g. Christy

et al. 2004, Downey et al. 2004, Picque et al. 2005, Liu et al. 2006, Ruoff et al. 2006, Galtier et al. 2007, Casale et al. 2010, Rohman and Man 2010).

Other widely used tools for the authentication of food products are DNA-based techniques. The polymerase chain reaction (PCR) technique allows detection of very low amounts of nucleic acid probes and the determination of their sequence via the amplification of individual segments of DNA or RNA (Ibañez and Cifuentes 2001). This technique is extensively used for the identification of animal and plant species and breeds/varieties in foods. PCR has the advantage of high sensitivity, rapid performance and the automatic processing of the high amount of samples (De La Fuente and Juárez 2005). The greatest amount of research on the application of PCR for the authentication of food involves the analysis of the products of animal origin. For example, PCR has been used to detect the adulteration of minced beef with chicken and pork (Calvo et al. 2002), the adulteration of minced lamb with beef (Sawyer et al. 2003), or of duck and goose meat with chicken (Rodriguez et al. 2003) and to differentiate species of fish in frozen or processed fish products (e.g. Terol et al. 2002, Comesana et al. 2003, Jerome et al. 2003). PCR has also been applied for the detection of cheap rice varieties in Basmati rice (Steele et al. 2008), identification of fruit species in fruit purees and juices (Scott and Knight 2009) and detection of common wheat in durum wheat flavour or semolina (von Buren et al. 2001, Alary et al. 2002, Terzi et al. 2003).

Mass spectrometry (MS) is one of the most frequently used techniques in food authenticity studies. It is a powerful analytical tool for measuring the mass-to-charge ratio of ions (Aebersold and Mann 2003). MS characterizes the composition of a sample by generating mass spectra representing individual organic compounds. This is achieved by ionising the compound, separating the ions of differing masses, and by recording the abundance of each molecular ion and its fragment ions. MS is often used in combination with different types of peripherals: gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), inductively coupled plasma-mass spectrometry (ICP-MS), and isotope ratio mass spectrometry (IRMS).

GC-MS and LC-MS are instrumental techniques, comprising a GC or LC, respectively, coupled to a mass spectrometer. These techniques can both qualitatively and quantitatively analyse complex mixtures containing numerous compounds. GC or LC separates the components of a mixture and MS characterises each of the

components individually (Luykx and van Ruth 2008). GC-MS is one of the most widely used techniques and represents the method of choice for the analysis of food volatiles because of its high reproducibility (Pillonel et al. 2003a). The main applications of GC-MS and LC-MS include varietal and geographical classification of olive oils, honey, dairy products, fruit juices and various other commodities (Radovic et al. 2001, Mauriello et al. 2003, Gómez-Ariza et al. 2005, Zhang et al. 2005, Cunha et al. 2006, Canabate-Diaz et al. 2007, Ruiz-Matute et al. 2007).

ICP-MS has many applications in food authentication studies. ICP-MS can screen the geographical origin of food products by the analysis of numerous chemical elements and obtaining fingerprints of the element pattern at trace (ppb-ppm) and ultra-trace (ppq-ppb) levels (Vanhoe 1993, Lachas et al. 2000, Ariyama and Yasui 2006). This technique has been successfully applied for the authentication of the geographical origin of vegetables, nuts, tea and wines (e.g. Moreda-Piñeiro et al. 2003, Coetzee et al. 2005, Gómez-Ariza et al. 2006, Ariyama et al. 2007).

Yet one of the most promising and specific MS techniques, which over the past few decades has been applied for the authentication of a wide range of foodstuffs, and is considered as one of the most powerful tools in food authenticity studies, is stable isotope ratio analysis (SIRA) (Kelly and Rhodes 2002, Kelly et al. 2005, Reid et al. 2006, Luykx and van Ruth 2008, Primrose at al. 2010, Chesson et al. 2011). Stable isotopes are used as recorders of various environmental, chemical and biological processes, and thus SIRA has been applied for the analyses of a vast range of food products: dairy (e.g. Rossmann et al. 2000, Pillonel et al. 2003b, Camin et al. 2004, Manca et al. 2006, Camin et al. 2008), meat and fish products (e.g. Schmidt et al. 2005b, Bell et al. 2007, Camin et al. 2007, Heaton et al. 2008, Thomas et al. 2008, Perini et al. 2009, Horacek and Min 2010, Rhodes et al. 2010), vegetables and fruits (e.g. Georgi et al. 2005, Rapisarda et al. 2005, Bateman et al. 2007, Rogers 2008, Rapisarda et al. 2010b, Camin et al. 2011, Flores et al. 2011, Šturm and Lojen 2011), edible oils (e.g. Spangenberg et al. 1998, Angerosa et al. 1999, Aramendia et al. 2007, Bontempo et al. 2009, Iacumin et al. 2009, Camin et al. 2010a, Camin et al. 2010b), wines (e.g. Rossmann et al. 1998, Ogrinc et al. 2001, Martinelli et al. 2003, Calderone et al. 2004, Hermann and Voerkelius 2008), fruit juices (e.g. Rossmann et al. 1997, Perez et al. 2006, Rummel et al. 2010), honey (e.g. Schellenberg et al. 2010) and various other commodities. Following sections will provide a more

detailed description and theoretical background of the use of stable isotope approach for food authentication purposes.

1.4. Stable isotope analyses for the authentication of food products

1.4.1. Introduction

Isotopes are atoms of a chemical element with different number of neutrons in the nucleus, and consequently, with slightly different masses. Stable isotopes are those isotopes of an element that do not undergo radioactive decay. Most elements have more than one stable isotope. The most widely measured stable isotope ratios include those of the 'light' elements (bioelements): carbon, nitrogen, oxygen, hydrogen and sulphur (e.g. Faure 1986, Peterson and Fry 1987, Kendall and Caldwell 1998, Dawson et al. 2002, West et al. 2006, Michener and Lajtha 2007, Allegre 2008). However, the isotopes of other elements (e.g. of strontium) have also been applied as recorders of natural processes (e.g. Almeida et al. 2001, Kawasaki et al. 2002, Fortunato et al. 2004).

Generally, the lighter isotope of an element is much more abundant than the heavier isotope. Stable isotope abundances are typically expressed relative to an internationally accepted standard and as ratios of the rare to the abundant isotope:

$$\delta E\ (‰) = ((R_{sample} - R_{standard})/R_{standard}) \cdot 1000 \qquad (1.1.)$$

where E is the element of interest, and R is the ratio of the rare to the abundant isotope. Absolute abundance ratios are often very small, therefore expressing isotope values relative to a standard and multiplying these by 1000 expresses the very small fractional differences in parts per thousand using 'per mil' (‰) notation (Faure 1986, Allegre 2008).

Accepted standards used by the international community include VSMOW (Vienna Standard Mean Ocean Water) for hydrogen and oxygen, VPDB (Vienna Pee Dee Belemnite) for carbon, atmospheric air for nitrogen and VCDT (Vienna Canyon Diablo Troilite) for sulphur. By definition, standards have a δ value of 0‰ (Coplen 1996, Werner and Brand 2001).

Stable isotopes can be quantified in different ways; the most common technique is isotope ratio mass spectrometry (IRMS). Samples are quantitatively converted to a pure gas (usually CO_2, CO, N_2, N_2O, O_2, H_2, or SO_2), which is

cryogenically or chromatographically purified and introduced into the mass spectrometer. There gas is ionized to produce positively charged species, which is followed by the dispersion of different masses in a magnetic field, the impaction of different masses on different collector cups, and measurement of the ratios of the isotopes in the ionized gas (Kendall and Caldwell 1998, West et al. 2006, Michener and Lajtha 2007).

Stable isotope ratios can be measured using IRMS either in entire (bulk) sample or in the individual compounds isolated from the sample prior to isotopic analyses. Bulk analyses can be performed using elemental analyser-IRMS (EA-IRMS). Compound specific isotopic analyses are usually carried out using gas chromatograph coupled to IRMS (GC-IRMS) (e.g. Spangenberg et al. 1998, Schmidt et al. 2004, Evershed et al. 2008, Spitzke and Fauhl-Hassek 2010, Elsner et al. 2012) or sometimes also using liquid chromatograph coupled to IRMS (LC-IRMS) (e.g. Cabañero et al. 2009, Wild et al. 2010, Leitner et al. 2012).

1.4.2. Isotope fractionation

The chemical behaviour of two isotopes of an element is qualitatively similar. However, differences in masses of two isotopes of an element lead to their different reaction rates and bond strength. This is because atomic mass determines the vibrational energy of the nucleus. As heavy atoms vibrate more slowly than lighter ones, the energy of the molecule with the heavy isotope is lower, and therefore it forms more stable, stronger bonds. Differences in bond strength and in the kinetics of chemical reactions between isotopes lead to a change in the partitioning of heavy and light isotopes between a source substrate and the product(s), and is termed isotope fractionation (e.g. Kendall and Caldwell 1998, Dawson et al. 2002, West et al. 2006, Michener and Lajtha 2007, Allegre 2008).

Isotope fractionations are categorized as primarily two types: equilibrium fractionations and kinetic fractionations. During equilibrium isotope fractionations the distribution of isotopes differs between chemical substances (substrate versus product) or phases (e.g., liquid versus vapour) when a reaction is in equilibrium. Such reactions are temperature dependent and occur in closed, well-mixed systems, where substrates and products remain in close contact, and the reverse reactions can take place. The heavier isotope tends to accumulate in the denser phase or in a compound where bonds are the strongest. Kinetic isotope fractionations occur when

the reaction is unidirectional and the reaction rates are mass-dependent. The lighter isotope of an element forms bonds that are more easily broken, it is more reactive and likely to be concentrated in the product faster than the heavier isotope. In biological systems kinetic fractionations are often catalyzed by an enzyme that discriminates between the isotopes in the mixture such that the substrate and product become isotopically distinct from one another (e.g. Kendall and Caldwell 1998, Dawson et al. 2002, West et al. 2006, Michener and Lajtha 2007, Allegre 2008).

Many biological and physico-chemical processes in the environment can lead to isotope fractionations. For example, difference in isotopic values can arise due to evaporation and condensation, diffusion, crystallisation and melting, absorption and desorption. The isotope ratio of any substance that is part of the reaction can act as a fingerprint for that resource or transitional form. It can therefore be used as a tracer to follow the reaction products through complex cycles and into diets (Kendall and Caldwell 1998, Dawson et al. 2002, Michener and Lajtha 2007). Table 1.1 provides a brief overview of the way in which stable isotope ratios of four major bioelements (C, H, O and N) are fractionated in the environment and how this can be exploited for food authentication (Kelly et al. 2005).

Table 1.1. The overview of the way in which stable isotope ratios of four major bioelements are fractionated in the environment and how this can be exploited for food authentication.

Isotope ratio	Fractionation	Information for food authentication
$^{13}C/^{12}C$	plant photosynthetic pathways	C3 versus C4/CAM plants, geographical origin, greenhouse versus outdoor cultivation
$^{2}H/^{1}H$ $^{18}O/^{16}O$	evaporation, condensation, precipitation	plant water uptake and transpiration, geographical origin
$^{15}N/^{14}N$	microbial N transformations, NH_3 volatilisation, trophic level	the use of organic versus synthetic N fertilisers, the use of N-fixing plants, animal diet

From the food authentication perspective, stable isotope analyses can be exploited to trace the geographical and varietal origin of plant and animal products, agricultural practise used, as well as indicate the difference in production techniques and processing methods of certain product types.

1.4.3. Stable carbon isotope analyses for food authentication

Stable carbon isotope ratios are widely applied in different research fields to differentiate plants with different photosynthetic systems. According to the photosynthetic carbon fixation system plants are divided into three types: C3 (the majority of cultivated plants, including wheat, rice, nuts), C4 (corn, sugar cane, millet, sorghum and many tropical grasses) and CAM plants (pineapple, many cacti and succulent plants) (Sage and Monson 1999, Leegood et al. 2000, Tipple and Pagani 2007).

The difference in the carbon isotope composition among these three plant groups occurs due to different enzymatic reactions involved in the fixation and incorporation of CO_2 molecules into organic acids during photosynthesis. C3 plants use carboxylating enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco), activity of which is associated with a substantial discrimination against ^{13}C. Bulk $\delta^{13}C$ values of C3 plants have a median of about -28‰ (O'Leary 1981, Farquhar et al. 1989, Brugnoli and Farquhar 2000, Marshall et al. 2007). C4 plants use a different enzyme for the initial CO_2 fixation – phosphoenol pyruvate carboxylase (PEP). A much smaller discrimination against ^{13}C is associated with PEP compared to Rubisco. The bulk $\delta^{13}C$ values for C4 plants cluster around -14‰ (O'Leary 1981, Farquhar 1983, Brugnoli and Farquhar 2000, Marshall et al. 2007, Tipple and Pagani 2007). CAM plants, similarly to C4 plants, use PEP for initial CO_2 fixation. Some facultative-CAM species are capable to switch between CAM and C3 photosynthesis. The $\delta^{13}C$ values of obligate CAM plants cluster around -11‰, while those of facultative CAM plants are between -11‰ and -28‰ (Farquhar 1983, O'Leary 1981, Brugnoli and Farquhar 2000).

Thus, there is a significant difference in stable carbon isotope values of C3, C4 and CAM plants. This difference is also preserved at higher trophic levels of the food chain such as that the $\delta^{13}C$ values of animal tissues and products of animal origin reflect the proportion of C3 and C4 plants ingested by these animals (Heaton et al. 2008). This allows using stable carbon isotope composition for the differentiation between C3 and C4 plants in a wide range of food products of both plant and animal origin.

Furthermore, the $\delta^{13}C$ values of plants are strongly influenced by different environmental factors, such as relative humidity, temperature, amount of precipitation, all of which affect the photosynthetic activity of plants (Smith and

Epstein 1971, O'Leary 1981, Farquhar et al. 1989, Tieszen 1991, Ehleringer and Dawson 1992, Martin and Martin 2003). In conditions of low relative humidity, high temperature or water deficit, plants generally close stomata in order to reduce transpiration rates and to preserve water. Partial or complete closure of stomata reduces the internal CO_2 concentration (c_i) and thus, decreases the net photosynthetic rate. On the other hand, low c_i has also a strong effect on the carbon isotope composition of the newly formed photosynthate. The isotopic discrimination against $^{13}CO_2$ during photosynthesis is inherent with the primary carboxylating enzyme (Rubisco/PEP). The discrimination against ^{13}C is great when the c_i is high, however little discrimination is observed in the case of low c_i, which can occur if stomatal conductivity is reduced (O'Leary 1981, Farquhar et al. 1982, Marshall et al. 2007). Therefore, stable carbon isotope ratios can provide additional information about the physiological response of plants to different environmental conditions and their water use efficiency (Smith and Epstein 1971, O'Leary 1981, Farquhar et al. 1982, Anyia and Herzog 2004), which can sometimes serve as a supporting evidence for the determination of the geographical and production origin of foods.

There are multiple examples of the successful application of stable carbon isotope analysis for the authentication of food products. The use of $^{13}C/^{12}C$ ratio analysis for the detection of food adulteration began in the 1970s when the relatively inexpensive high-fructose corn syrup was being widely added to honey (Antolovich et al. 2001). Stable carbon isotope analysis was used to determine sugar addition to Israeli orange juices (Nissenbaum et al. 1974) and adulteration of honey with cane sugar or corn syrup (Doner and White 1977). Since then, the technique has been successfully used in assessing the authenticity of apple juice (Doner et al. 1980, MAFF Food Surveillance Information 2000), orange juice (Doner and Bilss 1981, Simpkins et al. 2000), cranberry juice (Hong and Wrolstad 1986), maple products (Carro et al. 1980) and palm sugar (Kelly et al. 2009).

Carbon isotope ratios are also applied for the authentication of olive oils and various vegetable oils (Kelly et al. 1997, Spangenberg and Ogrinc 2001, Cristea et al. 2012). Differentiation between olive oil and other vegetable oils obtained from C3 plants such as sunflower oil, rapeseed oil, hazelnut oil is not possible on the basis of carbon isotope ratios, however successful distinction between corn (C4) and other C3 vegetable oils (e.g. olive, sunflower, hazelnut, soybean) has been obtained on the basis of $^{13}C/^{12}C$ ratios (Royer et al. 1999). For example, application of $^{13}C/^{12}C$

analysis was successful in the detection of corn oil adulteration with cheaper oils such as rapeseed oil (FSA Food Surveillance Information 2002).

Differentiation between C3 and C4 plants in animal diets has also been accomplished with the use of carbon isotope analyses. Stable carbon isotope ratios were used to verify that 'corn fed' chickens were fed a diet of at least 50% corn (C4 plant) during their rearing period (Rhodes et al. 2010). Furthermore, the geographical distribution of C3 and C4/CAM plants is different. C_3 plants predominate at higher latitudes whereas C_4 plants are more common in warmer climates at lower latitudes, thus there is a gradient of decreasing $^{13}C/^{12}C$ in plant material from the equator to the poles. This gives a possibility to use carbon isotope ratios as a proxy for the determination of the geographical origin of various food commodities. Carbon isotope composition (usually combined with other isotope ratios) was applied to verify the geographical origin of beef (Renou et al. 2004a, Heaton et al. 2008, Martinelli et al. 2011), lamb (Piasentier et al. 2003, Camin et al. 2007), butter (Rossmann et al. 2000) and cheese (Manca et al. 2001, Camin et al. 2004, Manca et al. 2006).

In addition, carbon isotope composition can sometimes be used to determine the growing regime of crops such as greenhouse vs. open field cultivation or organic vs. conventional systems. CO_2 used in the greenhouses for heating originates from the combustion of methane, which has a low $^{13}C/^{12}C$ ratio, and thus can be distinguished from atmospheric CO_2 (Schmidt et al. 2005a). This was, for example, successfully applied for the verification of greenhouse cultivation and the geographical origin of peppers from Holland and Spain (Förstel 2007). Differences in the carbon isotope composition of organically and conventionally grown plants have also been reported: organic plants sometimes had lower $\delta^{13}C$ values compared to conventional counterparts (Georgi et al. 2005). This has been explained by a possibly higher photosynthetic rate with lower discrimination of Rubisco against $^{13}CO_2$ in conventional crops and due to a higher microbial activity in organic fields resulting in more ^{13}C-depleted CO_2 respired, which then became available for re-fixation by plants (Högberg et al. 1995, Rapisarda et al. 2010a).

1.4.4. Stable oxygen and hydrogen isotope analyses for food authentication

Hydrogen and oxygen atoms in plant and animal tissues predominantly originate from water, consequently processes that affect the isotope ratios of water will influence the hydrogen and oxygen isotope composition of plant and animal matter. Stable hydrogen and oxygen isotopes are fractionated during the meteorological cycle of evaporation, condensation and precipitation. The evaporation of water from the oceans is a fractionating process, which decreases the concentration of heavy isotopomers of water ($^{1}H^{2}H^{16}O$, $^{1}H^{1}H^{18}O$) in the clouds compared to the water body. Heavier isotopes accumulate in the liquid phase and will fall first in the precipitation. As the clouds move inland and gain altitude further evaporation, condensation and precipitation events take place, and thus rain water becomes isotopically lighter moving from equatorial regions to higher latitudes and altitudes (Craig 1961, Mook 2000, Marshall et al. 2007). Generally, $\delta^{18}O$ and $\delta^{2}H$ values of meteoric water decrease with decreasing temperature, increasing distance from the sea and elevation, and increasing amounts of atmospheric precipitation (Dansgaard 1964, Yuntseover and Gat 1981).

Plants take up source water via xylem and incorporate H and O atoms into their synthesized compounds. When taken up by plants source water is not isotopically fractionated (Dawson et al. 2002). Source water exhibits similar systematic isotope variation with climate and geographical location as meteoric water and thus, is strongly latitude dependent (Dansgaard 1964, Yuntseover and Gat 1981). As a result, water in plant xylem carries the same isotopic signal as the source water and can therefore, serve as an indicator of climatic and geographical conditions which were present during plant growth (Dawson et al. 2002, Marshall et al. 2007, Iacumin et al. 2009). As water reaches sites of evaporation in the leaves fractionation does occur. Transpiration of water from leaves can significantly enrich the hydrogen and oxygen isotope composition of plant leaf water. The extent of this enrichment is related to several factors such as relative humidity and ambient temperature, which in turn depend on geographical location and climate. Low relative humidity and high temperature increase the rate of evapotranspiration affecting plants (Dawson et al. 2002, Marshall et al. 2007, Iacumin et al. 2009).

Similarly to plants, stable oxygen and hydrogen isotope composition of animal tissues reflects isotopic composition of drinking water and feedstuffs used by animals

(Clark and Fritz 1997, Schmidt et al. 2001), which is valuable for tracing the geographical origin of meat. Moreover, $\delta^{18}O$ and $\delta^{2}H$ composition of organic compounds present in animal products, such as milk, butter and cheese, also exhibits a strong correlation with the isotopic composition of water consumed by animals, and can provide valuable information for the geographical characterisation of these foodstuffs (Manca et al. 2001, Pillonel et al. 2003b, Camin et al. 2004).

The verification of geographical origin using stable oxygen or hydrogen isotope analyses has proved to be very promising for the authentication of a wide range of food products. Stable oxygen or hydrogen isotope composition has been used for the geographical classification of olive oils (Angerosa et al. 1999, Aramendia et al. 2007, Bontempo et al. 2009, Camin et al. 2010a), rice (Kelly et al. 2002), coffee (Weckerle et al. 2002), wine (Breas et al. 1994, Rossmann et al. 1999, Gremaud et al. 2004), beef (Renou et al. 2004a, Hegerding et al. 2002), milk (Renou et al. 2004b, Ritz et al. 2005), butter (Rossmann et al. 2000), cheese (Pillonel et al. 2003b, Camin et al. 2004, Manca et al. 2006) and various other commodities (e.g. reviews by Kelly et al. 2005, Luykx and van Ruth 2008, Primrose et al. 2010).

1.4.5. Stable nitrogen isotope analyses for food authentication

Nitrogen is an essential component of all amino acids and proteins. Main inorganic nitrogen forms include nitrogen gas (N_2), nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3), and ammonium (NH_4^+). Fractionations of stable nitrogen isotopes occur during biotic nitrogen transformations and abiotic processes. The former include microbial fixation of air nitrogen, nitrogen assimilation and mineralization, nitrification and denitrification processes. Each of these processes is associated with different fractionation factors (Kendall 1998, Marshall et al. 2007, Hoefs 2009). Abiotic fractionation of nitrogen isotopes can occur, for example, during the volatilization of ammonia and the diffusion of solutes (Högberg 1997, Kendall 1998, Evans 2007, Marshall et al. 2007).

Stable isotopes of nitrogen have been used to trace the flow and fate of nitrogen over many different scales ranging from pot experiments to tracing nitrogen through food webs and ecosystems (Bedard-Haughn 2003). From the food authentication perspective, stable nitrogen isotope ratios can provide valuable information about animal diet and agricultural practise (organic/conventional) used for crop and livestock production (Kendall 1998, Bateman et al. 2007, Marshall et al.

2007). Possibility to use nitrogen isotope composition for the differentiation between organic and conventional agricultural practises arises due to different nitrogen isotope compositions of synthetic and organic fertilisers. Synthetic nitrogen fertilisers are produced during the Haber-Bosch process from atmospheric nitrogen, which being an international standard, by definition has $\delta^{15}N$ value of 0‰ (Mariotti 1983). Due to little fractionation associated with the synthetic fertiliser production process, $\delta^{15}N$ composition of synthetic nitrogen fertilisers is generally similar to that of air nitrogen (Shearer et al. 1974, Freyer and Aly 1974, Kendall 1998, Vitoria et al. 2004, Bateman et al. 2007). In contrast, stable nitrogen composition of animal manures, composts and other fertilisers permitted in organic production systems is usually higher compared to that of synthetic fertilisers and can become ^{15}N-enriched due to preferential volatilisation of ^{15}N-depleted ammonia from organic fertilisers after their application to the field or during storage (Kendall 1998, Bateman and Kelly 2007).

The difference in the nitrogen isotope composition of synthetic and organic fertilisers applied to crops is likely to be reflected in the nitrogen isotope values of crops, and thus $\delta^{15}N$ values can be used as a marker to differentiate between organically and conventionally grown plants and plant products (Nakano et al. 2003, Choi et al. 2003, Bateman et al. 2005). Similarly, animals feeding on organic pastures or receiving organic feedstuffs have different nitrogen isotopic composition compared to conventionally raised animals, and thus nitrogen isotope analyses can be useful for the discrimination between organic and conventional products of animal origin (Kelly et al. 2005).

Stable nitrogen isotope analyses have been widely applied over the past several decades for the authentication of a wide range of food products. Nitrogen isotope ratios were used for the discrimination between conventional and organic vegetables (e.g. Georgi et al. 2005, Bateman et al. 2007, Camin et al. 2007, Rogers 2008, Flores et al. 2011, Šturm and Lojen 2011), cereals (Schmidt et al. 2005a, Choi et al. 2006, Suzuki et al. 2009), fruits (Rapisarda et al. 2005, Rapisarda et al. 2010b, Camin et al. 2011). Nitrogen isotope composition has also been analysed to determine the production origin of animal products, for example beef (Schmidt et al. 2005b, Bahar et al. 2008) and milk (Kornexl et al. 1997).

1.5. Current issues in food authentication

1.5.1. Virgin olive oils

On the basis of the expanding global market for olive oil, its authenticity has become an important issue from both a commercial and health perspective (Lai et al. 1994). Over the recent years global olive oil market has been gradually growing with the total amount of oil produced annually worldwide comprising on average more than 2.7 million tons (International Olive Oil Council 2012). Most of the global olive oil production (79%) and consumption (60%) takes place in the Mediterranean region. However, due to highly valued unique sensory characteristics, nutritional and health properties of olive oil (Harwood and Aparicio 2000, Lerma-García et al. 2010) olive cultivation and processing has been recently increasing in other regions of the world, such as North and South America, and Oceania (International Olive Oil Council 2012).

There is a big variation in olive oil prices depending on its grade, production technique, geographical and botanical origin, which has created an enticement for fraudulent activities (Lai et al. 1994). Olive oil is commercialised under different grades depending on the processing method. The highest quality and most valued is extra virgin olive oil. It is obtained from olive fruits solely by mechanical or other physical means under conditions that do not lead to alterations in the chemical composition of oil (Codex Alimentarius Commission 2003, International Olive Oil Council 2012). Lower olive oil grades include refined olive oil, lampante olive oil, which has a high acidity and is intended for refining or for technical use, and olive pomace oil, which is obtained by treating olive pomace with solvents or other physical treatments (International Olive Oil Council 2012).

Virgin olive oils are usually four to five times more expensive than common vegetable oils, such as corn or sunflower oil (European Commission 2003). As regards olive oil grades, extra virgin olive oil may cost nearly twice as much as refined olive oil (Lai et al. 1994). This can lead consumers to doubt the quality of commercialized olive oils and creates a temptation for dishonest producers and traders to gain financial profit from various sorts of adulteration, mislabelling and other mispresentation of genuine olive oil (Lai et al. 1994). Large profit can be made if high quality virgin olive oil is adulterated even with small amounts of cheaper seed oils (sunflower, corn, soy, etc.) or olive pomace oil (Marcos Lorenzo et al. 2002). In addition, European law (Council Regulation (EC) No 510/2006) provides oil

producers with the opportunity of indicating the geographical origin of extra virgin olive oil using the certified labels of protected denomination of origin (PDO) or the protected geographic indication (PGI), however it does not indicate specific analytical methods to check the authenticity of these indications. Thus, extra virgin olive oils with protected geographical and botanical origin bearing certified PDO and PGI labels are also subject to food scams (Camin et al. 2010a).

Multiple cases of olive oil fraud such as adulteration and false labelling have been detected in recent years in Italy (Shears 2010). For example, olive pomace oil, hazelnut oil and some other vegetable oils from Turkey have been frequently passed of as Italian premium virgin olive oil (Shears 2010). In 2008 Italian police launched operation ‘Golden Oil’, raiding nearly 100 olive producers, and found invoices to the EU for €6.5 million of subsidies and receipts for €39 million of 'Italian' oil made with non-Italian olives (Shears 2010).

Olive oil authentication, therefore, is currently of a great concern to consumers, as well as genuine oil processors. Continuous vigilance is required to control the adulteration and mislabelling of olive oil products. There is a need for the development of novel reliable analytical methods for the verification of geographical and production origin of olive oils (Aparicio 2000, Harwood and Aparicio 2000, Camin et al. 2010a, Camin et al. 2010b). The development of databases on the European and international level is urgently needed to provide information on the chemical composition of olive oils with different production, geographical and varietal origins (Aparicio and Aparicio-Ruiz 2000, Camin et al. 2010b).

1.5.2. Organic food products

The authenticity of organic products has recently become another highly important issue in food sector in Europe and worldwide (Beck 2012, van den Idsert 2012). According to the latest FiBL-IFOAM survey on certified organic agriculture worldwide (Willer and Kiltcher 2012) there has been a strong growth in the number of organically cultivated areas in Europe with an increase of 9% since 2009. Global organic food market has expanded more than three-fold since year 2000, with consumer demand for organic food being the highest in the US and the EU (Willer and Kiltcher 2012).

Market prices of organic food products are on average 50-75% higher compared to conventional counterparts (Brown and Sperow 2005,Winter and Davis

2006, Willer and Kilcher 2010), however depending on the product type the difference in prices can reach 175 % (Thompson and Kidwell 1998), 220% and even more (Oberholtzer et al. 2005). Even though there is such variation in prices on the market, consumers, who agree to pay a premium price for organic produce, have a right to be sure that they are purchasing high quality and authentic products. To know whether a particular product is of organic origin, a consumer must rely on the information from the product label or simply trust the retailer when no labelling is available, such as, for example, on farmers markets. With organic food selling at premium prices, there is a temptation for dishonest growers and retailers to sell their conventional produce as organic. Recently there have been a number of reported cases in the UK and the EU where conventional products were mislabelled, passed off as organic and priced accordingly (Beck 2012, Ferrante 2012, Huber 2012, Piva 2012). Some of the well known organic food fraud cases have recently taken place in Italy and Germany (Beck 2012). One of the biggest organic food fraud cases, which took place in Europe during five years from 2007 to 2011, has been recently discovered in Italy. Tons of organic produce, mainly wheat and other commodities of plant origin, having false certificates were imported from Romania and further exported to other countries of the EU where they were sold at premium prices (Beck 2012, Ferrante 2012, Huber 2012, Piva 2012).

To minimize and prevent organic food fraud it is crucial that organic food operators have quality control procedures based on systematic identification of critical processing steps. Further, they must guarantee that at all stages production and processing complies with the organic production rules and that the necessary steps are taken to prevent contamination, mixing or confusion with non-organic products. It is these procedures and their effectiveness that must be the subject of a detailed inspection (Beck 2012). The development of analytical tools for the verification of organic certification information is urgently needed. It is highly important to find specific markers for the differentiation between organic and conventional products to ensure the authenticity of organic produce in Europe and worldwide (Beck 2012, Huber 2012, van den Idsert 2012).

1.5.3. Rationale for the present work

With a number of recently reported severe fraud cases involving organic food products and virgin olive oils, there is currently a great need to develop robust analytical approaches for the authentication of these commodities. Finding novel specific markers for the authentication of organic products is complicated due to a great variability of both conventional and organic agricultural practises, either of which can use a wide range of different fertilisation and plant cultivation strategies. Similarly, chemical properties of virgin olive oils are subject to variability due to a large number of external factors, such as geographical and climatic conditions of the production location, different production techniques and olive varieties used, each of which needs to be taken into account in the search for novel authenticity markers.

Stable isotope ratio analyses so far have been one of the most successful and widely used analytical approaches in food authentication studies (Kelly and Rhodes 2002, Kelly et al. 2005, Reid et al. 2006, Luykx and van Ruth 2008, Primrose at al. 2010, Chesson et al. 2011). Stable isotopes have been applied in food studies as recorders of various environmental, chemical and biological processes. A vast number of studies apply isotopic analyses of bulk sample material, however this type of analysis reflects the contribution from different compound classes, which are present in a sample and may have distinct isotopic values. In contrast, compound-specific isotopic measurements provide information regarding individual compound(s) and can provide a more direct information about the impact of different external factors on the physiological and biochemical processes in plant tissues and, consequently, in plant products.

Furthermore, a vast number of food authenticity studies have focused on the stable isotope analysis of a single element, which can be an attractive and quick screening tool for the authenticity testing of food products. However, natural isotope variability, complexity of the chemical structure of the analysed food material, and the impact of a wide range of external factors can introduce large uncertainties to the interpretation of isotopic data and, thus require more robust analyses for the reliable verification of food authenticity. Recent technological advances in IRMS have introduced a possibility to perform simultaneous rapid analyses of multiple isotope ratios. The use of multi-element isotopic analyses as well as combination of isotopic techniques with other analytical methodologies (e.g. molecular analyses, trace element analyses, IR spectroscopy etc.) and chemometrics have been reported to

provide higher discriminative power and are currently gaining increased attention in food provenancing studies (Hölzl et al. 2004, Franke et al. 2005, Kelly et al. 2005, Rapisarda et al. 2010a, Camin et al. 2010a, Camin et al. 2010b, Camin et al. 2011).

The dissertation is focused on the application of novel compound-specific stable isotope approaches together with molecular techniques for studying the relationships between environmental conditions and the chemical composition of plants and plant products. The present work evaluates the potential of the novel analytical tools for the authentication of the geographical and production origin of plant products and aims to contribute to the search of novel food authenticity markers.

Chapter 2 and Chapter 3 focus on the application of stable isotope analyses for the differentiation between organically and conventionally cultivated crops. Along with bulk stable nitrogen isotope analyses, a novel approach – compound-specific stable oxygen and nitrogen isotope analysis of plant-derived nitrate using bacterial denitrifier method (Sigman et al. 2001, Casciotti et al. 2002) – is applied for the first time to plant material.

Chapter 2 describes stable isotope analyses of lettuces cultivated in the controlled greenhouse conditions and evaluates the potential of the novel compound-specific isotope analysis of nitrate for the differentiation between plants from conventional and organic fertiliser treatments. In addition, the intra-plant isotopic variation in lettuce plants, as well as the impact of the fertiliser application rate on the stable isotope composition of lettuce is addressed.

Chapter 3 evaluates the potential of the total nitrogen content, bulk nitrogen isotope analysis and the novel compound-specific oxygen and nitrogen isotope analyses of plant nitrate for the discrimination between field grown vegetables from conventional and two types of organic agricultural systems. I discuss which stable isotope parameters provide the most discriminative potential for the classification of vegetables according to their growing system. Further, the compound-specific isotope analysis of nitrate is applied to retail vegetable samples from different geographical locations and evaluated on its capability for the authentication of organic vegetables.

Chapters 4 to 6 focus on the molecular and stable isotope analyses of olives and extra virgin olive oils. For the first time, I perform compound-specific stable hydrogen and carbon isotope analyses of *n*-alkanes from olive oils, olive fruits and

leaves. Novel isotope approach is evaluated on its capability for the differentiation of geographical, varietal and production origins of oils.

Chapter 4 describes the molecular and hydrogen and carbon isotope compositions of *n*-alkanes in extra virgin olive oils from eight Mediterranean countries. I study the relationship between the environmental factors and the molecular and stable isotope compositions of *n*-alkanes in extra virgin olive oils and discuss which of the analysed molecular and stable isotope parameters provide the most discriminative potential for the classification of olive oils according to their geographical region.

In Chapter 5 the molecular and compound-specific carbon isotope analyses of *n*-alkanes are evaluated in respect to their capability to distinguish extra virgin olive oils produced with two different olive harvesting techniques: manual and mechanical. Molecular and, for the first time, carbon isotope compositions of *n*-alkanes in olive fruits and leaves from three Italian olive varieties (Frantoio, Leccino and Moraiolo) are also analysed and discussed here.

Chapter 6 studies the difference in the distribution and carbon isotope composition of *n*-alkanes in olives and leaves of the three Italian olive varieties (Frantoio, Leccino and Moraiolo) during different stages of olive fruit maturity (from July to November) and discusses the applicability of the molecular and compound-specific carbon isotope analyses of *n*-alkanes for varietal differentiation.

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CHAPTER 2

THE EFFECTS OF ORGANIC AND SYNTHETIC FERTILISATION ON THE STABLE ISOTOPE COMPOSITION OF LETTUCE IN THE GREENHOUSE CONDITIONS

Abstract

Organic cultivation systems exclude the use of synthetic fertilisers and pesticides. At present no analytical controls of fertiliser inputs are performed and fraudulent application of synthetic fertilisers to organic crops as well as substitution of organic products with conventional counterparts in the supply chains are difficult to detect. Therefore, there is currently an urgent need to develop novel robust approaches for the discrimination between organic and conventional foods.

This study focused on the application of stable isotope analysis for differentiating between lettuce plants grown with synthetic (KNO_3, urea) and organic (chicken manure) fertilisation under controlled greenhouse conditions. Stable nitrogen isotope composition of bulk plant matter ($\delta^{15}N_{bulk}$) and, for the first time, stable nitrogen and oxygen isotope composition of plant-derived nitrate ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) were evaluated for their capability to differentiate lettuces from three fertiliser treatments. Additionally, the impact of the fertiliser application rate (50 and 150 mg N/kg substrate) on the isotopic composition of lettuce was determined. Furthermore, leaves and roots of lettuce were analysed separately to study the intra-plant isotopic variation.

Bulk nitrogen isotope composition has been successful in differentiating lettuce plants grown with synthetic fertilisers from the plants grown with chicken manure. Lettuces fertilised with chicken manure have significantly higher ($p<0.05$) $\delta^{15}N_{bulk}$ values compared to lettuces grown with synthetic KNO_3 and urea, and reflect the difference in the nitrogen isotope compositions of the applied fertilisers.

The nitrogen and oxygen isotope compositions of lettuce nitrate have been capable of differentiating plants grown with synthetic nitrate from plants grown with manure. The $\delta^{15}N_{NO3}$ values of leaves from lettuces grown with chicken manure are significantly higher ($p<0.05$) than those of lettuce grown with synthetic KNO_3. In

contrast, the $\delta^{18}O_{NO3}$ values of lettuces fertilised with chicken manure are lower compared to those treated with synthetic nitrate fertiliser, and reflect the difference in the oxygen isotope compositions of sources of oxygen in synthetic and organic nitrate.

A strong effect of the fertiliser application rate on the isotopic composition of lettuce plants has been observed in the present study. In both synthetic and organic fertiliser treatments, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuces are higher when the fertiliser is applied at the lower rate compared to the higher application rate. An intra-plant isotopic variation has been observed in all fertiliser treatments with consistently higher $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce leaves compared to roots and substrates.

This has been the first time when compound-specific nitrogen and oxygen isotope analysis of plant-derived nitrate has been used for differentiating between conventional and organic cultivation. The oxygen isotope composition of nitrate has a potential for the discrimination between plants grown with synthetic nitrate and those fertilised with manure. Further analysis of a greater range of plant species from both greenhouse and field experiments and with different fertilisation strategies are needed to establish the scope of application and robustness of the novel approach.

2.1. Introduction

2.1.1. Stable nitrogen isotope analysis as a tool for the discrimination between organic and conventional food products

Consumer demand for organic food has grown tremendously over the past decade (Willer and Kilcher 2012). Significantly high prices for organic goods have created a financial enticement for dishonest growers and retailers to mislabel and try to sell cheaper conventional products as organic. Various severe fraud cases in the organic sector have been reported over the recent years in the EU and worldwide (e.g. Giannakas 2002, Beck 2012, Ferrante 2012, Piva 2012). This has resulted in pressure for the establishment of standards and labelling of organic products. Governments in the EU, the USA, Canada and in other countries have introduced various regulations for the standardization, certification and labelling of organic food (Giannakas 2002).

Organic cultivation systems exclude the use of any synthetic fertilisers or pesticides, which are extensively used in conventional agricultural practises. Soil fertility in organic agricultural systems is maintained through the use of crop rotations that include green manures and also by the application of selected fertilisers which may be permitted where the need is recognized by the inspecting authority. Fertilisers that may be permitted in organic agriculture include animal manures, composts and other products of plant and animal origin (Commission Regulation (EC) No. 889/2008).

It has been proposed that nitrogen isotope composition could be used as a tool to differentiate between organically and conventionally grown produce (Choi et al. 2003, Nakano et al. 2003). This is due to a difference in the type and nitrogen isotope composition of the fertilisers applied in conventional and organic agricultural systems. Synthetic nitrogen fertilisers are produced from atmospheric nitrogen in the Haber-Bosch process and generally have $\delta^{15}N$ values within a few per mil of zero (mean $\delta^{15}N$ = -3 to +3‰) reflecting their origin from air N_2 (Shearer et al. 1974, Freyer and Aly 1974, Hübner 1986, Kendall 1998, Vitoria et al. 2004, Bateman and Kelly 2007). In contrast, the stable nitrogen composition of animal manures, composts and other fertilisers permitted in organic production systems is significantly higher compared to that of synthetic nitrogen fertilisers (Kendall 1998, Bateman and Kelly 2007). The mean $\delta^{15}N$ values of organic fertilisers cluster around

+8‰, however some fertilisers can have values up to +35‰ and even higher, which occurs due to the preferential volatilization of ^{15}N-depleted ammonia from animal manures in the field (Bateman and Kelly 2007).

Over the past decade a number of studies have been conducted to determine whether stable nitrogen isotope composition could be used as an indicator of organically grown produce. Some studies reported no significant differences in the $\delta^{15}N$ values among certain plant species cultivated under synthetic and organic regimes (Choi et al. 2001, Bateman et al. 2007, Šturm and Lojen 2011). In contrast, other studies confirmed that fertilisation regime had a significant impact on the isotopic composition of cultivated crops, and resulted in significantly lower nitrogen isotope composition of plants cultivated with synthetic fertilisers compared to that of organically grown plants (Yoneyama et al. 1990, Choi et al. 2003, Nakano et al. 2003, Bateman et al. 2005, Georgi et al. 2005, Rapisarda et al. 2005, Schmidt et al. 2005, Camin et al. 2007, Flores et al. 2007, del Amor and Navarro 2008, Suzuki et al. 2009, Rapisarda et al. 2010, Camin et al. 2011, Šturm and Lojen 2011, Zhou et al. 2012, Yuan et al. 2012).

For example, Nakano et al. (2003) examined the effects of inorganic and organic fertilisers on the yields, $\delta^{15}N$ and $\delta^{13}C$ values of tomatoes. Authors found that the $\delta^{15}N$ values of soil, in which tomato plants were cultivated, and the tomato fruits themselves reflected the $\delta^{15}N$ value of the applied fertiliser. Nakano et al. (2003) concluded that the bulk nitrogen isotope analysis may be used for the indication of organic cultivation, however they also discussed that this approach may not work in all cases. As an example authors mentioned urine that is sometimes applied as a liquid fertiliser in organic agricultural farming and has nitrogen isotope values similar to those of synthetic nitrogen fertilisers.

Bateman et al. (2005) conducted a series of greenhouse experiments to investigate the effect of the type of fertiliser, growing medium and irrigation water on the $\delta^{15}N$ of carrots, tomatoes, and lettuces. Authors reported that fertiliser type was a significant factor influencing the nitrogen isotope composition of the crop. Under controlled conditions, the application of synthetic nitrogen fertilisers resulted in crops with lower $\delta^{15}N$ values. Authors also concluded that irrigation water and growing media could impact the $\delta^{15}N$ values of the cultivated crops.

Šturm et al. (2011) investigated the combined use of synthetic and organic fertilisers on the bulk nitrogen composition of pot grown lettuces. Authors reported

that the $\delta^{15}N$ values of whole plants treated with different fertilisers differed significantly when the fertiliser was applied in a single treatment. However, additional fertilisation (with isotopically the same or different fertiliser) did not cause a significant alteration of the plant $\delta^{15}N$. Authors suggested that the $\delta^{15}N$ value could be used as a rough marker to reveal the history of nitrogen fertilisation in the case of a single fertiliser application.

Zhou et al. (2012) conducted a greenhouse experiment to investigate the impact of compost and synthetic urea on the ^{15}N composition of soil and tomato plants. Authors reported significant difference in the $\delta^{15}N$ values of tomato plants cultivated with synthetic and organic fertilisers, and concluded that the $\delta^{15}N$ values of plants could reflect the application of compost or synthetic nitrogen fertiliser.

Thus, nitrogen isotope composition of crops has been shown to reveal differences between synthetic and organic fertilisation regimes. However, some authors report overlapping $\delta^{15}N$ values of crops from conventional and organic fertilisation and discuss that the difference between the nitrogen isotope composition of the introduced nitrogen fertiliser and the isotopic composition of substrate may be in some cases relatively small and this, in combination with nitrogen isotope fractionations that occur during nitrogen turnover among different pools in soil and further in plants, may introduce great uncertainties in the $\delta^{15}N$ data interpretation (Handley and Raven 1992, Evans 2001, Bedard-Haughn et al. 2003). In order for plant $\delta^{15}N$ to be used more generally as an indicator of agricultural regime, the impact of synthetic nitrogen fertiliser on the $\delta^{15}N$ value of a crop must predominate over many other factors which may also influence plant $\delta^{15}N$. Such factors include: i) variability in the $\delta^{15}N$ of synthetic nitrogen fertilisers; ii) the form of nitrogen in the applied synthetic fertiliser (nitrate, ammonium, urea); iii) variability in the $\delta^{15}N$ of organic nitrogen fertilisers; iv) timing of fertiliser application; v) the pedoclimatic conditions of the location; vi) the use of nitrogen-fixing plants for enhancing the nitrogen fertility of soils (Bateman et al. 2005).

Greater differences between the $\delta^{15}N$ values of crops from organic and conventional growing regimes are more likely to be seen in crops with high nitrogen requirements, crops grown under controlled greenhouse conditions or hydroponically, whereas less significant differences in the $\delta^{15}N$ values between two growing regimes can arise in the case of the field-grown crops with relatively low nitrogen requirements or long growth cycles (Bateman et al. 2007, Rogers 2008).

2.1.2. The potential of using stable oxygen isotope analysis of plant-derived nitrate for the discrimination between conventional and organic crops

The oxygen isotope analysis of nitrate in the atmosphere, hydrosphere, and geosphere can provide important information about the sources, transport and reactions of nitrate in the environment (Böhlke et al. 2003). Synthetic nitrate fertilisers, which are commonly used in conventional agriculture, have distinctive $\delta^{18}O_{NO3}$ values. During the manufacture of synthetic nitrate fertilisers, all three oxygen atoms of nitrate are derived from atmospheric oxygen ($\delta^{18}O$ = +23.5‰; Kroopnick and Craig 1972), and thus, $\delta^{18}O$ values of those fertilisers are similar to the $\delta^{18}O$ of air oxygen, and are in the range from +17‰ to +25‰ (Amberger and Schmidt 1987).

In contrast, when organic nitrogen fertilisers are used nitrate is produced during the process of nitrification by soil microorganisms. This microbially produced nitrate has two possible sources of isotopically distinctive oxygen: i) atmospheric oxygen and ii) oxygen from soil water. Aerobic nitrification pathway from ammonium (NH_4^+) to nitrate (NO_3^-) proceeds in two steps. Firstly, NH_4^+ oxidation to nitrite (NO_2^-) takes place, resulting in NO_2^- which contains one O atom from air O_2 and one from soil water. The following oxidation of NO_2^- to NO_3^- has been shown to incorporate O atoms from soil water only (Andersson and Hooper 1983, Kumar et al. 1983, Hollocher 1984, Dispirito and Hooper 1986). Therefore, the $\delta^{18}O$ of nitrate is interpreted as a mixture of two oxygen atoms from the ambient water and one from air oxygen:

$$\delta^{18}O_{NO3} = 2/3\ (\delta^{18}O_{H2O}) + 1/3\ (\delta^{18}O_{O2}) \qquad (2.1.)$$

where the $\delta^{18}O_{H2O}$ is stable oxygen composition of ambient water, and the $\delta^{18}O_{O2}$ is stable oxygen composition of air oxygen.

Four critical assumptions have been made for the above mentioned equation: i) the proportions of O originating from water and O_2 are the same in soils as observed in laboratory cultures; ii) incorporation of oxygen from H_2O or O_2 during nitrification occurs without fractionation; iii) the $\delta^{18}O$ of water used by the nitrifying microorganisms is equal to that of the bulk soil water; iv) the $\delta^{18}O$ of O_2 used by the nitrifying microorganisms is equal to that of atmospheric O_2 (Kendall, 1998).

Thus, nitrate produced during microbial nitrification should have a relatively narrow range of oxygen isotope values, with most of the variability being attributed to the oxygen isotopic composition of the local meteoric waters involved. Environmental waters typically have $\delta^{18}O$ values in the range between -25‰ and +4‰, while the $\delta^{18}O$ of atmospheric oxygen is +23.5‰ (Kroopnick and Craig 1972, Hollocher 1984, Durka et al. 1994). The average $\delta^{18}O$ values of microbially produced nitrate have been reported to lie between -10‰ and +10‰ (Kendall 1998, Kendall et al. 2007).

Fractionations of nitrogen and oxygen isotopes occur during nitrate assimilation by plants (Handley and Raven 1992, Evans et al. 1996, Kendall 1998, Robinson et al. 1998, Evans 2001), however significant difference in the ^{18}O signatures between synthetic and microbially produced nitrate is likely to be preserved in the nitrate derived from plant tissues. Thus, ^{18}O analysis of nitrate from plants may have a potential for the discrimination between plants grown under synthetic and organic fertilisation regimes.

2.1.3. Rationale for this study

For some crop species the nitrogen isotope composition of bulk matter proved to be rather a good tracer of the applied nitrogen fertiliser and allowed discrimination between conventionally and organically grown crops (e.g. Nakano et al. 2003, Georgi et al. 2005, Rapisarda et al. 2005, Camin et al. 2007, del Amor and Navarro 2008, Suzuki et al. 2009, Rapisarda et al. 2010, Šturm et al. 2011, Zhou et al. 2012, Yuan et al. 2012). However, in a large number of studies, the $\delta^{15}N$ values of organically and conventionally grown plants overlapped, and for some crop species $\delta^{15}N$ analysis alone was not able to discriminate between two agricultural regimes (Choi et al. 2001, Bateman et al. 2007, Šturm and Lojen 2011). Therefore, this single isotope approach cannot always ensure the complete discrimination between different nitrogen sources, and the interpretation of such results with overlapping values implies big uncertainties.

Some studies suggest that the oxygen isotope composition of nitrate may be a good tracer of nitrate sources (Durka et al. 1994, Wassenaar 1995, Kendall et al. 1995, Kendall 1998). Nitrate fertilisers are most common type of nitrogen-based fertilisers used in conventional agricultural systems in Western Europe, including UK (Kendall 1998). Ammonium nitrate and calcium ammonium nitrate, together

accounting for over 40% of the total fertiliser use. Solid urea and urea ammonium nitrate account for about 20-25% (Isherwood 2003, Chambers and Dampney 2009). Synthetic nitrate fertilisers have $\delta^{18}O$ values that are significantly higher from those of microbially produced nitrate (Durka et al. 1994, Kendall 1998, Kendall et al. 2007). This suggests that the oxygen isotope composition of nitrate may be used as a tracer for distinguishing between synthetic nitrates and organic fertilisers, and consequently between plants gown using these fertilisers.

For the isotopic analysis, nitrate present in the solution should be firstly converted to a gas, which then is introduced into the IRMS. Some existing techniques for sample preparation for the stable isotope analysis of nitrate are very labour-intensive and expensive, for example: "ion-exchange" or "$AgNO_3$ method" (Chang et al. 1999, Silva et al. 2000), "cadmium reduction" or "azide method" (McIlvin and Altabet 2005). The bacterial "denitrifier method" (Sigman et al. 2001, Casciotti et al. 2002), which is based on microbial conversion of nitrate to N_2O gas, is an efficient, less expensive and less laborious approach, which over the recent years has gained a growing attention in respect to the isotope analysis of nitrate in freshwater and seawater (Xue et al. 2009). To our knowledge, there are currently no published studies on the use of the denitrifier method for the isotopic analysis of nitrate from plant material.

This study is focused on the application of stable isotope analyses for differentiating lettuces grown under conventional and organic regimes in controlled greenhouse conditions. Along with bulk nitrogen isotope analysis, for the first time, the denitrifier method (Sigman et al. 2001, Casciotti et al. 2002) has been applied to analyse the oxygen and nitrogen stable isotope composition of the nitrate extracted from lettuce. The main objective of the study was to evaluate the capability of bulk and compound-specific approaches to differentiate lettuce plants grown with three different fertiliser treatments: synthetic potassium nitrate, urea and chicken manure. The second goal of the study was to evaluate the impact of two different application rates of each fertiliser (50 and 150 mg N/kg substrate) on the stable isotope composition of lettuces. Furthermore, leaves and roots of lettuce plants were analysed separately in order to study the intra-plant isotopic variation in lettuce plants.

2.2. Materials and methods

2.2.1. Greenhouse experiment

The lettuce pot experiment was conducted in the temperature and light-controlled greenhouse at the John Innes Centre (Norwich, UK) from April till June 2011. The following soilless seed compost formula was used for substrate preparation for the experiment: sand (William Sinclair Horticulture Ltd., Lincoln, UK) and Irish moss peat (Erin Horticulture Ltd., Derrinlough, Ireland; NO_3-N water extractable 6 mg/l, NH_4-N water extractable 4.1 mg/l; pH 4.6) were mixed in the ratio: 1:3 and subsequently amended with potassium sulphate (K_2SO_4, Vitax Ltd., Leics, UK) – 0.3 g/l, superphosphate ($Ca(H_2PO_4)_2$, Gem Gardening, Blackburn, UK) – 1.1 g/l, dolomite ($CaMg(CO_3)_2$, Omya Ltd., Derby, UK) – 2.9 g/l and calcium carbonate ($CaCO_3$, Omya Ltd., Derby, UK) – 2.3 g/l. No nitrogen fertiliser was added to the substrate at this stage.

Seeds of lettuce *(Lactuca sativa* L. cv. Little Gem) were sown in plug trays with the prepared substrate and placed in the greenhouse. After 30 days, at the four to six true leaf stage, seedlings were individually transplanted into 2 litre pots (ø 160 mm) that contained substrates to which different nitrogen fertilisers had been added (Figure 2.1). Three different fertiliser treatments were used in the experiment:

1) potassium nitrate (KNO_3; Kemira Chemicals Ltd., Goole, UK; N = 13%)
2) urea ($CO(NH_2)_2$; Yara, Grimsby, UK; N = 46%)
3) pelleted chicken manure (Vitax Ltd., Leics, UK; N = 3.5%)

Two different fertiliser concentrations were used for each fertiliser treatment: 50 mg N/kg substrate and 150 mg N/kg substrate. Levels of nitrogen application were chosen to represent those used in lettuce cultivation, which are typically 100-300 kg N/ha. Five lettuce plants were grown for each treatment type. Fertiliser amounts were calculated per 15 litres of substrate based on the amount (%) of nitrogen in the fertiliser (as indicated above). All three fertilisers were used in dry, fine powder form. Each fertiliser was thoroughly mixed with 15 litres of substrate in a 20 litre container and then distributed evenly to 2 litre pots. Clean containers were used for each fertiliser type. Fertilisers were added to substrates at the start of the experiment only, no subsequent addition was performed in the course of the experiment.

After the addition of fertilisers, plants were grown for 50 days and watered daily with ultrapure water (18MΩ cm, Millipore; NO_3^- not detected, $\delta^{18}O$ = -7.7 ± 0.0‰). Constant greenhouse conditions were maintained during the whole length of the experiment (light regime: 16 hours, 270 µMol $m^{-2} \cdot s^{-1}$ (HQI lamp, 400W), day temperature: +20 °C, night temperature: +18 °C. Photographs of the greenhouse grown lettuce plants are attached in the Appendix A (Figure A1).

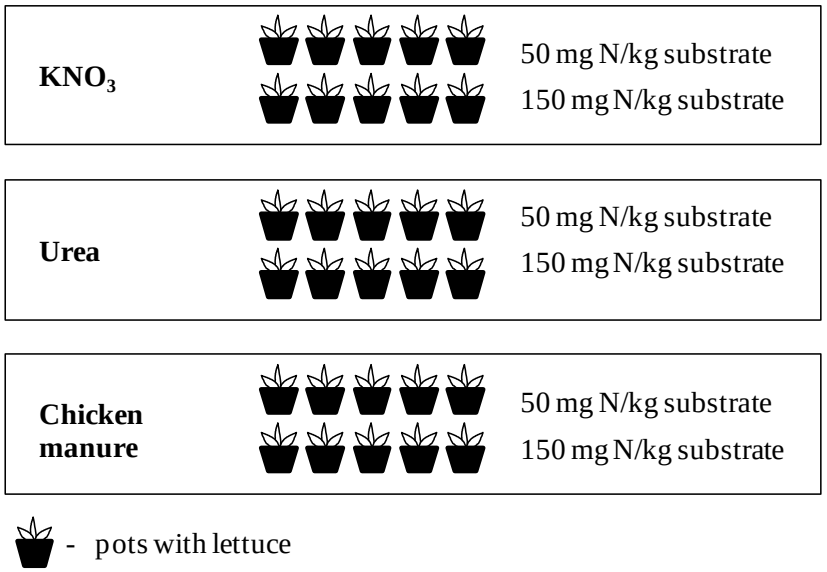

Figure 2.1. Experimental scheme of the lettuce pot experiment.

2.2.2. Sampling and sample preparation

Substrate samples were collected at the start (day 0) and at the end of the experiment (day 80). At the start of the experiment one sample of substrate containing a particular fertiliser was collected before distributing the substrate into pots. At the end of the experiment substrate samples were collected from all pots. Lettuce samples were collected at the end of the experiment; all plants were separated into leaves and roots. The number of fertiliser, substrate and lettuce samples collected from each fertiliser treatment is shown in Appendix A (Table A1).

Lettuce leaves were freeze-dried for 48h. Lettuce roots were gently rinsed with ultrapure water (18MΩ cm, Millipore) and then freeze-dried for 48h. Substrate samples were freeze-dried for 48h and sieved through 710 µm aperture sieve (ENDECOTTS Ltd., London, UK). Then both lettuce and substrate samples were prepared in a similar way – homogenized, ground to a fine powder using a ball mill

(Mixer/Mill® 8000-M230, SPEX SamplePrep LLC, New Jersey, USA) and stored in plastic containers at room temperature until further analyses. Due to a very small amount of freeze-dried lettuce root material, five samples of lettuce roots from each fertiliser treatment were combined in one sample to ensure sufficient amounts for the isotopic analyses.

2.2.3. Bulk nitrogen isotope analysis

Freeze-dried and ground samples were weighed into tin capsules to give 0.1 mg of N per analysis. Nitrogen isotope composition was determined using Costech elemental analyzer coupled to Thermo Finnigan Delta XP continuous flow isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were analysed in duplicate, and for the vast majority of samples the absolute difference between duplicate measurements was < 0.3‰, never exceeding 0.5‰.

Each batch of samples included replicate analyses of the in-house standard, casein ($\delta^{15}N$ = +6.15‰, previously calibrated against the International Atomic Energy Agency (IAEA) reference materials during an inter-laboratory comparison exercise as part of EU Project SMT4-CT98-2236), which was used for the drift correction of the raw data. The long-term performance of the mass spectrometer was monitored by analysis of a secondary reference material, collagen ($\delta^{15}N$ = +6.12‰), which was included to every batch of samples. Isotope ratios were reported with respect to the international standard – air nitrogen. Data were processed using Isodat NT 2.0 software (Thermo Scientific, Bremen, Germany).

2.2.4. Compound specific nitrogen and oxygen isotope analysis of nitrate

2.2.4.1. Nitrate extraction

Nitrate was extracted from freeze-dried samples of lettuce leaves, roots and substrates using hot water extraction procedure. Extraction was performed in 50 ml polypropylene centrifuge tubes (Corning®, Sigma-Aldrich, UK). In the case of lettuce leaves and roots, 20 ml of Ultrapure water (18MΩ cm, Purelab Ultra) was added to 0.8 g of dry plant material; in the case of substrates, 30 ml of Ultrapure water (18MΩ cm, Purelab Ultra) was added to 5 g of each substrate sample. Then

tubes were placed in a 90 °C hot water bath for 30 minutes. All extracts were cooled, centrifuged for 10 min at 4500 rpm, filtered through 0.2 micron filter (Minisart®, Sartorius, UK) and stored at -20 °C.

2.2.4.2. The denitrifier method

The denitrifier method (Sigman et al. 2001, Casciotti et al. 2002), which was originally developed for the isotopic analysis of nitrate in sea- and fresh-water, was used for the oxygen and nitrogen isotope analysis of nitrate extracted from vegetable matter. The method is based on the reduction of nitrate (NO_3^-) to nitrous oxide (N_2O) using a bacterial strain *Pseudomonas aureofaciens* (ATCC no. 13985, recently reclassified as a strain of *P. chlororaphis*) that is lacking nitrous oxide reductase, thereby preventing further reduction to N_2:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \,||\, (N_2 \text{ not produced}) \qquad (2.2.)$$

This allows simultaneous determination of both $\delta^{15}N$ and $\delta^{18}O$ values of the sample NO_3^- by measuring $\delta^{15}N$ and $\delta^{18}O$ of the N_2O produced by *P. aureofaciens*. A detailed description of the denitrifier method for the sample preparation for the nitrogen and oxygen isotope analysis of nitrate can be found in Casciotti et al. (2002). The method was adapted with minor adaptations. Bacteria were cultivated at room temperature on tryptic soy agar (Difco™, Becton, Dickinson and Company, NJ, USA) containing 10 mM KNO_3, 1.9 mM $(NH_4)_2SO_4$, and 37 mM K_2HPO_4. Further, a single bacterial colony was transferred to a starter tube containing tryptic soy broth solution (9 ml) (Bacto™, Becton, Dickinson and Company, NJ, USA) containing the same nitrate and ammonium amendments as the agar medium. After overnight incubation on a reciprocal shaker an aliquot from the starter tube was used to inoculate presealed sterile media bottles (2.7 ml bacterial medium per 445 ml bottle). Bottles were then incubated for 3-10 days under anaerobic conditions, at room temperature on a reciprocal shaker. After the incubation period bacteria were concentrated by centrifugation and resuspension in 80 ml of nitrate free media, to which an antifoaming agent (~1 ml; Antifoam B Emulsion, Sigma-Aldrich, UK) was added to avoid excessive bubbling. Then one ml aliquots of bacterial cell solution were injected into 20 ml headspace vials which then were crimp sealed using butyl rubber septa and aluminium crimp seal caps and purged with pure He gas for 45

minutes to remove the N_2O produced from the original 10 mM nitrate media and to ensure anaerobic conditions. After the He purge, the vials were stored overnight on a reciprocal shaker table. On the following day purging with He was repeated, after which the injection of the nitrate extracts followed. Nitrate extracts from lettuce leaves and roots were injected at the volume of 0.2 ml, nitrate extracts from substrates were injected at the volume of 1 ml. Vials without sample addition were used as "bacterial blanks". Three international nitrate reference materials: IAEA-NO-3 ($\delta^{15}N = 4.7 \pm 0.1‰$, $\delta^{18}O = 25.61 \pm 0.2‰$), USGS-34 ($\delta^{15}N = -1.8 \pm 0.1‰$, $\delta^{18}O = -27.93 \pm 0.3‰$) and USGS-35 ($\delta^{15}N = 2.7 \pm 0.1‰$, $\delta^{18}O = 57.50 \pm 0.2‰$) (Böhlke et al. 2003) were used as standards and injected in a similar way as the samples. All vials were left inverted overnight to enable bacterial culture to convert nitrate to nitrous oxide. Afterwards 0.1 ml of 6M NaOH solution was added into each vial to lyse the bacterial cells and scavenge CO_2. Samples were analysed on the isotope ratio mass spectrometer within the following few days.

2.2.4.3. Nitrogen and oxygen isotope analysis

N_2O produced by the denitrifying bacteria was stripped from each sample vial, purified and then analyzed on a PDZ Europa GEO 20-20 mass spectrometer (PDZ Europa Ltd., Northwich, UK), which was coupled with an ANCA TG II system (PDZ Europa Ltd., Northwich, UK) and Gilson head-space autosampler. The N_2O from the sample vial was purged via an autosampler needle with a helium carrier stream. The outflow from the needle passed through a Nafion drier (Perma Pure Inc., MD-050-72S-1) and a chemical trap (containing magnesium perchlorate and carbosorb) to remove water and most of the by-product CO_2. The sample gas was trapped in a stainless steel loop immersed in liquid N_2 to condense N_2O (as well as any remaining H_2O and CO_2), while the uncondensed gases (He, N_2) vented to the atmosphere. The sample gas was then focused into a smaller volume using a second cryogenic trap and then released under the low-flow He into the gas chromatographic columns, held at 37 °C (CP-Poraplot-Q 12.5 m x 0.32 mm; ChromoPack and HP-Plot-Q 30 m x 0.53 mm; Agilent Technologies Inc.), where N_2O was separated from residual CO_2. The outflow from the GC columns entered the isotope ratio mass spectrometer used in continuous-flow mode to measure the m/z = 45/44 and 46/44 ratios of analyte N_2O.

The samples were measured against a laboratory standard (20 ppm N_2O) and each batch of samples included replicate analyses of three international nitrate reference materials: IAEA-NO-3 ($\delta^{15}N = 4.7 \pm 0.1‰$, $\delta^{18}O = 25.61 \pm 0.2‰$), USGS-34 ($\delta^{15}N = -1.8 \pm 0.1‰$, $\delta^{18}O = -27.93 \pm 0.3‰$) and USGS-35 ($\delta^{15}N = 2.7 \pm 0.1‰$, $\delta^{18}O = 57.50 \pm 0.2‰$) (Böhlke et al. 2003). Data were processed using Callisto software (Sercon, Crewe, UK) and corrected for drift, blank and oxygen isotopic exchange with the parent water oxygen (Sigman et al. 2001, Casciotti et al. 2002, Böhlke et al. 2003, Kaiser et al. 2007). The precision of the analyses was generally better than 0.3‰ for both ^{15}N and ^{18}O measurements, based on the quadruplicate analyses of the three international standard materials. Isotope ratios were reported with respect to the international standards: Vienna Standard Mean Ocean Water (VSMOW) for $\delta^{18}O$ and atmospheric nitrogen (air) – for $\delta^{15}N$.

2.2.5. Statistical analyses

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). All data were checked for normality and homogeneity of variances. Data were normally distributed unless stated otherwise. One-way analysis of variance (ANOVA) compares the mean scores of three or more groups on a given variable. ANOVA was applied to study the difference among three fertiliser treatments on the basis of: i) the mean $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce leaves; ii) the mean $\delta^{15}N_{bulk}$ values of lettuce roots ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of roots from three fertiliser treatments were not subjected to the comparison due to the lack of replicate samples as explained in section 2.2.2); iii) the mean $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of substrates (day 80). ANOVA was also applied to test the difference in the mean $\delta^{15}N_{bulk}$ values among lettuce leaves, roots and substrates. Differences in the mean values were evaluated for significance with Tukey and Games-Howell *post hoc* comparisons; differences were considered to be significant at $p < 0.05$.

Independent sample t-test compares the mean scores of two groups on a given variable. The test was applied to study the difference i) between two application rates of fertilisers on the basis of $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuces and substrates; ii) between lettuce leaves and substrates on the basis of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values. Differences were considered to be significant at $p < 0.05$.

2.3. Results

2.3.1. Stable isotope composition of fertilisers

Two synthetic (KNO_3 and urea) and one organic fertiliser (chicken manure) were used in the greenhouse pot experiment. Table 2.1 shows the bulk nitrogen isotope composition ($\delta^{15}N_{bulk}$) and the nitrogen and oxygen isotope composition of nitrate ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3,}$ respectively) in the three fertilisers. Chicken manure has the highest $\delta^{15}N_{bulk}$ value (+4.5‰), whereas the $\delta^{15}N_{bulk}$ value of urea is the lowest (-4.5‰). The $\delta^{15}N_{bulk}$ value of KNO_3 is 0.0‰ (Table 2.1).

Table 2.1. Stable nitrogen and oxygen isotope composition of the fertilisers used in the lettuce pot experiment.

Fertiliser	$\delta^{15}N_{bulk}$	$\delta^{15}N_{NO3}$	$\delta^{18}O_{NO3}$
KNO_3	0.0	-0.2	+20.7
Urea	-4.5	n.a.	n.a.
Chicken manure	+4.5	+0.7	+16.2

n.a. (not applicable) indicates that the stable isotope composition of nitrate could not be determined due to the absence of nitrate in the fertiliser.

The nitrogen and oxygen isotope composition of nitrate also differs between synthetic and organic fertilisers. The $\delta^{15}N_{NO3}$ of KNO_3 fertiliser is lower than that of chicken manure: -0.2‰ and +0.7‰, respectively. In contrast, the $\delta^{18}O_{NO3}$ value of KNO_3 is higher compared to that of manure: +20.7‰ and +16.2‰, respectively (Table 2.1). The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of urea could not be determined due to the absence of nitrate in this fertiliser.

2.3.2. Stable isotope composition of substrates

Bulk nitrogen isotope composition as well as oxygen and nitrogen isotope values of nitrate in substrates treated with different fertilisers at the start of the experiment (day 0) are shown in Table 2.2. The $\delta^{15}N_{bulk}$ value of the initial substrate with no added fertiliser is -1.4 ‰. The $\delta^{15}N_{bulk}$ values of substrates with added fertilisers are very similar at both low (50 mg N/kg substrate) and high (150 mg N/kg substrate) application rates of a particular fertiliser. Substrates with added urea have the lowest $\delta^{15}N_{bulk}$ values (mean $\delta^{15}N_{bulk}$ of two application rates = -2.2‰), while the

highest $\delta^{15}N_{bulk}$ values are in substrates treated with chicken manure ($\delta^{15}N_{bulk}$ = -0.1‰). The $\delta^{15}N_{bulk}$ of substrates with added KNO_3 is -1.5‰ (Table 2.2).

The $\delta^{15}N_{NO3}$ value of the initial substrate with no added fertiliser at the beginning of the experiment is +8.7 ‰. Substrates treated with chicken manure have the highest $\delta^{15}N_{NO3}$ values (mean $\delta^{15}N_{NO3}$ of two application rates = +9.5‰). The mean $\delta^{15}N_{NO3}$ values of substrates with added KNO_3 and urea are +4.0‰ and +5.7‰, respectively.

Table 2.2. Stable nitrogen and oxygen isotope composition of substrates treated with KNO_3, urea and chicken manure at the start of the lettuce pot experiment.

Applied fertiliser	Fertiliser application rate, mg N/kg substrate	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
No fertiliser	0	-1.4	+8.7	+22.6
KNO_3	50	-1.5	+4.4	+20.1
	150	-1.5	+3.5	+19.9
	Mean	**-1.5**	**+4.0**	**+20.0**
Urea	50	-2.1	+5.2	+20.4
	150	-2.2	+6.1	+21.9
	Mean	**-2.2**	**+5.7**	**+21.2**
Chicken manure	50	-0.1	+9.1	+18.4
	150	-0.1	+9.9	+21.2
	Mean	**-0.1**	**+9.5**	**+19.8**

The $\delta^{18}O_{NO3}$ value of the initial substrate with no added fertiliser at the beginning of the experiment (day 0) is +22.6 ‰. Substrates treated with chicken manure have the lowest mean $\delta^{18}O_{NO3}$ of two application rates (+19.8‰). The mean $\delta^{18}O_{NO3}$ values of substrates treated with KNO_3 and urea are +20.0‰ and +21.2‰, respectively (Table 2.2).

Table 2.3 shows the mean $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of substrates after lettuce cultivation at the end of the experiment (day 80). The stable isotope values of the individual samples are included in the Appendix A (Table A2). The mean $\delta^{15}N_{bulk}$ values of substrates treated with synthetic fertilisers are significantly lower ($p<0.05$) compared to $\delta^{15}N_{bulk}$ values of substrates with chicken manure (Table 2.3). The mean $\delta^{15}N_{bulk}$ values are -2.6‰, -2.6‰ and -1.9‰, for KNO_3, urea and manure treatments, respectively (values are the average of two fertiliser application rates). No significant differences ($p>0.05$) in the $\delta^{15}N_{bulk}$ values of substrates are observed between two application rates of three fertilisers (Table 2.3).

The difference between the $\delta^{15}N_{NO3}$ values of substrates treated with synthetic fertilisers and chicken manure is not significant ($p>0.05$) at the end of the experiment. However, the $\delta^{18}O_{NO3}$ values of substrates amended with synthetic fertilisers differ significantly ($p<0.05$) from those containing chicken manure (Table 2.3). The $\delta^{18}O_{NO3}$ values of substrates with chicken manure (mean $\delta^{18}O_{NO3}$ of two fertiliser application rates = +17.9‰) are significantly lower ($p<0.05$) compared to those of substrates with KNO_3 and urea treatment (mean $\delta^{18}O_{NO3}$ of two fertiliser application rates = +31.7‰ and +37.8‰, respectively).

Table 2.3. Stable nitrogen and oxygen isotope composition of substrates treated with different fertilisers at the end of the lettuce pot experiment.

Applied fertiliser	Fertiliser application rate, mg N/kg substrate	$\delta^{15}N_{bulk}$, ‰		$\delta^{15}N_{NO3}$, ‰		$\delta^{18}O_{NO3}$, ‰	
		Mean (n=5)	SD	Mean (n=5)	SD	Mean (n=5)	SD
KNO_3	50	-2.6a	0.1	7.6*	2.7	27.4a*	4.0
	150	-2.6b	0.3	15.0a*	4.9	35.9b*	3.1
Urea	50	-2.5c	0.3	6.9	2.7	45.0a*	3.4
	150	-2.7d	0.4	5.1a	2.4	30.5c*	3.1
Chicken manure	50	-2.2a,c	0.2	5.8	2.2	18.8a	1.2
	150	-1.5b,d	0.4	9.5	7.8	16.9b,c	5.5

Different letters denote significant difference ($p<0.05$) at one fertiliser application rate within a column. * - significant difference ($p<0.05$) in the mean values between two fertiliser application rates. SD - standard deviation (n=5).

Significant difference ($p<0.05$) between two fertiliser application rates is found in the $\delta^{18}O_{NO3}$ values of substrates treated with KNO_3 and urea. The $\delta^{18}O_{NO3}$ values of substrates are lower at the lower KNO_3 application rate (50 mg N/kg substrate) than those at the higher application rate (150 mg N/kg substrate). The $\delta^{18}O_{NO3}$ values of substrates treated with urea are higher at the lower application rate (50 mg N/kg substrate) compared to the higher application rate.

2.3.3. Stable isotope composition of lettuce

The nitrogen isotope compositions of bulk lettuce leaves and roots are shown in Table 2.4 and in Figure 2.2. The $\delta^{15}N_{bulk}$ values of the individual samples are included in the Appendix A (Tables A3-A4). The $\delta^{15}N$ values of lettuce leaves grown with chicken manure are significantly higher ($p<0.05$) than those treated with KNO_3

(difference is significant for both fertiliser application rates) and with urea (the difference is significant at the application rate of 150 mg N/kg substrate) (Table 2.4). Similarly to lettuce leaves, the $\delta^{15}N_{bulk}$ values of lettuce roots from chicken manure treatment are significantly higher ($p<0.05$) compared to those from either KNO_3 or urea treatment at both fertiliser application rates (Table 2.4).

Table 2.4. The nitrogen isotope composition of bulk lettuce leaves and roots from different fertiliser treatments.

Applied fertiliser	Fertiliser application rate mg N/kg substrate	$\delta^{15}N_{bulk}$, ‰ Leaves		$\delta^{15}N_{bulk}$, ‰ Roots	
		Mean (n=5)	SD	Mean (n=5)	SD
KNO_3	50	+4.8a,x*	0.4	+1.6a,x	0.6
	150	+2.4b*	0.5	+0.7b	2.0
Urea	50	+5.4x*	1.7	+0.5a,x*	0.2
	150	+2.2c,x*	0.3	+0.2c,x*	0.2
Chicken manure	50	+8.1a,x*	0.3	+4.2a,x	0.6
	150	+5.9b,c,x*	1.3	+3.9b,c,x	0.7

Different letters denote significant difference ($p<0.05$) at one fertiliser application rate within a column (a,b,c) or within a row (x). * - significant difference ($p<0.05$) in the mean values between two fertiliser application rates. SD - standard deviation (n=5).

$\delta^{15}N_{bulk}$ values of lettuce roots are lower compared to the values of leaves (Figure 2.2). The difference is significant ($p<0.05$) for all three fertiliser treatments at both fertiliser application rates (except for KNO_3, where the difference is significant only at the application rate of 50 mg N/kg substrate).

In all three fertiliser treatments, the $\delta^{15}N_{bulk}$ values of leaves and roots are higher when the fertiliser is applied at the lower rate (50 mg N/kg substrate) compared to the higher rate (150 mg N/kg substrate). The difference is significant ($p<0.05$) for all fertiliser treatments in lettuce leaves and for urea treatment – in lettuce roots (Table 2.4).

The $\delta^{15}N_{NO3}$ values of lettuce leaves and roots are shown in Table 2.5 and Figure 2.3 (A). The $\delta^{15}N_{NO3}$ values of the individual samples are shown in Tables A3 and A4 (Appendix A).

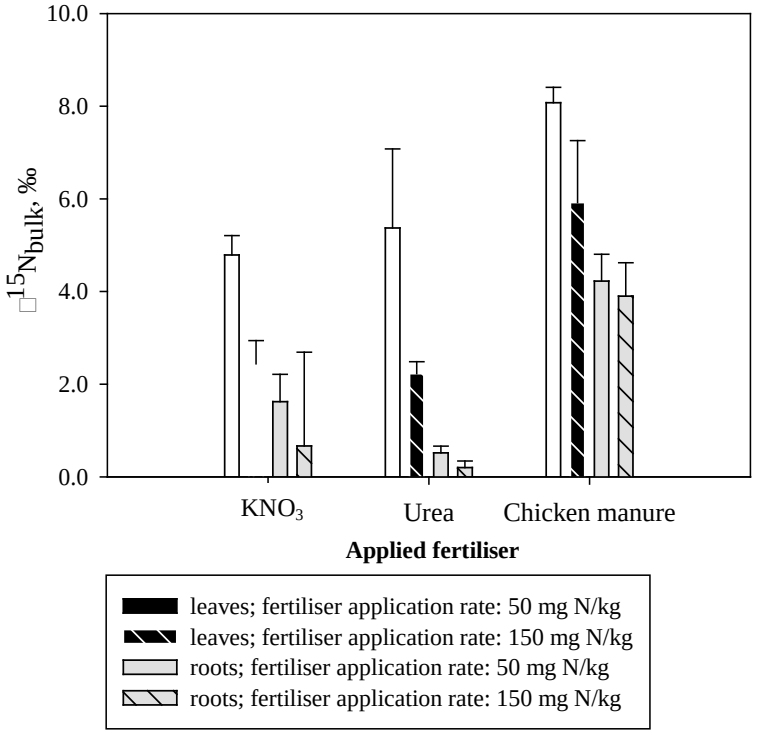

Figure 2.2. Bulk $\delta^{15}N$ values of lettuce leaves and roots from different fertiliser treatments. The error bars represent standard deviation values based on five replicate samples.

Similarly to the $\delta^{15}N_{bulk}$ values, the $\delta^{15}N_{NO3}$ values of lettuce leaves from chicken manure treatment are significantly higher ($p<0.05$) than those from either KNO_3 (in both low and high application rates) or urea (at application rate of 150 mg N/kg substrate) treatment (Table 2.5).

Table 2.5. The $\delta^{15}N_{NO3}$ values of lettuce leaves and roots from different fertiliser treatments.

Fertiliser applied	Application rate, mg N/kg substrate	$\delta^{15}N_{NO3}$, ‰ Leaves		Roots	
		Mean (n=5)	SD	Combined (n=1)	SD
KNO_3	50	+23.3a	1.1	+18.3	n.a.
	150	+21.9b	1.6	+19.5	n.a.
Urea	50	+25.5	6.8	+17.7	n.a.
	150	+20.1c	1.5	+18.3	n.a.
Chicken manure	50	+35.4a*	2.3	+19.6	n.a.
	150	+27.4b,c*	0.8	+19.5	n.a.

Different letters denote significant difference ($p<0.05$) at one fertiliser application rate within a column. * - significant difference ($p<0.05$) in the mean values between two fertiliser application rates. SD - standard deviation (n=5); n.a. – not applicable; SD was not calculated for roots samples as five replicates were combined into one (n=1) due to small amount of sample material.

The $\delta^{15}N_{NO3}$ values of lettuce leaves grown with chicken manure are higher by 12.5‰ and 5.7‰ than those of lettuce treated with KNO_3 at the application rates of 50 and 150 mg N/kg substrate, respectively, and higher by 10.3‰ and 7.5‰ than the $\delta^{15}N$ values of lettuce leaves grown with urea at the application rates of 50 and 150 mg N/kg substrate, respectively.

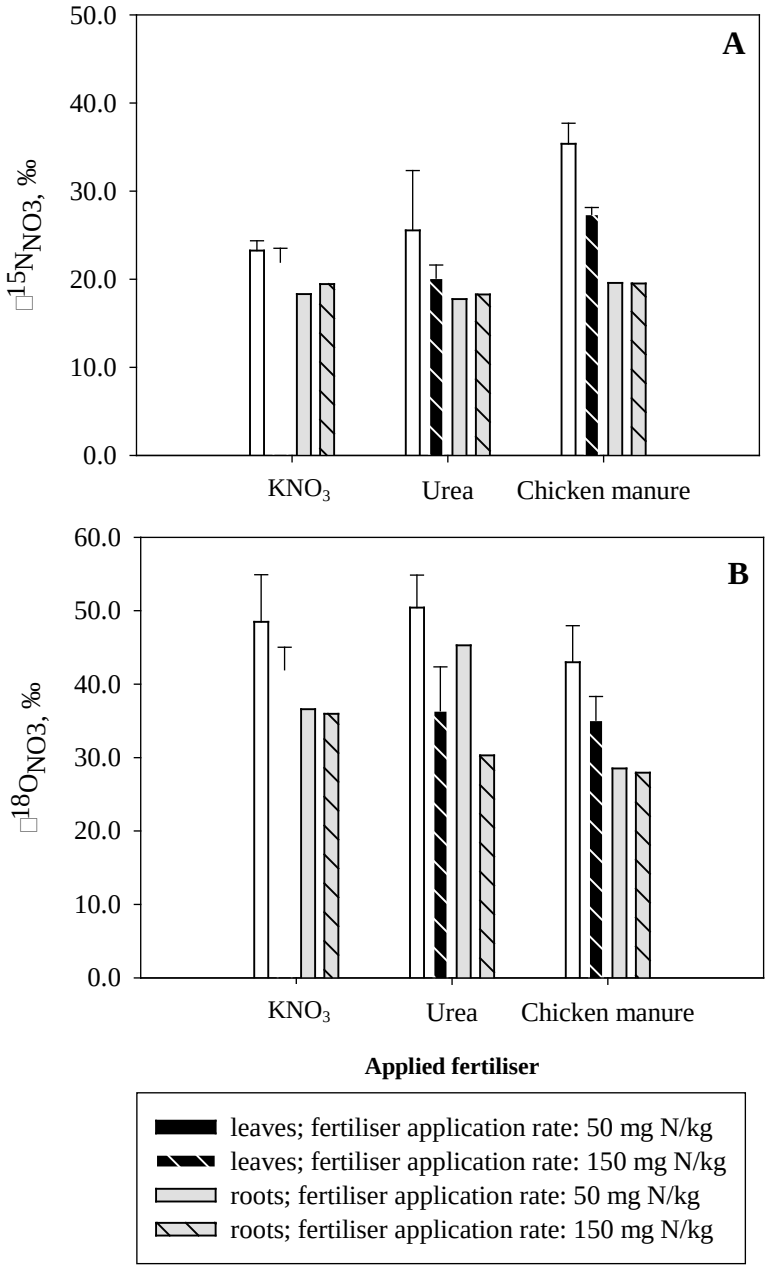

Figure 2.3. The $\delta^{15}N_{NO3}$ (A) and $\delta^{18}O_{NO3}$ (B) values of lettuce leaves and roots from different fertiliser treatments. The error bars represent standard deviation values based on five replicate samples.

The $\delta^{15}N_{NO3}$ values of lettuce leaves in all three fertiliser treatments are higher when the fertiliser is applied at the lower rate (50 mg N/kg substrate) compared to the higher rate (150 mg N/kg substrate); the difference is significant ($p<0.05$) for manure treatment.

Table 2.6 and Figure 2.3 (B) show the $\delta^{18}O_{NO3}$ values of lettuce leaves and roots at the end of the experiment. The $\delta^{18}O_{NO3}$ values of the individual samples are shown in Tables A3 and A4 (Appendix A). The $\delta^{18}O_{NO3}$ values of lettuces grown with chicken manure are lower than those of lettuces fertilised with synthetic N fertilisers. The difference is significant ($p<0.05$) between manure and KNO_3 treatment at the application rate of 150 mg N/kg substrate. The $\delta^{18}O_{NO3}$ values of lettuce leaves grown with chicken manure are lower by 5.5‰ and 6.9‰ than those grown using KNO_3 (application rate: 50 and 150 mg N/kg substrate, respectively), and lower by 7.4‰ and 1.3‰ than $\delta^{18}O_{NO3}$ values of lettuce leaves grown using urea (application rate: 50 and 150 mg N/kg substrate, respectively).

Table 2.6. The $\delta^{18}O_{NO3}$ values of lettuce leaves and roots from different fertiliser treatments.

Fertiliser applied	**Application rate, mg N/kg substrate**	**$\delta^{18}O_{NO3}$, ‰**			
		Leaves		**Roots**	
		Mean (n=5)	SD	Combined (n=1)	SD
KNO_3	50	+48.5	6.4	+36.6	n.a.
	150	+42.0a	3.1	+36.0	n.a.
Urea	50	+50.4*	4.4	+45.3	n.a.
	150	+36.4*	6.0	+30.3	n.a.
Chicken manure	50	+43.0*	5.0	+28.5	n.a.
	150	+35.1a*	3.2	+27.9	n.a.

Different letters denote significant difference ($p<0.05$) at one fertiliser application rate within a column. * - significant difference ($p<0.05$) in the mean values between two fertiliser application rates. SD - standard deviation (n=5); n.a. – not applicable. SD was not calculated for roots samples as five replicates were combined in one (n=1) due to small amount of sample material.

Similarly to leaves, lettuce roots from chicken manure treatment have lower $\delta^{18}O_{NO3}$ values compared to the roots treated with either KNO_3 or urea. For the equivalent amounts of nitrogen applied, lettuces grown with chicken manure have $\delta^{18}O_{NO3}$ values of roots that are lower by 8.1‰ than those of roots grown using KNO_3 (value is equal for both application rates), and lower by 16.8‰ and 2.4‰ than

$\delta^{18}O_{NO3}$ values of roots treated with urea (application rate: 50 and 150 mg N/kg substrate, respectively). The highest $\delta^{18}O_{NO3}$ values of lettuce leaves and roots among the three treatments are in urea treatment at both application rates.

In respect to differences between two fertiliser application rates, in all three fertiliser treatments the $\delta^{18}O_{NO3}$ values of leaves are higher when the fertiliser is applied at the lower rate (50 mg N/kg substrate) compared to the higher rate (150 mg N/kg substrate); the difference is significant ($p<0.05$) in the case of urea and chicken manure treatments.

2.4. Discussion

This study evaluated the effect of the synthetic and organic fertiliser treatments on the bulk nitrogen isotope composition of lettuce plants, and, for the first time, on the nitrogen and oxygen isotope composition of nitrate extracted from lettuce. Firstly, I discuss here the differences in the isotopic composition of the applied fertilisers. Then I address the differences observed in the isotopic composition of lettuces and substrates depending on the applied fertilisation as well as discuss the impact of the two different fertiliser application rates on the stable isotope composition of lettuce. Finally, based on the stable isotope values of substrates, lettuce leaves and roots, the intra-plant isotopic variation in lettuces is discussed.

2.4.1. Stable isotope composition of fertilisers

Three nitrogen fertilisers were used in the present experiment: KNO_3 and urea were selected as synthetic nitrogen sources that are widely used in conventional agricultural practise, and chicken manure was used as an organic fertiliser. The bulk $\delta^{15}N$ values of synthetic fertilisers (0.0‰ and -4.5‰ for KNO_3 and urea, respectively) are lower compared to the values of chicken manure (+4.5‰).

The major difference in the $\delta^{15}N_{bulk}$ values between synthetic fertilisers and chicken manure is caused by the nitrogen isotope values of their sources. Synthetic nitrogen fertilisers are derived from air nitrogen, which being an international standard, has the accepted $\delta^{15}N$ value of 0.0‰ (Mariotti 1983). Due to little fractionation involved in the production process of synthetic nitrogen fertilisers, their $\delta^{15}N_{bulk}$ values are generally close to that of air nitrogen (Vitoria et al. 2004, Bateman and Kelly 2007). The nitrogen isotope composition of bulk manure is the combination of nitrogen isotope values of a wide range of organic and inorganic compounds present in the manure, such as organic nitrogen compounds, ammonia, ammonium, hydroxylamine, nitrous and nitric oxide, nitrous and nitric acid, nitrite and nitrate ions (United States Department of Agriculture, Natural Resources Conservation Service 2007). The $\delta^{15}N$ values of animal manure are therefore, higher compared to those of synthetic nitrogen fertilisers. Moreover, preferential volatilization of ^{15}N-depleted ammonia from manure after its application to plants, results in further ^{15}N-enrichment of manure and increases the difference in the

$\delta^{15}N_{bulk}$ values between manure and synthetic fertilisers (Choi et al. 2003, Bateman and Kelly 2007).

The $\delta^{15}N_{bulk}$ values of fertilisers from the present study are in agreement with the nitrogen isotope data of fertilisers reported previously (Vitoria et al. 2004, Bateman and Kelly 2007). Vitoria et al. (2004) performed the multi-elemental stable isotope analysis of a variety of synthetic fertilisers and reported that the $\delta^{15}N$ values of bulk nitrate fertilisers (ammonium and calcium nitrates) were in the range from -0.7‰ to +3.9‰, while the $\delta^{15}N_{bulk}$ value of urea was lower (-1.1‰). Bateman and Kelly (2007) analysed a wide range of synthetic and organic fertilisers, including all three types of fertilisers used in the present study. The $\delta^{15}N_{bulk}$ values of KNO_3 fertilisers reported by Bateman and Kelly (2007) ranged from -1.5‰ to -1.0‰, the values of urea, similarly to the present findings, were generally lower (-5.9‰ to -0.8‰), while the $\delta^{15}N_{bulk}$ values of chicken manure pellets were significantly higher than those of synthetic fertilisers (+4.8‰ to +8.4‰), which is in accordance with the present findings.

The nitrogen isotope value of nitrate extracted from KNO_3 fertiliser is -0.2‰, which is close to that obtained from bulk ^{15}N analysis (0.0‰). The $\delta^{15}N_{NO3}$ of chicken manure is higher compared to that of KNO_3, which reflects the difference in the nitrogen isotope signature of sources of these two fertilisers, as has been discussed above in relation to the $\delta^{15}N_{bulk}$ values. The bulk nitrogen isotope value of manure is higher than its $\delta^{15}N_{NO3}$ value. This can be explained by the presence of other more ^{15}N-enriched compounds in the manure, such as ^{15}N-enriched ammonia and volatile amines, which shift the $\delta^{15}N$ of bulk manure to a more positive value.

On the other hand, the $\delta^{18}O_{NO3}$ value of chicken manure is more negative compared to the $\delta^{18}O_{NO3}$ of KNO_3 fertiliser (+16.2‰ and +20.7‰ for manure and KNO_3, respectively). This is due to the difference in the sources of nitrate oxygen atoms in these two types of fertilisers. The $\delta^{18}O_{NO3}$ of KNO_3 is higher than that of manure because all three oxygen atoms of synthetic nitrate are derived from atmospheric O_2 and thus, the $\delta^{18}O$ value of nitrate fertiliser is similar to $\delta^{18}O$ of air oxygen ($\delta^{18}O$ = +23.5‰; Kroopnick and Craig 1972). The $\delta^{18}O_{NO3}$ values of some synthetic fertilisers were reported by Vitoria et al. (2004). Authors found that the $\delta^{18}O_{NO3}$ values of synthetic ammonium nitrate and calcium nitrate were +25.1‰ and +22.1‰, respectively, which is in agreement with the results of the present study.

2.4.2. Bulk nitrogen isotope composition of substrates and lettuce plants treated with synthetic and organic fertilisers

The bulk nitrogen isotope composition of the applied fertiliser is reflected in the $\delta^{15}N_{bulk}$ values of substrates and lettuce plants. At the start of the experiment, the $\delta^{15}N_{bulk}$ value of the substrate amended with chicken manure is higher compared to that of substrates treated with either KNO_3 or urea (Table 2.2). The impact of the fertiliser on the $\delta^{15}N_{bulk}$ values of substrates is observed also at the end of the lettuce pot experiment – the $\delta^{15}N_{bulk}$ values of substrates treated with chicken manure are significantly higher ($p<0.05$) compared to those of substrates with added synthetic fertilisers (Table 2.3). Similar pattern is observed in the bulk $\delta^{15}N$ values of lettuce plants. Both leaves and roots of lettuces grown with chicken manure are significantly higher ($p<0.05$) than the $\delta^{15}N_{bulk}$ values of lettuces grown with either KNO_3 or urea. These differences in the $\delta^{15}N_{bulk}$ values of lettuces from synthetic and organic fertiliser treatments suggest a strong impact of the bulk nitrogen isotope composition of fertilisers on the $\delta^{15}N_{bulk}$ values of lettuce in the controlled greenhouse conditions.

Present results are in a good agreement with the findings of previous studies that determined the nitrogen isotope composition of greenhouse crops grown with different fertilisation strategies (e.g. Yoneyama et al. 1990, Nakano et al. 2003, Bateman et al. 2005, Yun et al. 2006, Del Amor et al. 2008, Šturm et al. 2011, Yuan et al. 2012, Zhou et al. 2012). For example, Yoneyama et al. (1990) reported variations in the $\delta^{15}N_{bulk}$ of several crop species (corn, spinach, potato, wheat and barley) grown with different fertilisers at a number of agricultural experimental stations across Japan. Their results showed that the $\delta^{15}N_{bulk}$ values of crops were generally lower when synthetic nitrogen fertilisers were applied and higher when animal manures were used. Nakano et al. (2003) conducted a greenhouse experiment in which tomato plants were cultivated using three different fertiliser treatments: synthetic ammonium nitrate ($\delta^{15}N = +0.8‰$), fertigation (drip irrigation of a nutrient-containing solution) with a synthetic nitrogen fertiliser ($\delta^{15}N = 0.0‰$), and fertigation with organic corn steep liquor ($\delta^{15}N = +8.5‰$). Authors reported that the $\delta^{15}N_{bulk}$ values of both substrates and tomato plants reflected the $\delta^{15}N_{bulk}$ values of the applied fertilisers, and were the highest in the case of organic fertiliser treatment. Yun et al. (2006) conducted a pot experiment to investigate whether N fertiliser source – composted manure ($\delta^{15}N = +16.4‰$) and urea ($\delta^{15}N = -0.7‰$) – would

impact the $\delta^{15}N_{bulk}$ values of Chinese cabbage plants. Authors reported that the $\delta^{15}N$ of bulk cabbage treated with compost was higher (> +9.0%) than that (< +1.0%) treated with urea, reflecting the effect of the isotopically different nitrogen sources. Šturm et al. (2011) tested whether $\delta^{15}N$ could be a potential marker for identifying the use of synthetic nitrogen fertilisation in lettuce plants. In their experiment pot-grown lettuce was treated with synthetic $CaNO_3$ ($\delta^{15}N$ = +5.7‰) and a commercial organic fertiliser ($\delta^{15}N$ = +14.8‰) in a single and split application. Authors found that the single application of organic fertiliser resulted in significantly higher $\delta^{15}N_{bulk}$ values of lettuce plants compared to synthetic fertiliser application. Zhou et al. (2012) reported that the application of organic compost and urea resulted in significant difference in the $\delta^{15}N_{bulk}$ values of substrates and tomato plants in the greenhouse conditions. Substrate treated with compost had the highest $\delta^{15}N$ values, whereas $\delta^{15}N$ values of substrates amended with either urea or compost-urea mixture were significantly lower.

Thus, data from the present experiment support the findings of other authors and confirm that under controlled conditions, where factors other than fertiliser type (light and temperature regimes, substrate characteristics, irrigation water) are kept the same, the $\delta^{15}N$ values of plants are significantly affected by the $\delta^{15}N$ values of the applied fertilisers, and are higher for plants fertilised with manure compared to plants treated with synthetic fertilisers.

2.4.3. The nitrogen and oxygen isotope composition of nitrate from substrates and lettuce plants treated with synthetic and organic fertilisers

Similarly to the bulk nitrogen isotope composition, the $\delta^{15}N_{NO3}$ values of substrates at the start of the experiment are higher for manure treatment compared to the treatments with synthetic KNO_3 or urea (Table 2.2). This is also reflected in the $\delta^{15}N_{NO3}$ values of lettuce leaves and roots, which are also higher in manure treatment compared to synthetic fertiliser treatments (Figure 2.3 (A) and Table 2.5).

The main goal of the compound-specific isotope analysis of nitrate, however, was to evaluate the capability of the $^{18}O_{NO3}$ composition to distinguish different fertilisation regimes. The $\delta^{18}O_{NO3}$ values of substrates and lettuce plants, in contrast with $\delta^{15}N_{NO3}$ values, are higher in the case of synthetic fertiliser applications and lower for chicken manure treatment. At the end of the experiment, the $\delta^{18}O_{NO3}$ values

of substrates with added KNO_3 or urea are significantly higher ($p<0.05$) in both fertiliser application rates than those of substrates treated with chicken manure (Table 2.3). The effect of the fertilisation regime is also observed in the $\delta^{18}O_{NO3}$ values of lettuce plants. Both leaves and roots of lettuce grown with synthetic KNO_3 or urea are higher than those from chicken manure treatment, and the difference is significant ($p<0.05$) between manure and KNO_3 treatment at the application rate of 150 mg N/kg substrate.

Thus, the compound-specific isotope analysis of nitrate reveals differences between synthetic and organic fertilisation regimes. The observed difference in the $\delta^{18}O_{NO3}$ values of substrates and lettuce plants treated with synthetic KNO_3 and chicken manure reflects the differences in the oxygen sources of these fertilisers. When manure is applied, nitrification process results in more ^{18}O-depleted nitrate compared to the nitrate from synthetic KNO_3. This is because oxygen in synthetic nitrate originates mainly from air (with $\delta^{18}O$ = +23.5‰, Kroopnick and Craig 1972), whereas during the process of nitrification of manure oxygen is taken from both air and substrate water (Kumar et al. 1983, Andersson and Hooper 1983, Hollocher 1984). The $\delta^{18}O$ of the ultrapure water from Norwich, which has been used for the irrigation of lettuce plants throughout the greenhouse experiment, is -7.7‰. This is much lower compared to the $\delta^{18}O$ of air, and thus shifts the $\delta^{18}O$ of nitrate microbially produced from manure towards more negative values.

The present experiment shows that for both fertiliser application rates $\delta^{18}O_{NO3}$ values of lettuce treated with manure are lower compared to plants treated with KNO_3. $^{18}O/^{16}O$ ratio analysis of nitrate has a potential for the differentiation between plants grown with synthetic nitrate and organic fertilisation regimes. The approach may be less useful for differentiating plants treated with synthetic fertilisers other than nitrates, such as urea and various ammonium containing fertilisers, because they contain no synthetic nitrate. In the present experiment, the $\delta^{18}O_{NO3}$ values of lettuce leaves treated with urea are not significantly different from values of organically grown lettuce. Nitrogen from urea has to be first converted to plant available N form (mainly NO_3^-), and therefore NO_3^- in both synthetic urea and organic manure treatments could have mainly been produced during nitrification in soil and subsequently fractionated during assimilation by lettuce plants, which resulted in less distinct $\delta^{18}O_{NO3}$ values of lettuces between chicken manure and urea treatments.

This is the first time when $\delta^{18}O$ analysis of plant derived nitrate has been applied to study the difference between plants treated with different fertilisers. Further analysis of a wider range of plant species from both greenhouse and field experiments and with a wider range of organic and synthetic nitrate fertilisers are needed to evaluate the discriminative potential and robustness of the approach for the differentiation between plants from conventional and organic agricultural systems.

2.4.4. The impact of the fertiliser application rate on the stable isotope composition of lettuce

In this study fertilisers were applied at two different rates: 50 mg N/kg substrate and 150 mg N/kg substrate. A consistent difference in the $\delta^{15}N_{bulk}$ values of lettuce leaves and roots has been observed between two different application rates of each fertiliser. In all three fertiliser treatments, the $\delta^{15}N_{bulk}$ values of lettuce leaves are significantly higher ($p<0.05$) when the fertiliser is applied at the lower rate (50 mg N/kg substrate) compared to the higher application rate (150 mg N/kg substrate) (Table 2.4 and Figure 2.2). Similar differences between two application rates of fertilisers are observed in the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce. The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce leaves are higher at the lower fertiliser application rate compared to the higher application rate (Tables 2.5 and 2.6, Figure 2.3).

Thus, a strong impact of the fertiliser application rate on the $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce has been observed in the present study. Observed differences in the stable isotope values of lettuce between two fertiliser application rates may be explained by a greater discrimination against ^{15}N and ^{18}O when lettuces were grown at a higher fertiliser rate (150 mg N/kg substrate) compared to the lettuces grown at a lower fertiliser concentration. This is in agreement with the findings of several other studies (Shearer and Legg 1975, Mariotti et al. 1982, Yoneyama and Koneko 1989, Bateman et al. 2005) that report a positive correlation between the rate of the discrimination against ^{15}N and the amount of fertiliser in the growth medium. For example, Shearer and Legg (1975) measured the $\delta^{15}N_{bulk}$ values in wheat plants grown on experimental plots at five locations in Pennsylvania and treated with synthetic fertiliser at various application rates (from 0 to 168 kg N/ha as $(NH_4)_2SO_4$). Their results showed decreasing $\delta^{15}N_{bulk}$ values of wheat with increasing amounts of nitrogen applied. Yoneyama and Koneko (1989) reported a decrease in

the $\delta^{15}N$ values of nitrate by 1.6 and 3.7 ‰ for leaves and petioles of kamatsuna plants, respectively, when the concentration of the applied nitrate was increased from 0.2 mM to 12.0 mM.

Thus, a significant isotope discrimination against ^{15}N and ^{18}O is observed when fertilisers are in plentiful amounts for the plant uptake, however smaller discrimination against ^{15}N and ^{18}O occurs in the case of limited fertiliser amounts. This is important and has to be taken into account when differentiating between organic and conventional crops cultivated under different fertilisation strategies. For example, if conventional crops fertilised with low fertiliser application rates are compared to organic samples with high fertiliser application rates, the difference between two agricultural practises can be less significant than in the case when equal fertilisation rates are applied to both type of crop samples.

2.4.5. Intra-plant isotopic variation in lettuce plants

In addition to stable isotope analysis of lettuce leaves, the analysis of lettuce roots was also performed in the present study. An intra-plant isotopic variation has been observed between lettuce roots and leaves. Figure 2.4 illustrates the difference in the $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values among lettuce leaves, roots and substrates for each fertiliser treatment. Stable isotope data show similar trends at both fertiliser application rates, and are shown here only for the lower rate (50 mg N/kg substrate); stable isotope data for the fertiliser application rate of 150 mg N/kg substrate are shown in Figure A2 (Appendix A).

The results of the bulk nitrogen isotope analysis show that in all three fertiliser treatments there is a consistent ^{15}N-enrichment of lettuce roots compared to substrates, and further ^{15}N-enrichment of lettuce leaves compared to substrates and roots. Similar trend is observed for the nitrogen and oxygen isotope composition of nitrate, with nitrate being more ^{15}N- and ^{18}O-enriched in lettuce leaves compared to lettuce roots and substrates (Figure 2.4).

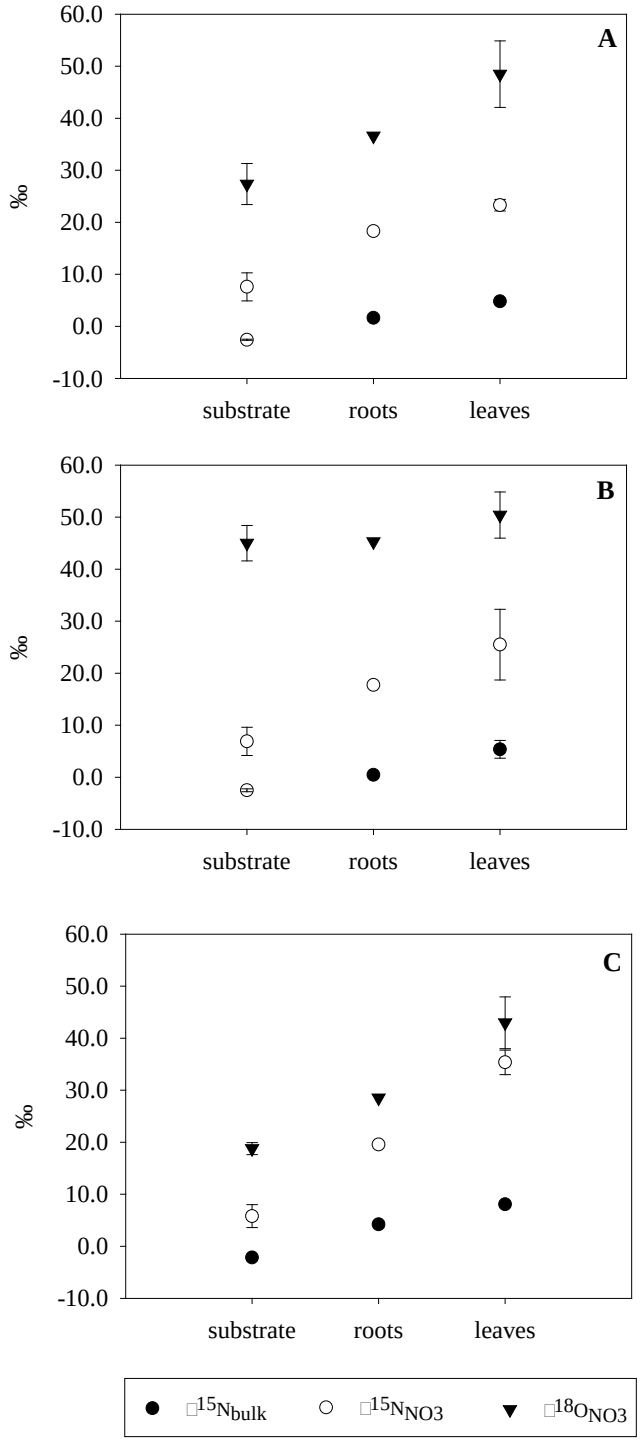

Figure 2.4. Stable nitrogen and oxygen isotope composition of lettuce leaves, roots and substrates treated with KNO_3 (A), urea (B) and chicken manure (C) at the application rate of 50 mg N/kg substrate. Values are the means of five replicate samples. The error bars represent standard deviations based on five replicate samples.

The observed isotopic variation likely results from nitrogen and oxygen isotope fractionations that occur during the assimilation of nitrate in roots and leaves

of lettuce. Lettuce plants take up nitrogen from the soil solution, mainly in the form of nitrate, and assimilate it into organic compounds in both roots and leaves (Hufton et al. 1996). Nitrate assimilation occurs by the nitrate reductase-nitrite reductase pathway, and is associated with a significant fractionation, with the reported discrimination against ^{15}N being on average 15‰ (Handley and Raven 1992, Yoneyama et al. 1993). As discussed by Mariotti et al. (1982), during nitrate assimilation some fraction of nitrate remains unassimilated, and due to the preferential use of ^{14}N during assimilation, this unassimilated nitrate becomes significantly ^{15}N-enriched. Therefore, the observed higher $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of roots compared to the substrate can arise due to fractionations during assimilation of nitrate in roots, which would isotopically enrich the remaining nitrate (which is unassimilated and available for extraction and analysis) and result in the elevated values of roots compared to the substrates.On the other hand, nitrate, which is not assimilated in roots, can be transported further to the assimilation sites in leaves (Yoneyama and Kaneko 1989, Evans et al. 1996). This would result in further isotopic enrichment of nitrate remaining in leaves and available for the extraction, and can account for the higher $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of leaves in comparison to roots and substrate, as observed in the present study.

Results of the present study are in agreement with the findings reported in several other studies, which addressed the intra-plant isotopic variation in plants in respect to bulk nitrogen isotope composition (Yoneyama and Kaneko 1989, Evans et al. 1996). Yoneyama and Kaneko (1989) analyzed nitrogen isotope composition of the komatsuna plants and observed that $\delta^{15}N_{bulk}$ values of komatsuna leaf blades were up to 7.8‰ enriched compared to roots. Evans et al. (1996) reported that the $\delta^{15}N_{bulk}$ values of tomato leaves were up to 5.8‰ higher than those of roots in controlled hydroponic experiment.

The intra-plant isotopic variation illustrates that differences in the stable isotope values can be present among different organs of the same plant species. This has to be taken into account when applying bulk nitrogen and compound-specific oxygen isotope analysis to crops, edible parts of which represent different plant organs.

2.5. Conclusions

A strong impact of the nitrogen isotope composition of bulk fertilisers on the $\delta^{15}N_{bulk}$ values of lettuces under controlled greenhouse conditions has been observed in the present study. Chicken manure ($\delta^{15}N_{bulk}$ = +4.5‰) is more ^{15}N-enriched compared to synthetic KNO_3 and urea fertilisers ($\delta^{15}N_{bulk}$ = 0.0‰ and -4.5‰, respectively), which is due to the difference in the nitrogen isotope composition of the synthetic and organic fertiliser sources. The difference in the bulk nitrogen isotope composition among synthetic and organic fertilisers is reflected in the nitrogen isotope composition of bulk substrates and lettuce plants. Both leaves and roots of lettuces grown with chicken manure have significantly higher ($p<0.05$) $\delta^{15}N_{bulk}$ values compared to lettuces grown with either KNO_3 or urea.

In this study, for the first time, compound-specific nitrogen and oxygen isotope analysis of nitrate has been applied to study the differences between plants grown with different fertilisation regimes. Similarly to the results of the bulk $\delta^{15}N$ analysis, compound specific isotope analysis of nitrate reveals differences between synthetic and organic fertilisation. The $\delta^{15}N_{NO3}$ value of chicken manure is higher than that of KNO_3 (+0.7‰ and -0.2‰, respectively). The $\delta^{15}N_{NO3}$ values of lettuce leaves and roots are also higher in manure treatment compared to synthetic fertiliser treatments.

In contrast, the $\delta^{18}O_{NO3}$ value of chicken manure (+16.2‰) is more negative compared to the $\delta^{18}O_{NO3}$ of synthetic KNO_3 fertiliser (+20.7‰), which is a result of different sources of oxygen atoms (air oxygen versus soil water oxygen) in synthetic and organic nitrates and due to different oxygen isotope compositions of these sources. The effect of the fertilisation regime is further observed in the $\delta^{18}O_{NO3}$ values of lettuce plants. Both leaves and roots of lettuce grown with synthetic KNO_3 or urea are higher than those from chicken manure treatment, and the difference is significant ($p<0.05$) between manure and KNO_3 treatment at the application rate of 150 mg N/kg substrate.

A strong effect of the fertiliser application rate on the isotopic composition of lettuce plants is observed in this study. In all three fertiliser treatments $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuces are higher when the fertiliser is applied at the lower rate (50 mg N/kg substrate) compared to the higher application rate (150 mg N/kg substrate), which indicates that a greater discrimination against ^{15}N and ^{18}O occurs when fertiliser concentration in the substrate is high.

In all three fertiliser treatments, there has been observed an intra-plant isotopic variation with a consistent increase in the $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce leaves compared to those of roots and substrates, which can result from nitrogen and oxygen isotope fractionations that occur during the assimilation of nitrogen fertiliser in roots and leaves of lettuce.

The results of the present study suggest that $^{18}O/^{16}O$ analysis of plant derived nitrate has a potential for differentiation between plants grown with synthetic nitrate fertilisers and manure. Further analysis of a greater number of plant species from both greenhouse and field experiments and with different fertilisation strategies are needed to establish the scope of application and robustness of the compound-specific approach.

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CHAPTER 3

DIFFERENTIATION BETWEEN ORGANIC AND CONVENTIONAL FIELD GROWN AND RETAIL VEGETABLES USING BULK AND COMPOUND-SPECIFIC STABLE ISOTOPE ANALYSIS

Abstract

Organic food market has been rapidly growing over the past decade. Increasing demand for organically grown food, the introduction of certification and premium prices of organic goods have created a financial enticement to mislabel and try to pass off cheaper conventional products as organic. A number of severe fraud cases in organic food sector have been recently reported. Authentication of organic food products is currently strained due to the lack of specific markers for organic food products. Search for the novel markers and the development of new analytical approaches to distinguish organic and conventional products are urgently needed for authentication purposes.

This study focused on the analyses of total nitrogen (TN) content, nitrogen isotope composition of bulk vegetable matter ($\delta^{15}N_{bulk}$) as well as, for the first time, nitrogen and oxygen isotope composition of vegetable-derived nitrate ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) for the discrimination between vegetables grown in the field in three different agricultural systems: conventional with the application of synthetic NPK fertilisers, organic with the application of animal manure (OA system) and organic with the use of cover crops (OB system). The discriminative power of the compound-specific oxygen isotope analysis of nitrate was further evaluated in retail conventional and organic tomato and potato samples from different geographical locations.

Organic potatoes, cabbages and onions from OA system have significantly higher ($p<0.05$) $\delta^{15}N_{bulk}$ values compared to the corresponding samples from conventional system, and reflect the difference in the nitrogen isotope composition of the organic and synthetic NPK fertilisers applied in the field. In contrast, significant difference ($p<0.05$) in the $\delta^{15}N_{bulk}$ values between conventional and organic OB system is observed only in onions, thus revealing the limitations of the bulk nitrogen

isotope composition in differentiating between agricultural systems with synthetic fertilisation versus the application of cover crops.

Stable oxygen isotope analysis of vegetable derived nitrate has introduced a significant improvement to the discrimination between conventional and organic potatoes. The $\delta^{18}O_{NO3}$ values of conventional potatoes are significantly higher ($p<0.05$) compared to those from OA and OB systems. Canonical discriminant analysis (CDA) of potatoes with TN, $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ used as predictor variables results in 86.1% of cross-validated potato samples being correctly classified and shows that $\delta^{18}O_{NO3}$ values have the greatest discriminative power. Similarly, the $\delta^{18}O_{NO3}$ values of retail conventional potatoes and tomatoes are significantly higher ($p<0.05$) compared to organic counterparts. The CDA of retail potato samples with $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ used as predictor variables results in 84.8% of correctly classified samples.

Probability densities of the normal distribution curves of $\delta^{18}O_{NO3}$ values for the expected entire organic and conventional potato populations have been generated and suggest that compound-specific oxygen isotope approach can be a useful tool for the discrimination between organic and conventional potatoes; low $\delta^{18}O_{NO3}$ values (<13‰) are typical of organic samples, while high $\delta^{18}O_{NO3}$ values (>24‰) are more typical of conventional potato samples.

3.1. Introduction

3.1.1. The application of stable isotope analyses for authentication of organic vegetables

Organic food market has been rapidly growing over the past decade (Willer and Kilcher 2012). Market prices of organically grown food are higher compared to those of conventional produce. An average price difference between organic and conventional products is in the range from 20 to 100% (e.g. Lin et al. 2008, Willer and Kilcher 2012), however depending on the product type price premium for organic foods can be much higher, sometimes being more than 200% (Kenanoğlu and Karahan 2002, Oberholtzer et al. 2005). This difference in prices and the lack of reliable markers for the authentication of organic products makes organic market highly susceptible to attempted fraud (Beck 2012).

Numerous attempts have been made to find specific compounds that could be used as markers for organically grown crops, however so far no specific and individual markers for organic production have been identified (Woese et al. 1997, Bourn and Prescott 2002, Hoefkens et al. 2009). This is presumably due to the natural variation in the chemical composition of organically produced food of plant origin caused by various factors, such as plant species, soil fertility, climate, fertilization strategy as well as pest and weed control (Tilman et al. 2002, Pretty 2008). In the recent past, the analytical controls performed on organic crops involved the search for pesticide residues. A number of studies showed that the content of pesticide residues is systematically lower in organic than conventional plants (Woese et al. 1997, Bourn and Prescott 2002, Hoefkens et al. 2009). However, random pesticide residue testing of organic plant products cannot ensure correct organic production practice as often only a limited amount of pesticide residues are tested compared to the large range of possible contaminants. Furthermore, pesticide residue contents are frequently below the analytical limit of detection, even in conventional plant products (Hoefkens et al. 2009). Studies on the nutrient content of organically and conventionally grown crops have reported contradicting and not consistent results. Systematic review by Dangour et al. (2009) indicated that there were no significant differences in the chemical composition between foods derived from organic and conventional growing systems, except that conventionally produced crops had significantly higher concentration of nitrogen, and organically produced

crops had significantly higher concentration of phosphorus and higher titratable acidity. Similar findings were reported earlier in reviews by Woese et al. (1997) and Bourn and Prescott (2002). While there are reports indicating that organic and conventional fruits and vegetables may differ on a variety of sensory qualities and vitamin content, the findings are inconsistent (Bourn and Prescott 2002).

Stable isotope analysis has been reported as one of the most promising analytical techniques for the discrimination between organic and conventional crops (Camin et al. 2007). The technique allows determining isotopic fingerprint of plants by tracing isotope fractionation processes that occur continuously in biological systems, including agricultural systems. Stable nitrogen isotope analysis has been most widely applied to study the differences between organically and conventionally grown crops (e.g. Bateman et al. 2007, Camin et al. 2007, Rapisarda et al. 2010, Camin et al. 2011, Flores et al. 2011, Šturm and Lojen 2011, Zhou et al. 2012). The nitrogen isotope approach relies on the differences in the nitrogen composition of fertilisers in conventional and organic systems, which is expected to be further reflected in the nitrogen isotope composition of crops (Choi et al. 2003, Bateman et al 2007).

Over the past decade, various studies applied bulk stable nitrogen isotope analysis to discriminate between crops grown in organic and conventional agricultural systems, with the majority of analyses performed on vegetables (e.g. Bateman et al. 2005, Georgi et al. 2005, Yun et al. 2006, Flores et al. 2007, Lim et al. 2007, del Amor and Navarro 2008, Flores et al. 2011, Šturm et al. 2011, Zhou et al. 2012, Yuan et al. 2012), cereals (Schmidt et al. 2005, Choi et al. 2006, Suzuki et al. 2009) and fruits (Rapisarda et al. 2005, Rapisarda et al. 2010, Camin et al. 2011). These studies mainly compared conventional crops treated with synthetic nitrogen fertilisers and organic crops grown with organic manures or compost, and reported that organically grown plants were generally characterised by higher $\delta^{15}N$ values.

Greater differences in the nitrogen isotope composition of organically and conventionally grown crops have been observed in controlled greenhouse conditions, however the analyses of the field grown and commercial crop samples have revealed that nitrogen isotope approach could not always provide sufficient discrimination between two growing systems (Bateman et al. 2007, Camin et al. 2007, Rogers 2008, Camin et al. 2011). Despite the $\delta^{15}N$ values of organic crops from the field trials in most cases being higher compared to those of conventional counterparts, results

showed overlapping $\delta^{15}N$ values of conventional and organic samples, which did not allow the complete discrimination of two groups.

Figure 3.1 shows the $\delta^{15}N$ data of certain retail organic and conventional vegetables and fruits summarized from the most recent studies. An overlap in the $\delta^{15}N$ values of conventional and organic samples can be seen in many of the analysed crops (lettuces, carrots, peppers, cauliflower, parsley, garlic, onion, oranges and clementines), suggesting that the discriminative power of the method varies greatly with the growing conditions as well as with the plant species (Bateman et al. 2007, Rogers 2008, Kelly and Bateman 2010, Šturm and Lohen 2011).

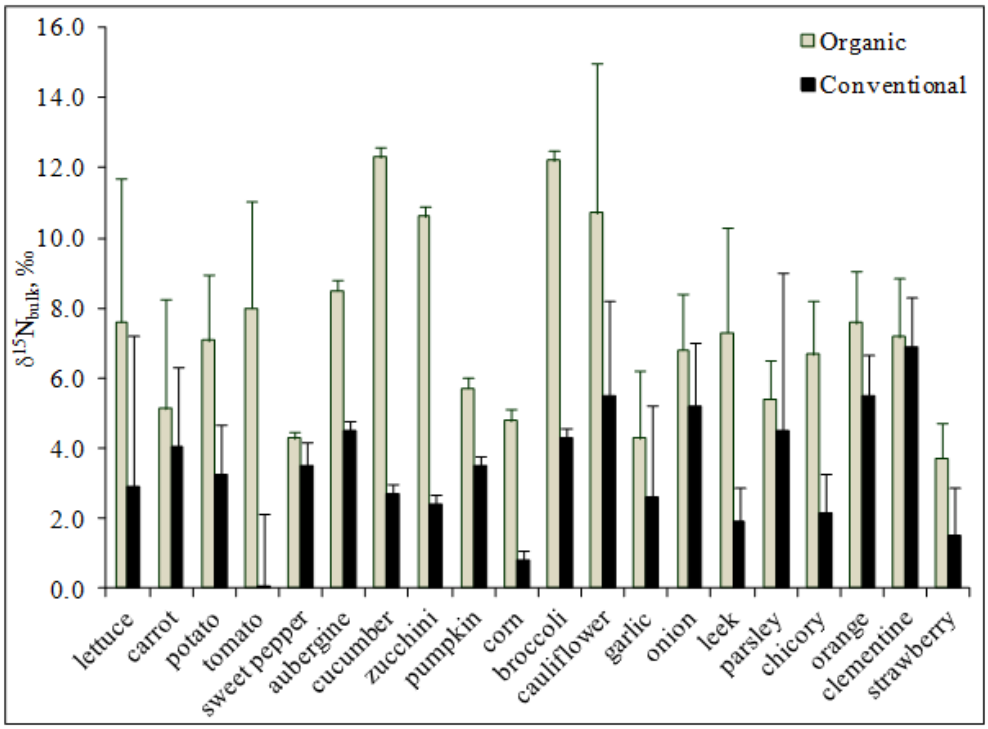

Figure 3.1. The nitrogen isotope composition of commercially produced organic and conventional vegetables and fruits. Data is compiled from Bateman et al. 2007, Camin et al. 2007, Rogers 2008, Camin et al. 2011, Šturm and Lojen 2011. The error bars represent pooled standard deviation values based on the number of samples compiled from the above studies. The number of samples and the $\delta^{15}N$ values of each vegetable species are attached in the Appendix B, Table B19).

In the study by Rogers (2008) only fast growing crops, such as zucchini and cucumber, could be successfully discriminated using $\delta^{15}N$ values. Šturm and Lohen (2011) reported that only six out of 14 analyzed organic vegetable species were successfully differentiated from their conventional counterparts on the basis of $\delta^{15}N$ values. Kelly and Bateman (2010) reported that the combination of trace element and nitrogen isotope data improved the correct classification of organic and conventional

tomato samples, however the approach appeared to have a limited effect on lettuces. Thus, nitrogen isotope analysis can be a useful tool for the discrimination of fast growing crops and crops cultivated under controlled greenhouse conditions, but the approach may have limited applicability to the field grown crops with long growth cycles.

Furthermore, the discrimination between organic and conventional crops on the basis of nitrogen isotope composition becomes very challenging if organic cultivation practises use nitrogen-fixing plants in order to increase the nitrogen content of soil, either together with main crops or in crop rotations. Nitrogen-fixing plants derive most of their nitrogen by fixing it from air and have $\delta^{15}N$ values that are close to those of air nitrogen ($\delta^{15}N$ = 0.0‰) (Handley and Raven 1992, Högberg 1997, Bedard-Haughn et al. 2003) and thus, are similar to the $\delta^{15}N$ values of synthetic nitrogen fertilisers used in the conventional systems. Moreover, conventional cropping systems, similarly to organic practises, sometimes use animal manures as the main nitrogen source, which again would diminish the differences in nitrogen isotope values between organic and conventional crops (Bateman et al. 2005).

To achieve more successful discrimination between organic and conventional systems, some studies combined nitrogen isotope analysis with the isotope analysis of other elements: carbon, oxygen, hydrogen and sulphur. Schmidt et al. (2005) for example, analyzed stable C, N and S isotopes to discriminate organic and conventional vegetables (lettuce, onions, cabbage and Chinese cabbage) and wheat. Rapisarda et al. (2010) performed multi-element H, C, O, N and S isotope analyses to study the differences in the isotope composition between organic and conventional oranges. Both above studies reported that only $\delta^{15}N$ values were suitable for the authentication of organic samples. Georgi et al. (2005) analysed bulk N, C and S isotope compositions and O isotope composition of leaf water in cabbage, onion, lettuce and Chinese cabbage samples. Authors reported that organic vegetables were significantly^{15}N-enriched as well as had lower $\delta^{13}C$ values compared to conventional vegetables. The difference in the carbon isotope composition between organically and conventionally cultivated vegetables in their study was explained by the higher microbial activity in organic soils, which resulted in more ^{13}C-depleted CO_2 respired from soils and subsequently refixed by crops. Camin et al. (2011) combined multi-element H, C, O, N and S isotope analyses with analyses of a wide range of

chemical/physical characteristics (e.g. pH, total soluble solids, colorimetric characteristics, antioxidant activity, the content of total nitrogen, organic acids, sugars, anthocyanins and polyphenols) for the investigation of potential markers for organic fruits: oranges, clementines, strawberries and peaches. The combination of nitrogen isotope values with several other parameters (the content of ascorbic acid and total soluble solids) was shown to be the most successful for distinguishing between organically and conventionally cultivated fruits.

Thus, the analyses of multiple isotope ratios and the combination of stable isotope analysis with other analytical methods may provide higher discrimination between organic and conventional foods (Camin et al. 2011). Further research in order to find novel markers for the differentiation between organic and conventional crops is currently needed.

3.1.2. Rationale for this study

A great number of studies have applied nitrogen isotope analysis for the evaluation of its capability to discriminate between organic and conventional crops in the controlled greenhouse conditions (e.g. Choi et al. 2002, Bateman et al. 2005, Flores et al. 2007, del Amor and Navarro 2008, Flores et al 2011, Šturm et al. 2011, Yuan et al. 2010, Zhou et al. 2012). Fewer studies compared the nitrogen isotope composition of crops grown in the field (Choi et al. 2006, Camin et al. 2007, Rapisarda et al. 2010, Camin et al. 2011) or performed retail crop sample analysis (Bateman et al. 2007, Rogers 2008, Camin et al. 2011, Šturm and Lojen 2011).

In the existing field studies, conventional crops were always fertilised with synthetic nitrogen fertilisers, while crops grown organically were usually treated with either animal manure or compost. Organic cropping systems, however, very often rely on the use of nitrogen fixing crops, which can be cultivated together with the main crop or in crop rotations, to increase the nitrogen content of soil and crops. Studies, analysing isotope composition of crops from different organic systems are currently needed to evaluate the capability of the isotopic approach for the differentiation between those crops and their conventional counterparts.

The combination of nitrogen isotope analysis with the analysis of isotope ratios of other elements as well as with other analytical techniques may result in higher discrimination between organically and conventionally field grown crops (Camin et al. 2011). A new promising tool for distinguishing between crops grown with

synthetic and organic fertilisers may be compound specific stable oxygen isotope analysis of plant derived nitrate ($\delta^{18}O_{NO3}$). This approach is based on the difference in the oxygen isotope signature of nitrate in conventional and organic agricultural systems. The oxygen isotope composition of synthetic nitrate fertilisers reflects the oxygen isotope composition of air oxygen, from which they are produced, and is generally in the range between +17‰ and +25‰ (Amberger and Schmidt 1987). In contrast, nitrate produced in soil during microbial nitrification of organic fertilisers derives oxygen atoms from two sources: air and soil water (Andersson and Hooper 1983, Kumar et al. 1983, Hollocher 1984, Dispirito and Hooper 1986, Wassenaar 1995, Böhlke et al. 1997, Kendall 1998, Mayer et al. 2001), and has lower $\delta^{18}O$ values, which on average are in the range between -10 and +10‰ (Kendall 1998, Kendall et al. 2007).

The stable isotope analysis of the plant derived nitrate has not been previously applied in organic food authentication studies. The technique has shown a potential for differentiating conventional and organic lettuces from the greenhouse experiment described in Chapter 2. In the present study I have applied compound-specific oxygen and nitrogen analysis of nitrate together with the analysis of total nitrogen content and bulk nitrogen isotope composition for the discrimination between organic and conventional vegetables. In comparison with studies where a single conventional system was compared with a single organic growing regime, present field trials included two different organic systems: the first system where crops were treated with animal manure and the second organic system where crops did not receive any fertiliser and relied on the nitrogen supplied by the leguminous cover crops.

The main goal of the study was to evaluate whether total nitrogen content and bulk nitrogen isotope composition can discriminate organic and conventional samples of five vegetable species (potato, faba bean, cabbage, carrot, onion) from the field trials and whether the discrimination of the samples according to their cultivation system could be improved on the basis of oxygen isotope composition of nitrate. The second objective of this study was to apply the oxygen isotope analysis of plant derived nitrate to the retail conventional and organic potatoes from different geographical locations (UK, Jersey, Israel, Egypt) as well as to conventional and organic tomatoes from UK, and to evaluate the capability of the compound-specific $\delta^{18}O_{NO3}$ analysis for the authentication of organic vegetables.

3.2. Materials and methods

3.2.1. Field trials

The samples of potatoes (*Solanum tuberosum* cv. Sava), carrots (*Daucus carota* cv. Bolero), onions (*Allium cepa* cv. Hytech), white cabbages (*Brassica oleracea* convar. *capitata* var. *alba* L. cv. Impala) and faba beans (*Vicia faba* L. cv. Columbo) were obtained from the field trials undertaken in 2007 and 2008 in Denmark, which were part of the long-term CropSys and VegQure crop rotation experiments (http://www.cropsys.elr.dk/uk; http://www.vegqure.elr.dk/uk). The samples were kindly provided by Prof. Dr. Søren Husted (Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen).

Field trials included four different field sites: Flakkebjerg, Foulum, Jyndevad (potato and faba bean plots) and Aarslev (cabbage, carrot and onion plots). Crops were grown in 120 m^2 plots in Aarslev and in 169 m^2, 216 m^2 and 378 m^2 plots in Flakkebjerg, Foulum and Jyndevad, respectively. Field trial conditions are described in Olesen et al. (2000), Soltoft et al. (2010) and Laursen et al. (2011) and are summarized in the Appendix B (Table B1).

Crops were cultivated in three different agricultural systems:

i) conventional system (C);

ii) organic system with the application of animal manure (OA);

iii) organic system with the application of cover crops (OB).

For each vegetable species three replicate plots representing each agricultural system were established, resulting in 9 plots per crop per year. In the conventional system, pesticides and inorganic nitrogen/phosphorus/potassium fertilisers (NPK) were used according to the usual conventional cultivation in Denmark. The organic systems were managed in full compliance with the guidelines for organic farming (European Community Council Regulation EEC 2091/91 and EC 834/2007), administered by the Danish Plant Directorate (http://pdir.fvm.dk). The OA system relied on the input of animal manure, while in the OB system the nutrient supply was based on the use of cover crops: hairy vetch (*Vicia villosa* L.), white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.), winter rye (*Secale cereale* L.), fodder radish (*Raphanus sativus oleiformis* L.), chicory (*Cichorium intybus* L.) and perennial ryegrass (*Lolium perenne* L.). Cover crops were grown in autumn after the main crops and incorporated into the soil in spring before sowing of the next

spring-sown main crop. Due to the high nitrogen demand of white cabbage and onions, animal manure was also applied to these crops in the OB system. The types and application rates of fertilisers used in the three agricultural systems are summarized in the Appendix B (Table B2).

3.2.2. Sampling and preparation of crops from the field trials

The sampling of crops was performed by Kristian Holst Laursen (Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen) together with Malene Søltoft, Emese Kápolna, Alicja B. Mark, Sidsel B. Schmidt, Tine Thach, Hanne C. Thomsen, Svend R. Madsen and Birgitte K. Herbst (University of Copenhagen, Technical University of Denmark, and Aarhus University, Denmark). Sampling and further preparation of crops for analysis is described in detail in Soltoft et al. (2010) and Laursen et al. (2011). In brief, representative sampling of all crops was performed on the same day for all agricultural systems at each location and growing year. Only the edible parts of the crops (t.i. potato tubers, onion bulbs, carrot roots, faba bean seeds, cabbage heads) were collected. A 15 kg sample of potatoes, carrots and onions, a 5 kg sample of faba bean pods and 8 whole cabbages (2.5-4.5 kg) were collected from each plot and stored at 4 ^{o}C.

For the analyses 1 kg of bean seeds and 5 kg of other vegetable material were washed in Milli-Q water, peeled (only carrots and potatoes), cut into 0.5 cm thick slices (all crops except faba beans), and freeze-dried for 1-2 days at a commercial freeze-drying company (Danish Freeze-Dry A/S, Kirke Hyllinge, Denmark). Then all samples were crushed, homogenized to a fine powder, and stored at -20 ^{o}C in an inert nitrogen atmosphere until analyses.

In total, 108 vegetable samples were obtained and used for analyses in the present study: 36 potato samples (12 samples from each agricultural system) and 18 samples of cabbages, faba beans, carrots and onions (six samples from each agricultural system for each vegetable species). Additionally, 14 samples of fertilisers were collected – nine samples of synthetic NPK fertilisers and five samples of animal manures. Synthetic fertilisers were kept at room temperature in plastic containers. Animal manures were kept in plastic containers at -20 ^{o}C. The type and number of vegetable and fertiliser samples obtained from each location are shown in the Appendix B (Table B3).

3.2.3. Retail potato and tomato samples

Retail potato samples (18 conventional and 15 organic) were obtained from the pesticide residue study (Fera Potato PRC Q2 2010-2011 survey) conducted at the Food and Environment Research Agency (Fera, UK). Samples were kindly provided by Dr. Helen Barker (Fera, UK).

Retail tomato samples (10 conventional and 15 organic samples) were obtained from local supermarkets and certified organic growers by Dr. Alison Bateman and Dr. Simon Kelly. The detailed collection of tomato samples is described in Bateman et al. (2007). Information regarding sample origin and variety is included in the Attachment B (Tables B15-B16).

Potato samples were homogenized in the presence of dry ice by Melissa Bowen (Fera, UK) and stored in -20 ^{o}C. Tomato samples were freeze-dried, homogenized, and ground to a fine powder by Dr. Alison Bateman (Bateman et al. 2007) and stored in amber glass vials at room temperature.

3.2.4. Elemental nitrogen analysis

Total nitrogen (TN) content was determined in the vegetable samples from the field trials by Kristian Holst Laursen and Anja Hect Ivø (Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen). Dried plant material (4 mg) was weighed into tin capsules, followed by the analysis on the Europa Scientific ANCA-SL elemental analyzer (Sercon Ltd., Crewe, U.K.). Samples were analysed in duplicate. Quality assurance was performed by the analysis of the certified reference materials: NIST-1515 apple leaves, NIST-8436 durum wheat and NIST-141d acetanilide (National Institute of Standards and Technology, NIST, Gaithersburg, MD, USA).

3.2.5. Bulk nitrogen isotope analysis of fertilisers from the field trials

Animal manure samples were homogenised, while synthetic fertiliser samples were homogenized and ground to a fine powder using a ball mill (Mixer/Mill® 8000-M230, SPEX SamplePrep LLC, New Jersey, USA). Then all fertiliser samples were weighed into tin capsules to give 0.1 mg of N per analysis. Nitrogen isotope composition ($\delta^{15}N_{bulk}$) was determined using Costech elemental analyzer coupled to

Thermo Finnigan Delta XP continuous flow isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were analysed in duplicate, and for the vast majority of samples the absolute difference between duplicate measurements was < 0.3‰, never exceeding 0.5‰.

Each batch of samples included replicate analyses of the in-house standard, casein ($\delta^{15}N$ = +6.15‰, previously calibrated against the International Atomic Energy Agency (IAEA) reference materials during an inter-laboratory comparison exercise as part of EU Project SMT4-CT98-2236), which was used for the drift correction of the raw data. The long-term performance of the mass spectrometer was monitored by analysis of a secondary reference material, collagen ($\delta^{15}N$ = +6.12‰), which was included to every batch of samples. Isotope ratios were reported with respect to the international standard – air nitrogen. Data were processed using Isodat NT 2.0 software (Thermo Scientific, Bremen, Germany).

3.2.6. Bulk nitrogen isotope analysis of vegetable samples from the field trials

Bulk stable nitrogen isotope analysis of vegetable samples was performed by Kristian Holst Laursen and Anja Hect Ivø (Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen) and is described in detail in Soltoft et al. (2010) and Laursen et al. (2011). In brief, 4 mg of freeze-dried samples were weighed into tin capsules and introduced into the Europa Scientific ANCA-SL elemental analyzer coupled to Europa Scientific 20-20 Tracermass isotope ratio mass spectrometer (Sercon Ltd., Crewe, UK). Samples were measured in duplicate. Accuracy and precision were monitored by the analysis of the certified reference materials: IA-R001 wheat flour (Iso-Analytical Limited, Crewe, UK), NIST-1515 apple leaves, NIST-8436 durum wheat and NIST-141d acetanilide (National Institute of Standards and Technology, NIST, Gaithersburg, MD, USA). The standard deviation of the long-term stability of the reference materials never exceeded 0.4‰. Isotope ratios were reported with respect to the international standard – air nitrogen.

3.2.7. Compound specific nitrogen and oxygen isotope analysis of nitrate

3.2.7.1. Nitrate extraction

Hot water extraction was used to obtain nitrate from the vegetable samples from the field trials as well as from the retail vegetable samples. Extraction was performed in 50 ml polypropylene centrifuge tubes (Corning®, Sigma-Aldrich, UK). In the case of field trial vegetable samples, 20 ml of Ultrapure water (18MΩ cm, Purelab Ultra) was added to the 0.5 g (potato, cabbage samples) or 1.0 g (faba bean, carrot, onion samples) of freeze-dried vegetable material. In the case of retail vegetable samples, 20 ml of Ultrapure water (18MΩ cm, Purelab Ultra) were added to the 4 g of frozen potato material and to 1.0 g of freeze-dried tomato material. Further hot water extraction conditions were the same for the field trial and retail vegetable samples. Centrifuge tubes were placed in a 90 °C hot water bath for 30 minutes. Then all vegetable extracts were cooled, centrifuged for 10 min at 4500 rpm, filtered through 0.2 micron filter (Minisart®, Sartorius, UK), and stored at -20 °C.

3.2.7.2. Sample preparation – the denitrifier method

For the calculation of the exact amount of vegetable extracts needed for the denitrifier method, the nitrate concentration in the extracts was measured by ion chromatography system Dionex ICS-2000 (Dionex Ltd., Camberley, UK). The system was equipped with Dionex AG18 guard column (50 mm x 2 mm) and Dionex AS18 analytical column (250 mm x 2 mm). Potassium hydroxide was used as the eluent. A range of sodium nitrate standards (concentration range from 0.1 to 15 mg/l) and a Ultrapure water blank were analysed at the beginning and at the end of each sample batch for the calibration of the instrument and to account for any instrument drift during the run. The data were processed using Chromeleon software 6.6 (Dionex Ltd., Camberley, UK). Assistance with analysis was provided by Kimberly Goodey (University of East Anglia, School of Environmental Sciences).

A detailed description of the denitrifier method for the sample preparation for the nitrogen and oxygen isotope analysis of nitrate can be found in Casciotti et al. (2002). The method was adapted with minor adaptations. Bacteria were cultivated at room temperature on tryptic soy agar (Difco™, Becton, Dickinson and Company,

NJ, USA) containing 10 mM KNO_3, 1.9 mM $(NH_4)_2SO_4$, and 37 mM K_2HPO_4. Further, a single bacterial colony was transferred to a starter tube containing tryptic soy broth solution (9 ml) (Bacto™, Becton, Dickinson and Company, NJ, USA) containing the same nitrate and ammonium amendments as the agar medium. After overnight incubation on a reciprocal shaker an aliquot from the starter tube was used to inoculate presealed sterile media bottles (2.7ml bacterial medium per 445 ml bottle). Bottles were then incubated for 3-10 days in anaerobic conditions, at room temperature on a reciprocal shaker. After the incubation period bacteria were concentrated by centrifugation and resuspension in 80 ml of nitrate free media, to which an antifoaming agent (~1 ml; Antifoam B Emulsion, Sigma-Aldrich, UK) was added to avoid excessive bubbling. One ml aliquots of bacterial cell solution were injected into 20 ml headspace vials which then were crimp sealed using butyl rubber septa and aluminium crimp seal caps and purged with pure He gas for 45 minutes to remove the N_2O produced from the original 10 mM nitrate media and to ensure anaerobic conditions. After the He purge, the vials were stored overnight on a reciprocal shaker table. On the following day purging with He was repeated, after which the injection of the nitrate extracts followed. The injection volumes of samples were calculated on the basis of nitrate concentration in the extracts to ensure 20 nM nitrate concentration in each vial. Vials without sample addition were used as "bacterial blanks". Three international nitrate reference materials: IAEA-NO-3 ($\delta^{15}N = 4.7 \pm 0.1$‰, $\delta^{18}O = 25.61 \pm 0.2$‰), USGS-34 ($\delta^{15}N = -1.8 \pm 0.1$‰, $\delta^{18}O = -27.93 \pm 0.3$‰) and USGS-35 ($\delta^{15}N = 2.7 \pm 0.1$‰, $\delta^{18}O = 57.50 \pm 0.2$‰) (Böhlke et al. 2003) were used as standards and injected in a similar way as the samples. All vials were left inverted overnight to enable bacterial culture to convert nitrate to nitrous oxide. Afterwards 0.1 ml of 6M NaOH solution was added into each vial to lyse the bacterial cells and scavenge CO_2. Samples were analysed on the isotope ratio mass spectrometer within the following few days.

Sample amounts used for the nitrate extraction, nitrate concentration in the extracts and calculated extract volumes needed for the denitrifier method are shown in the Appendix B (Tables B5-B9).

3.2.7.3. Nitrogen and oxygen isotope analysis

N_2O produced by the denitrifying bacteria was stripped from each sample vial, purified and then analyzed on a PDZ Europa GEO 20-20 mass spectrometer (PDZ Europa Ltd., Northwich, UK), which was coupled with an ANCA TG II system (PDZ Europa Ltd., Northwich, UK) and Gilson head-space autosampler.

The samples were measured against a laboratory standard (20 ppm N_2O in nitrogen) and each batch of samples included replicate analyses of three international nitrate reference materials: IAEA-NO-3 ($\delta^{15}N = 4.7 \pm 0.1‰$, $\delta^{18}O = 25.61 \pm 0.2‰$), USGS-34 ($\delta^{15}N = -1.8 \pm 0.1‰$, $\delta^{18}O = -27.93 \pm 0.3‰$) and USGS-35 ($\delta^{15}N = 2.7 \pm 0.1‰$, $\delta^{18}O = 57.50 \pm 0.2‰$) (Böhlke et al. 2003). Data were processed using Callisto software (Sercon, Crewe, UK) and corrected for drift, blank and oxygen isotopic exchange with the parent water oxygen (Sigman et al. 2001, Casciotti et al. 2002, Böhlke et al. 2003, Kaiser et al. 2007). The precision of the analyses was generally better than 0.3‰ for both ^{15}N and ^{18}O measurements, based on the quadruplicate analyses of the three international standard materials. Isotope ratios were reported with respect to the international standards: Vienna Standard Mean Ocean Water (VSMOW) for $\delta^{18}O$ and atmospheric nitrogen (air) – for $\delta^{15}N$.

3.2.8. Statistical analyses

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). All data were checked for normality and homogeneity of variances. Data were normally distributed unless stated otherwise. Independent sample t-tests were applied to study the difference between i) conventional and organic fertilisers from the field trials on the basis of the mean $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values; ii) conventional and organic retail potato and tomato samples on the basis of the mean $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values. For retail tomato samples nonparametric two independent sample t-test was applied (Mann-Whitney U test) as the data distribution was not normal. In both parametric and nonparametric t-tests applied the difference between two groups was considered to be significant at $p < 0.05$.

One-way analysis of variance (ANOVA) was applied to study the difference among conventional, OA and OB agricultural systems based on the mean TN,

$\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of vegetables. Differences in the mean values were evaluated for significance with Tukey and Games-Howell *post hoc* comparisons; differences were considered to be significant at $p < 0.05$.

3.2.8.1. Canonical discriminant analysis

Stepwise canonical discriminant analysis (CDA) was applied to potato samples from the field trials and to retail potato samples in order to determine which of the analysed parameters can contribute most to the successful classification of samples into conventional and organic agricultural systems. For the field trial potato samples four predictor variables were analysed: total nitrogen content (TN), $\delta^{15}N_{bulk}$, $\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$. For the retail potato samples $\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$ were used as predictor variables. A brief introduction to CDA summarised from Miller and Miller (2010) is given below.

CDA is a multivariate statistical technique which, given a set of independent predictor variables, attempts to find linear combinations of those variables that best separate the groups of cases. These combinations are called discriminant functions and have the form displayed in the equation:

$$D = a + b_1X_1 + b_2X_2 + b_3X_3 + \dots b_nX_n \quad (3.1.)$$

where D is the discriminant score for a subject or case, a is a constant, X_1 through X_n represent the predictor variables and b_1 through b_n are the discriminant function coefficients (weights) for each predictor variable.

The number of discriminant functions is equal to the number of groups minus one (degrees of freedom). The procedure automatically chooses the first function that will separate the groups as much as possible. It then chooses the second function that is both uncorrelated with the first function and provides as much further separation as possible between the groups of interest. The procedure continues adding functions in this way until reaching the maximum permissible number of functions.

Stepwise CDA enters the predictor variables into the model step by step. At each step all variables are reviewed and evaluated to determine which one will provide the greatest discrimination between the groups. That variable is then included in the model, and the process starts again. The procedure continues until all

possible variables are selected or the remaining variables do not contribute to the discrimination of the groups. Variable selection is based on Wilks' lamda, which indicates the significance of the discriminant function. At each step, the variable that minimized the overall Wilks' lamda value is selected. Additional variables are only entered into the model if the significance level of its F-statistic value is less than 0.05.

Several parameters in the CDA can assess the contribution of each analyzed variable to the model. These parameters include: i) the tests of equality of group means, ii) the discriminant function coefficients, and iii) the structure matrix. The tests of equality of group means measure each predictor variable's discriminative potential before the model is created and display the results of a one-way ANOVA for the predictor variable using the grouping variable as the factor. If the significance value is greater than 0.10, the variable most likely does not contribute to the model. The discriminant function coefficients are represented by eigenvalues that show the % of variance which each discriminant function is capable to explain. The structure matrix shows the correlation of each predictor variable with the discriminant function.

The model provides a classification table which shows how many of the original grouped and cross-validated cases are correctly classified. The cross validation procedure classifies all cases but one to develop a discriminant function and then categorises the case that was left out. This process is repeated with each case left out in turn. The cross-validation produces a more reliable function, and is a more rigorous presentation of the power of the discriminant function than that provided by the original classification.

3.2.8.2. Probability density of the normal distribution curves

The $\delta^{18}O_{NO3}$ data of the field grown and retail potato samples were combined to create two bigger datasets of conventional (n = 30) and organic potatoes (n = 39). Further the created datasets were used to generate the probability densities of the normal distribution curves for the expected $\delta^{18}O_{NO3}$ of entire population of conventional and organic potatoes. This type of data analysis is described in detail by Bateman et al. (2007). In brief, the expected normal distributions of the $\delta^{18}O_{NO3}$ values for the organic and conventional potatoes were constructed using Microsoft

Excel NORMDIST function, which produced a normal distribution for a specified mean and standard deviation. The normal distribution was described by the following equation:

$$y = \frac{1}{\sigma\sqrt{2\pi}} e^{-0.5z^2} \qquad (3.2.)$$

where z = (x - μ)/σ; y = frequency, σ = standard deviation, x = $\delta^{18}O_{NO3}$ value, μ = mean.

The mean (x) of each group (organic/conventional) of the analysed potato samples was used as an estimate of μ (the mean for the entire population), and the sample standard deviation of each group (σ^{n-1}) gave an estimate of σ (the standard deviation for the entire population). For the normal distribution, z scores of 1.96 and 2.58 were the limits on either side of a population mean, within which lie respectively, 95 and 99% of all observations. To compensate for the uncertainty in the estimates of μ and σ, the values of 1.96 and 2.58 were increased by replacing z scores with t values, which were calculated based on the known number of potato samples (30 conventional and 39 organic). The critical t values for the known number of conventional and organic samples at two confidence levels (p=0.05 and p=0.01) were calculated using Microsoft Excel TINV function and used in the following formula:

$$t = \frac{x - \bar{x}}{\sigma^{n-1}} \qquad (3.3.)$$

where x = value of an individual observation ($\delta^{18}O_{NO3}$ of a sample), $\bar{x}$ = mean of the sample set, and σ^{n-1} = standard deviation of the sample set.

From the above formula x values were expressed to obtain the lower and upper limits of the $\delta^{18}O_{NO3}$ values, within which 95 and 99% of all conventional and organic potato samples would be expected to lie. The probability densities were used to suggest a threshold for the $\delta^{18}O_{NO3}$ values of the organic potato population.

3.3. Results

3.3.1. Stable isotope composition of fertilisers used in the field trials

Nine synthetic and five organic fertilisers were used in the field trial study. Bulk nitrogen isotope composition of synthetic NPK fertilisers (mean $\delta^{15}N_{bulk}$ = -0.3‰) is significantly lower ($p<0.05$) than that of manures (mean $\delta^{15}N_{bulk}$ = +5.8‰) (Table 3.1). The $\delta^{15}N_{bulk}$ values of synthetic fertilisers range from -2.1‰ to +0.7‰, while those of animal manures are in the range between +2.6‰ and +12.5‰.

Table 3.1. Stable isotope composition of synthetic and organic fertilisers used in the field trials.

Parameter analysed		Synthetic NPK fertilisers	Animal manures
$\delta^{15}N_{bulk}$	**Mean**	**-0.3a**	**+5.8a**
	SD	0.9	4.2
	Minimum	-2.1	+2.6
	Maximum	+0.7	+12.5
$\delta^{15}N_{NO3}$	**Mean**	**+2.2b**	**+8.8b**
	SD	2.7	6.1
	Minimum	-1.5	+3.0
	Maximum	+7.7	+18.9
$\delta^{18}O_{NO3}$	**Mean**	**+23.8c**	**+15.9c**
	SD	1.9	4.5
	Minimum	+20.4	+10.7
	Maximum	+27.1	+19.8

Different letters denote significant difference ($p<0.05$) in one of the analysed parameters between synthetic fertilisers and animal manures. SD – standard deviation based on the number of samples (n): for synthetic fertilisers n = 9, for animal manures n= 5.

Compound specific stable isotope analysis revealed that both the $\delta^{15}N$ and $\delta^{18}O$ values of nitrate in synthetic NPK fertilisers differ significantly from those of animal manures. Similarly to $\delta^{15}N_{bulk}$ values, $\delta^{15}N_{NO3}$ values are significantly higher ($p<0.05$) in manures (mean $\delta^{15}N_{NO3}$ = +8.8‰) compared to synthetic fertilisers (mean $\delta^{15}N_{NO3}$ = +2.2‰), whereas $\delta^{18}O_{NO3}$ values of manures (mean $\delta^{18}O_{NO3}$ = +15.9‰) are significantly lower ($p<0.05$) than those of synthetic fertilisers (mean $\delta^{18}O_{NO3}$ = +23.8‰) (Table 3.1). Stable isotope compositions of the individual fertiliser samples are included in Appendix B, Table B4.

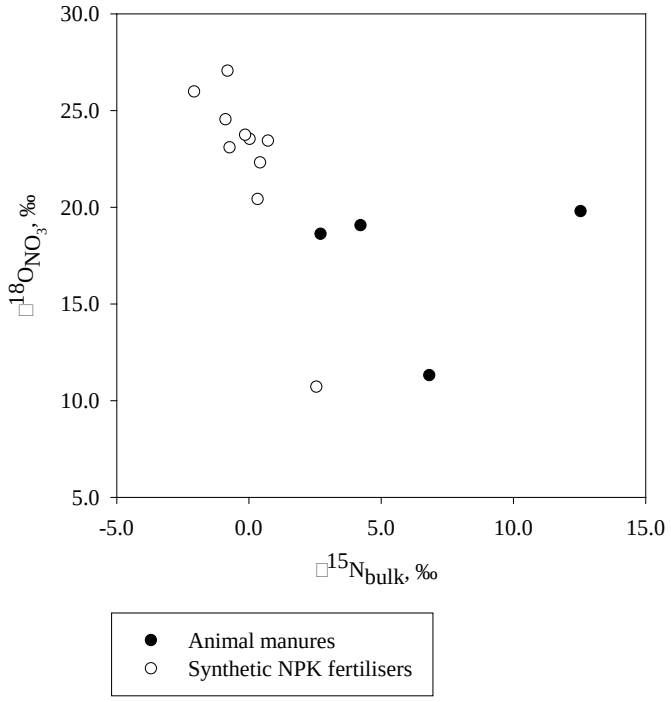

Figure 3.2. Scatter plot of the $\delta^{15}N_{bulk}$ and $\delta^{18}O_{NO3}$ values of fertilisers used in the field trials.

A scatter plot of the $\delta^{15}N_{bulk}$ and $\delta^{18}O_{NO3}$ values of the fertilisers is presented in Figure 3.2. There is a good separation of two groups of fertilisers on both axes. Analysed conventional NPK fertilisers have $\delta^{18}O_{NO3}$ values higher than +20‰ and $\delta^{15}N_{bulk}$ values lower than +1‰. In contrast, the $\delta^{18}O_{NO3}$ values of animal manures are lower than +20‰, while their $\delta^{15}N_{bulk}$ values are higher than 2.5‰ (Figure 3.2 and Table B4, Appendix B).

3.3.2. The elemental and stable nitrogen isotope composition of bulk vegetable samples from the field trials

Total nitrogen (TN) content of the analysed vegetable species is presented in Figure 3.3 and Table 3.2. TN content of the individual vegetable samples is shown in Tables B10-B14 (Appendix B). Out of five analysed vegetable species, faba beans have the highest TN content. TN values of faba beans (5.0-5.1%) are more than five times higher than those of carrots which have the lowest TN values (0.7-0.9%).

Significant difference ($p<0.05$) in the TN values among three agricultural systems is observed in carrot samples. Conventional carrot samples have the highest mean TN value (TN = 1.0%), while the lowest TN content is in carrots from organic system with the application of animal manure (OA system) (TN = 0.7%).

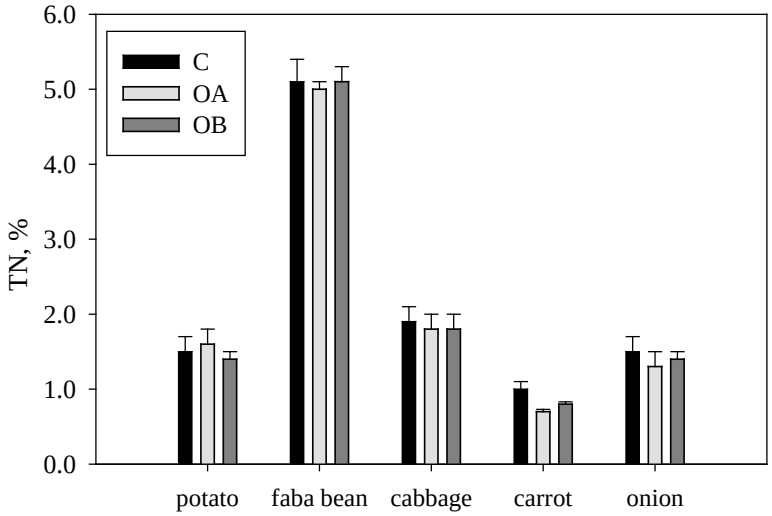

Figure 3.3. Total nitrogen content of vegetables from the field trials. Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. The error bars represent standard deviations based on the n number of samples from each agricultural system: for potatoes n=12, for all other crops n=6.

Higher TN content in crops from conventional system compared to organic systems is observed also in cabbage and onion samples, however, the difference between the samples from different agricultural systems is not significant ($p>0.05$).

Table 3.2. The TN content of vegetable samples from the field trials.

Crop	Agricultural system	Number of samples (n)	TN, %			
			Mean	SD	Minimum	Maximum
Potato	C	12	1.5	0.2	1.1	1.8
	OA	12	1.6a	0.2	1.5	2.1
	OB	12	1.4a	0.1	1.3	1.7
Faba bean	C	6	5.1	0.3	4.8	5.7
	OA	6	5.0	0.1	4.9	5.2
	OB	6	5.1	0.2	4.9	5.4
Cabbage	C	6	1.9	0.1	1.7	2.1
	OA	6	1.8	0.2	1.6	2.0
	OB	6	1.8	0.2	1.6	2.1
Carrot	C	6	1.0b	0.1	0.8	1.1
	OA	6	0.7b	0.0	0.7	0.8
	OB	6	0.8b	0.0	0.8	0.8
Onion	C	6	1.5	0.2	1.2	1.7
	OA	6	1.3	0.2	1.0	1.6
	OB	6	1.4	0.1	1.2	1.4

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Different letters (a-b) denote significant difference ($p<0.05$) in the mean TN values among three agricultural systems. SD - standard deviations based on the n number of samples.

The average nitrogen isotope composition of bulk vegetable samples is shown in Figure 3.4 and Table 3.3. The $\delta^{15}N_{bulk}$ values of the individual vegetable samples are included in the Appendix B (Tables B10-B14).

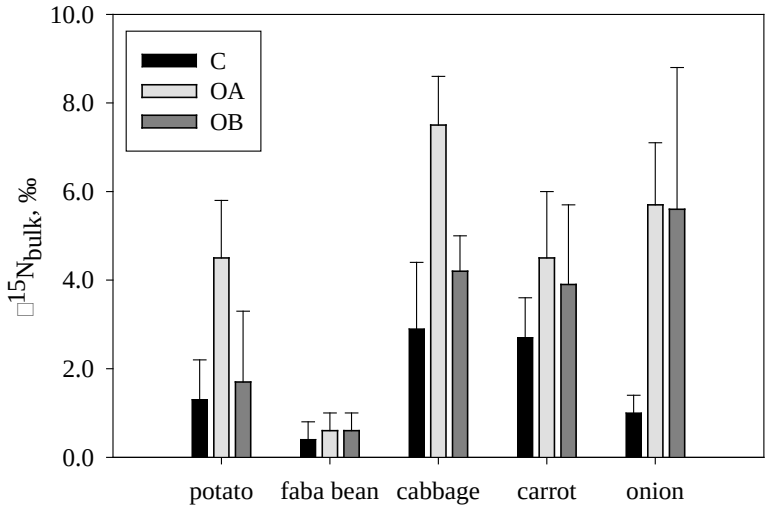

Figure 3.4. Stable nitrogen isotope compositions of bulk vegetable samples from the field trials. Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. The error bars represent standard deviations based on the n number of samples from each agricultural system: for potatoes n=12, for all other crops n=6.

In all vegetable species, the $\delta^{15}N_{bulk}$ values of samples from conventional agricultural system are lower compared to those of samples from organic systems (Figure 3.4). Significant difference ($p<0.05$) between conventional and OA system is observed in potato, cabbage and onion samples (Table 3.3). The difference between conventional and OB system is significant ($p<0.05$) only in the case of onion samples. The $\delta^{15}N_{bulk}$ values of faba beans and carrots do not differ significantly ($p>0.05$) between the three agricultural systems (Table 3.3).

Figure 3.5 presents scatter plots of total nitrogen versus bulk stable nitrogen isotope composition of five analysed vegetable species. Potato samples from conventional and OA system can be discriminated on the basis of $\delta^{15}N_{bulk}$ values, however a big overlap in the $\delta^{15}N_{bulk}$ values of conventional and OB system is observed. No discrimination is observed on the basis of TN or $\delta^{15}N_{bulk}$ values in faba beans. In case of cabbage, there is one conventional sample that has $\delta^{15}N_{bulk}$ value in the range of OB samples, which does not allow the complete discrimination between conventional and organic samples (Figure 3.5). In onions, conventional and OA samples are well discriminated on the basis of $\delta^{15}N_{bulk}$ values, conventional and OB

samples have a little overlap of $\delta^{15}N_{bulk}$ values. Carrots are the only vegetable species where TN values contribute significantly to the discrimination between conventional and organic samples compared to $\delta^{15}N_{bulk}$ values (Figure 3.5).

Table 3.3. The nitrogen isotope composition of bulk vegetable samples from the field trials.

Crop	Agricultural system	Number of samples (n)	$\delta^{15}N_{bulk}$, ‰			
			Mean	SD	Minimum	Maximum
Potato	C	12	+1.3a	0.9	+0.2	+3.3
	OA	12	+4.5ab	1.3	+1.9	+6.2
	OB	12	+1.7b	1.6	-0.1	+4.2
Faba bean	C	6	+0.4	0.4	-0.2	+0.8
	OA	6	+0.6	0.4	+0.2	+1.0
	OB	6	+0.6	0.4	+0.3	+1.5
Cabbage	C	6	+2.9c	1.5	+2.0	+6.0
	OA	6	+7.5cd	1.1	+5.9	+8.8
	OB	6	+4.2d	0.8	+3.5	+5.6
Carrot	C	6	+2.7	0.9	+1.3	+3.9
	OA	6	+4.5	1.5	+3.2	+7.4
	OB	6	+3.9	1.8	+1.4	+5.4
Onion	C	6	+1.0ef	0.4	+0.7	+1.8
	OA	6	+5.7e	1.4	+3.9	+7.8
	OB	6	+5.6f	3.2	+2.0	+10.1

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Different letters (a-f) denote significant difference ($p<0.05$) in the mean $\delta^{15}N_{bulk}$ values among three agricultural systems. SD - standard deviations based on the n number of samples.

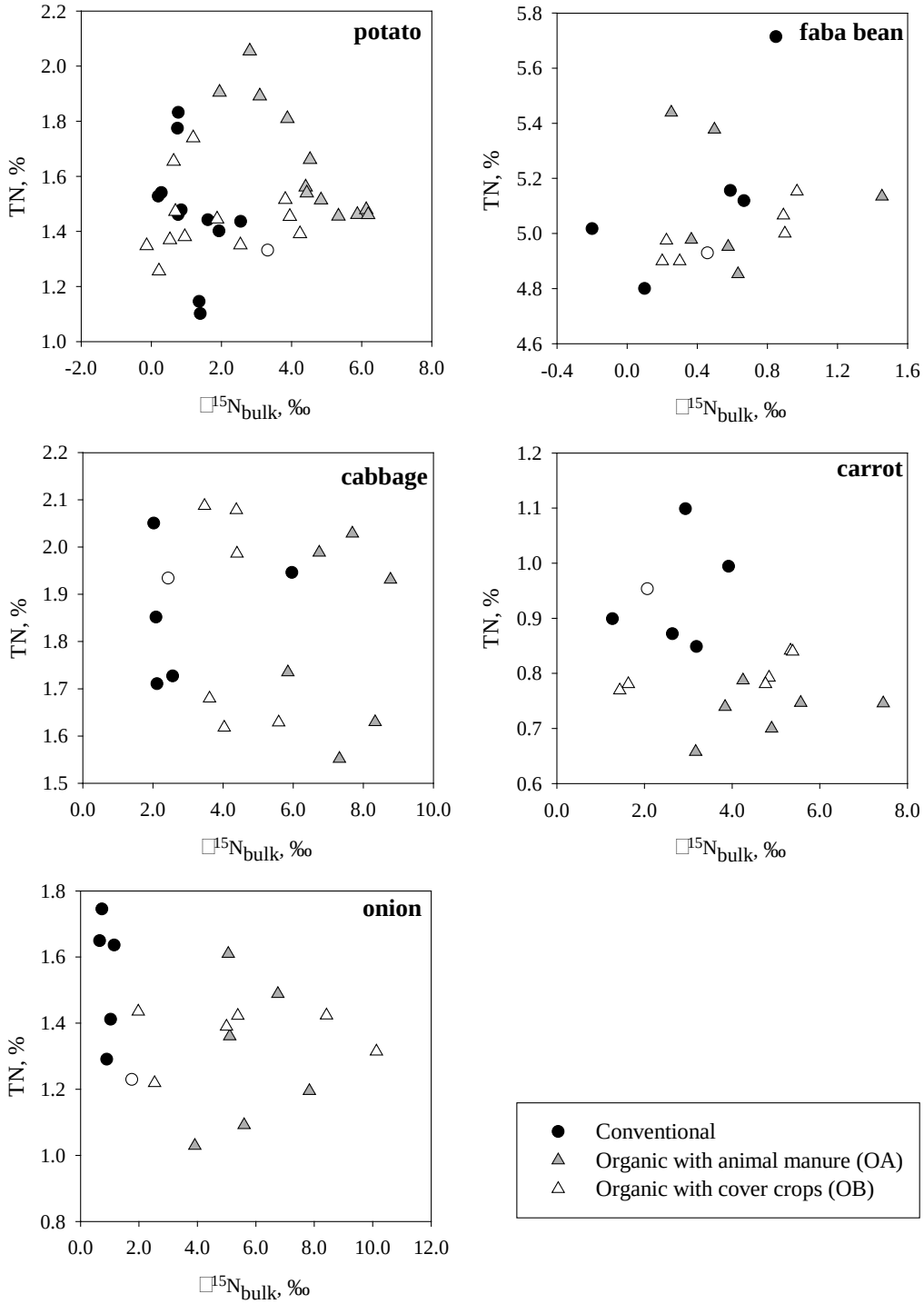

Figure 3.5. The scatter plots of total nitrogen content versus bulk nitrogen isotope composition of vegetable samples from the field trials.

3.3.3. Nitrogen and oxygen isotope composition of nitrate extracted from vegetable samples from the field trials

The mean nitrogen and oxygen isotope composition of nitrate extracted from vegetable samples from the field trials is presented in Table 3.4. The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of the individual vegetable samples are shown in Tables B10-B14 (Appendix B).

The nitrogen isotope composition of vegetable nitrate shows less significant differences between conventional and organic systems than bulk nitrogen isotope composition (Tables 3.3 and 3.4). A significant difference ($p<0.05$) in the mean $\delta^{15}N_{NO3}$ values is found between conventional and OA potato samples as well as between conventional and OB onion samples. In carrots, the difference in $\delta^{15}N_{NO3}$ values is significant between two organic systems.

Table 3.4. The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of the vegetable samples from the field trials.

Crop	Agricultural system	Number of samples (n)	$\delta^{15}N_{NO3}$, ‰		$\delta^{18}O_{NO3}$, ‰	
			Mean	SD	Mean	SD
Potato	C	12	+20.7a	2.8	+23.4de	2.8
	OA	12	+23.4a	2.3	+18.1d	2.7
	OB	12	+21.2	2.3	+17.9e	1.4
Faba bean	C	6	+11.9	1.9	+29.1	4.5
	OA	6	+11.0	1.7	+31.5	6.2
	OB	6	+11.5	3.7	+28.8	4.5
Cabbage	C	6	+24.5	2.7	+21.2	3.3
	OA	6	+27.6	3.7	+19.1	3.9
	OB	6	+23.4	3.7	+17.8	4.1
Carrot	C	6	+30.0	2.8	+26.9	1.1
	OA	6	+29.1b	1.1	+26.8	1.9
	OB	6	+33.9b	4.1	+26.4	2.8
Onion	C	6	+37.6c	4.6	+35.6	4.1
	OA	6	+31.5	6.0	+35.3	3.4
	OB	6	+28.3c	3.9	+35.8	2.2

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Different letters (a-e) denote significant difference ($p<0.05$) in the mean $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values among three agricultural systems. SD - standard deviations based on the n number of samples.

Oxygen isotope composition of nitrate shows significant difference ($p<0.05$) between conventional and both organic systems in potato samples (Table 3.4 and Figure 3.6). The $\delta^{18}O_{NO3}$ values of conventional potato samples (mean $\delta^{18}O_{NO3}$ =

+23.4‰) are significantly higher ($p<0.05$) than those of potatoes from OA (mean $\delta^{18}O_{NO3}$ = +18.1‰) and OB (mean $\delta^{18}O_{NO3}$ = 17.9‰) systems (Table 3.4). For other vegetable species, the difference in the $\delta^{18}O_{NO3}$ values between conventional and organic systems is not significant ($p>0.05$).

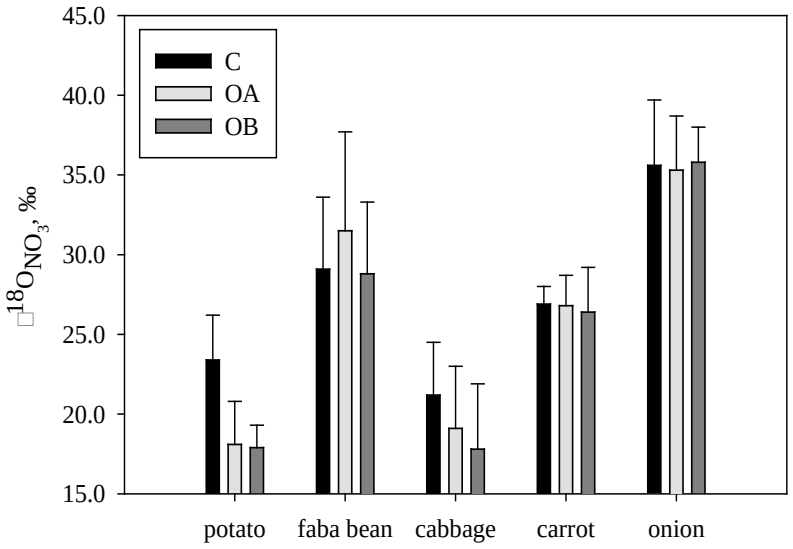

Figure 3.6. The oxygen isotope composition of nitrate extracted from vegetable samples from the field trials. Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. The error bars represent standard deviations based on the n number of samples from each agricultural system: for potatoes n=12, for all other crops n=6.

Figure 3.7 presents scatter plots of the $\delta^{18}O_{NO3}$ values versus $\delta^{15}N_{NO3}$ values of vegetable samples from the field trials. The $\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$ values of potato samples discriminate conventional system from two organic systems significantly better compared to TN and $\delta^{15}N_{bulk}$ values. In other vegetable species, the combination of $\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$ values does not result in any significant discrimination between conventional and organic cultivation systems.

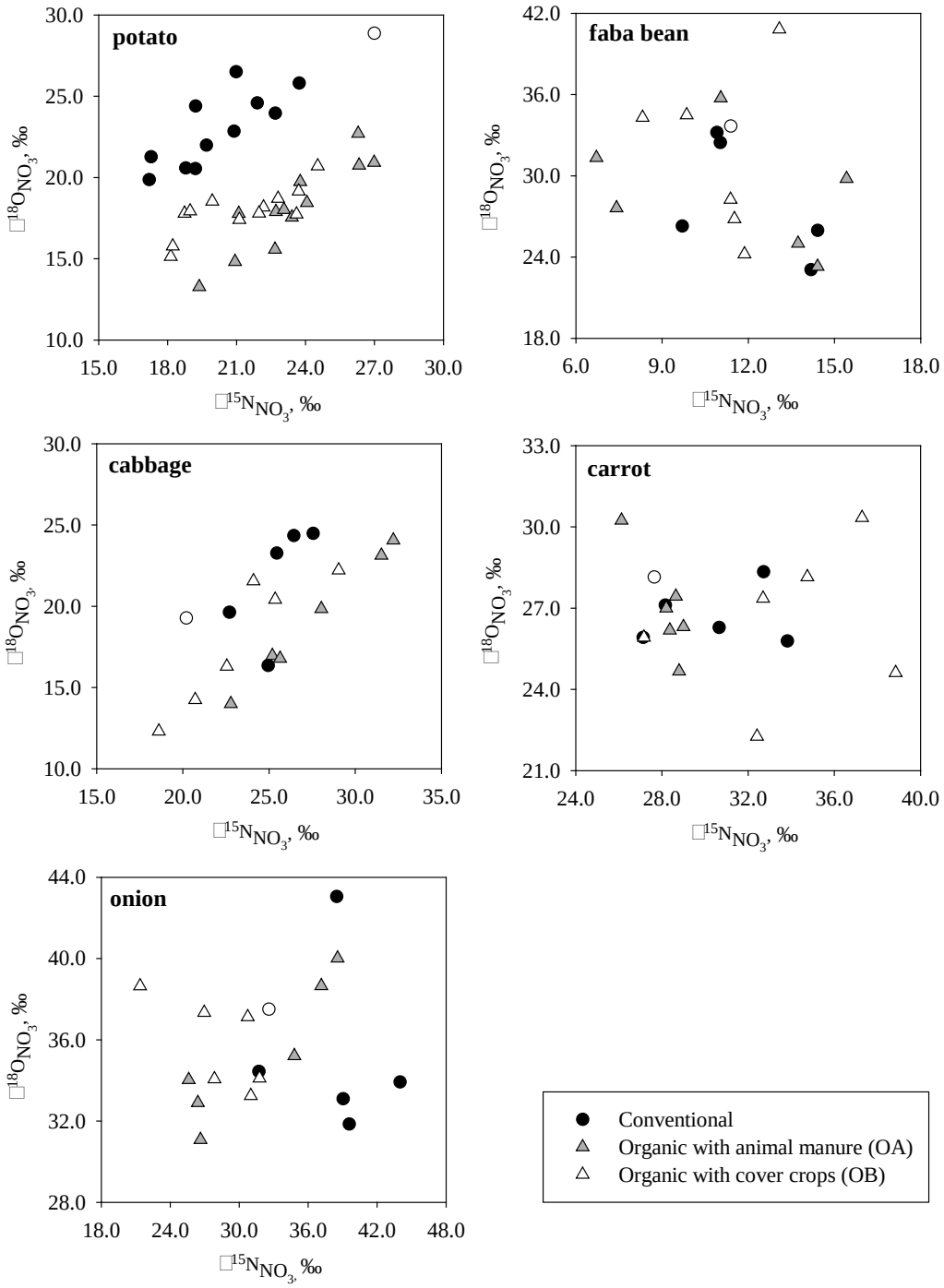

Figure 3.7. The scatter plots of $\delta^{18}O_{NO3}$ versus $\delta^{15}N_{NO3}$ values of vegetable samples from the field trials.

3.3.4. Canonical discriminant analysis of potato samples from the field trials

Canonical discriminant analysis (CDA) was performed on potato samples (n=36) from the field trials with TN, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ as predictor variables to test whether the combination of the analysed parameters can successfully discriminate the potato samples into one of the three agricultural systems. CDA was initially performed only with TN and $\delta^{15}N_{bulk}$ as predictor variables, and then rerun using all four analysed variables.

The results from entering the TN and $\delta^{15}N_{bulk}$ of potatoes as predictor variables in the CDA are shown in the Table 3.5. The cross-validated classification results in 50.0% of potato samples being correctly classified. All OA samples (100%) are classified correctly, 66.7% of conventional samples are mis-classified as OB samples; OB samples are classified correctly in only 16.7% of all cases, with 25% being mis-classified as OA and 58.3% – as conventional.

Table 3.5. Classification results from the canonical discriminant analysis of potato samples from the field trials. Predictor variables: TN and $\delta^{15}N_{bulk}$. Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops.

		Agricultural system	Predicted Group Membership			Total
			C	OA	OB	
Original	Count	C	6	0	6	12
		OA	0	12	0	12
		OB	6	3	3	12
	%	C	50.0	0.0	50.0	100.0
		OA	0.0	100.0	0.0	100.0
		OB	50.0	25.0	25.0	100.0
Cross-validated	Count	C	4	0	8	12
		OA	0	12	0	12
		OB	7	3	2	12
	%	C	33.3	0.0	66.7	100.0
		OA	0.0	100.0	0.0	100.0
		OB	58.3	25.0	16.7	100.0

58.3% of original grouped cases correctly classified.
50.0% of cross-validated grouped cases correctly classified.

The use of all four measured parameters (TN, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) as predictor variables in the CDA has greatly improved the classification results of

potato samples (Table 3.6). The results of the cross-validated classification show that overall 86.1% of potato samples are now correctly classified with 100% of conventional samples, 83.3% of OA samples and 75% of OB samples being classified correctly.

Table 3.6. Classification results from the canonical discriminant analysis of potato samples from the field trials. Predictor variables: TN, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$. Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops.

		Agricultural system	Predicted Group Membership			Total
			C	OA	OB	
Original	Count	C	12	0	0	12
		OA	0	11	1	12
		OB	0	3	9	12
	%	C	100.0	0.0	0.0	100.0
		OA	0.0	91.7	8.3	100.0
		OB	0.0	25.0	75.0	100.0
Cross-validated[a]	Count	C	12	0	0	12
		OA	0	10	2	12
		OB	0	3	9	12
	%	C	100.0	0.0	0.0	100.0
		OA	0.0	83.3	16.7	100.0
		OB	0.0	25.0	75.0	100.0

88.9% of original grouped cases correctly classified.
86.1% of cross-validated grouped cases correctly classified.

The tests of equality of group means and the structure matrix of CDA model are shown in Table 3.7 and Table 3.8, respectively. The tests of equality of group means assess variable's discriminative potential before the discriminative model is created. Out of four predictor variables, only two variables – $\delta^{15}N_{bulk}$ and $\delta^{18}O_{NO3}$ – have low Wilks' Lambda values and highly significant ($p<0.0001$) discriminative potential (Table 3.7).

Table 3.7 The tests of equality of group means from the CDA of vegetables from the field trials.

Predictor variable	Wilks' Lambda	F	Significance
TN	0.800	4.125	0.025
$\delta^{15}N_{bulk}$	0.444	20.656	0.000
$\delta^{18}O_{NO3}$	0.442	20.798	0.000
$\delta^{15}N_{NO3}$	0.805	3.991	0.028

Two discriminant functions were created in the CDA model. Figure 3.8 illustrates how each of the functions separates potato samples from the three agricultural systems. Function 1 separates conventional samples and the samples from two organic systems, whereas Function 2 contributes to the separation of OA and OB potatoes.

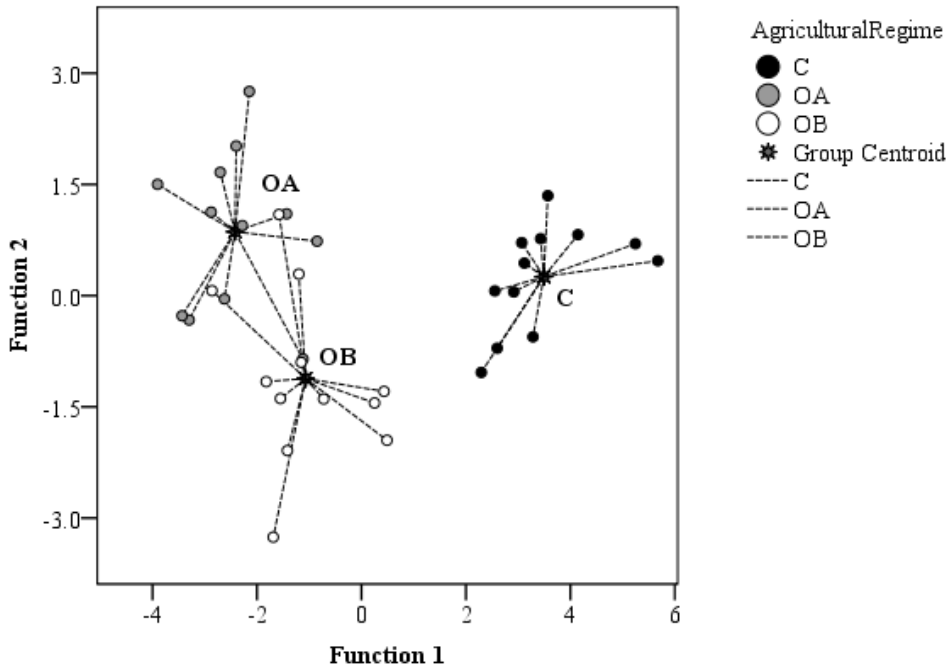

Figure 3.8. Canonical discriminant functions from the CDA of potato samples from the field trials. Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops.

The structure matrix of the model indicates that $\delta^{18}O_{NO3}$ has the highest correlation with Function 1 (correlation coefficient 0.411). The $\delta^{15}N_{bulk}$ shows the next highest correlation with Function 1 (correlation coefficient -0.321). The highest correlation with Function 2 shows $\delta^{15}N_{bulk}$ (Table 3.8).

Table 3.8. The structure matrix of the model from the CDA of potato samples from the field trials.

Predictor variable	Function	
	1	2
$\delta^{18}O_{NO3}$	0.411*	0.327
$\delta^{15}N_{bulk}$	-0.321	0.844*
$\delta^{15}N_{NO3}$	-0.146	0.353
TN[a]	-0.076	0.108

Variables ordered by absolute size of correlation within function.
* Largest absolute correlation between a variable and any discriminant function
[a] This variable was not used by the model.

3.3.5. Compound specific stable isotope analysis of retail potato and tomato samples

Stable oxygen isotope analysis of nitrate has significantly improved the discrimination of organic and conventional field grown potatoes. Here I evaluate the capability of the oxygen isotope composition of nitrate to discriminate between conventional and organic retail potato samples as well as retail tomato samples.

The results of the compound-specific isotope analysis of nitrate from retail potato and tomato samples show a significant difference ($p<0.05$) in the $\delta^{18}O_{NO3}$ values between conventional and organic samples. The $\delta^{18}O_{NO3}$ values of conventional potato samples are significantly higher ($p<0.05$) than those of organic potato samples (mean $\delta^{18}O_{NO3}$ = +22.6‰ and +15.1‰, respectively). Similarly, conventional retail tomatoes have significantly higher ($p<0.05$) $\delta^{18}O_{NO3}$ values compared to organic tomato samples (mean $\delta^{18}O_{NO3}$ = +45.3‰ and 27.3‰, respectively) (Table 3.9 and Figure 3.9).

The $\delta^{15}N_{NO3}$ values of either potato or tomato samples do not differ significantly ($p>0.05$) between conventional and organic production systems (Table 3.9). The $\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$ values of the individual retail potato and tomato samples are shown in Tables B15 and B16 (Appendix B).

Table 3.9. The mean stable nitrogen and oxygen isotope composition of nitrate from retail potato and tomato samples.

Parameter analysed		Potato		Tomato	
		conventional (n=18)	organic (n=15)	conventional (n=10)	organic (n=15)
$\delta^{15}N_{NO3}$	**Mean**	**+21.3**	**+19.0**	**+27.8**	**+24.4**
	SD	4.8	5.4	3.5	8.2
	Minimum	+13.6	+9.7	+23.8	+10.8
	Maximum	+29.4	+25.1	+33.3	+38.2
$\delta^{18}O_{NO3}$	**Mean**	**+22.6a**	**+15.1a**	**+45.3b**	**+27.3b**
	SD	4.1	2.3	5.2	5.9
	Minimum	+15.5	+9.6	+32.1	+18.5
	Maximum	+29.5	+20.3	+50.4	+38.0

Different letters (a-b) denote significant difference ($p<0.05$) in the mean $\delta^{18}O_{NO3}$ values between conventional and organic samples. SD - standard deviations based on the n number of samples.

Figure 3.10 shows the scatter plots of $\delta^{18}O_{NO3}$ values versus $\delta^{15}N_{NO3}$ values of potato and tomato samples. With an exception of one sample, which has the $\delta^{18}O_{NO3}$ value of +32.1‰, all analysed conventional tomato samples have $\delta^{18}O_{NO3}$ values that are higher than +40‰, whereas organic samples lie below the +38‰ margin.

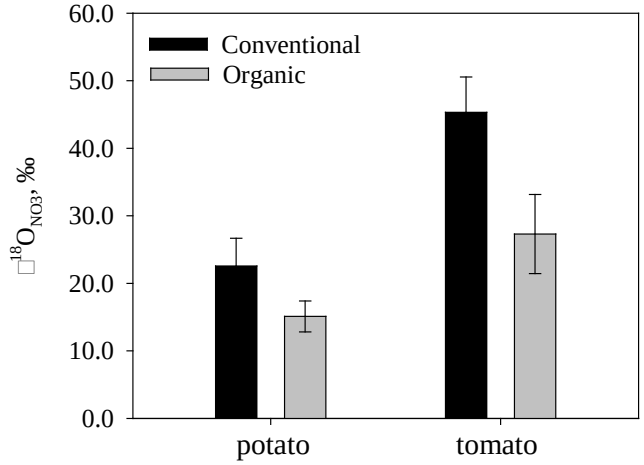

Figure 3.9. The mean $\delta^{18}O_{NO3}$ values of retail potato and tomato samples. The error bars represent standard deviations based on the n number of samples: for conventional and organic potatoes n = 18 and 15, respectively; for conventional and organic tomatoes n = 10 and 15, respectively.

The majority of conventional potato samples have $\delta^{18}O_{NO3}$ values that lie above +20‰ margin, while the $\delta^{18}O_{NO3}$ values of most organic samples are below +20‰. However, a complete discrimination between two types of potato samples on the basis of $\delta^{18}O_{NO3}$ values is not possible due to the overlap in $\delta^{18}O_{NO3}$ values of several samples from two agricultural systems.

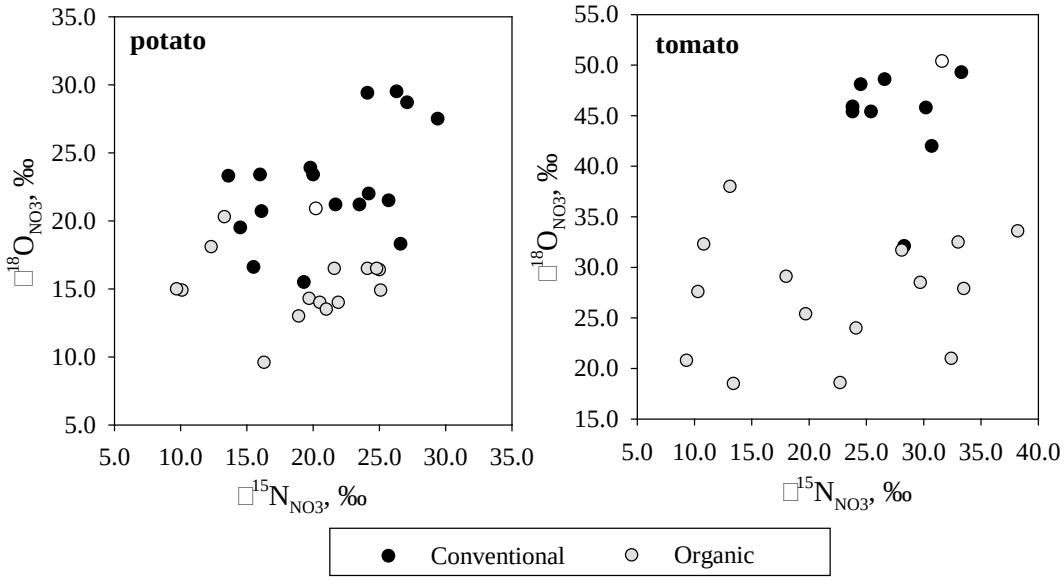

Figure 3.10. The scatter plots of $\delta^{18}O_{NO3}$ versus $\delta^{15}N_{NO3}$ values of retail potato and tomato samples.

Results from entering $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ of potato samples as predictor variables in the CDA are shown in the Table 3.10. The results of the tests of equality of group means, the discriminant function coefficients, and the structure matrix table from the CDA analysis of retail potato samples are attached in Appendix B (Table B17). The cross-validated classification shows that overall 84.8% of potato samples are correctly classified. Organic samples are classified correctly in 86.7% cases, whereas conventional samples – in 83.3% cases.

Table 3.10. Classification results from the canonical discriminant analysis of retail potato samples. Predictor variables: $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$.

		Agricultural system	Predicted Group Membership		Total
			Conventional	Organic	
Original	Count	Conventional	15	3	18
		Organic	1	14	15
	%	Conventional	83.3	16.7	100.0
		Organic	6.7	93.3	100.0
Cross-validated	Count	Conventional	15	3	18
		Organic	2	13	15
	%	Conventional	83.3	16.7	100.0
		Organic	13.3	86.7	100.0

87.9% of original grouped cases correctly classified
84.8% of cross-validated grouped cases correctly classified

3.3.6. Probability densities of the normal distribution curves for the organic and conventional potato populations

Using the mean $\delta^{18}O_{NO3}$ and standard deviation values of the combined datasets of the field grown and retail organic (n=39) and conventional (n=30) potatoes probability densities of the normal distribution curves for the expected entire population of organic and conventional potatoes were generated. Modelled normal distribution curves for the organic and conventional potato populations are shown in Figure 3.11. Table 3.11 presents the calculated upper and lower limits of the $\delta^{18}O_{NO3}$ values at 0.05 and 0.01 confidence levels. Critical t values for conventional and organic potato samples are attached in Appendix B (Table B18).

Ninety five percent (p = 0.05) of the modelled organic population would be expected to have $\delta^{18}O_{NO3}$ values in the range between +11.6‰ and +22.2‰ (Table 3.11 and Figure 3.11).

Table 3.11. Calculated upper and lower limits of the $\delta^{18}O_{NO3}$ values for organic and conventional potato datasets.

Agricultural system	Number of samples	Degrees of freedom	Lower limit $\delta^{18}O_{NO3}$, ‰		Upper limit $\delta^{18}O_{NO3}$, ‰	
			(p=0.05)	(p=0.01)	(p=0.05)	(p=0.01)
conventional	30	29	+15.6	+13.0	+30.3	+32.8
organic	39	38	+11.6	+9.8	+22.2	+24.0

A potato with a $\delta^{18}O_{NO3}$ value of >22.2‰ can be described as statistically unlikely to be drawn from a population with the same mean as set for organic potatoes analyzed in this study. Of the remaining 5% of the population, 2.5% would be expected to fall in each of the distributions tails; that means that five of 200 organic samples could be expected to have $\delta^{18}O_{NO3}$ values > 22.2‰.

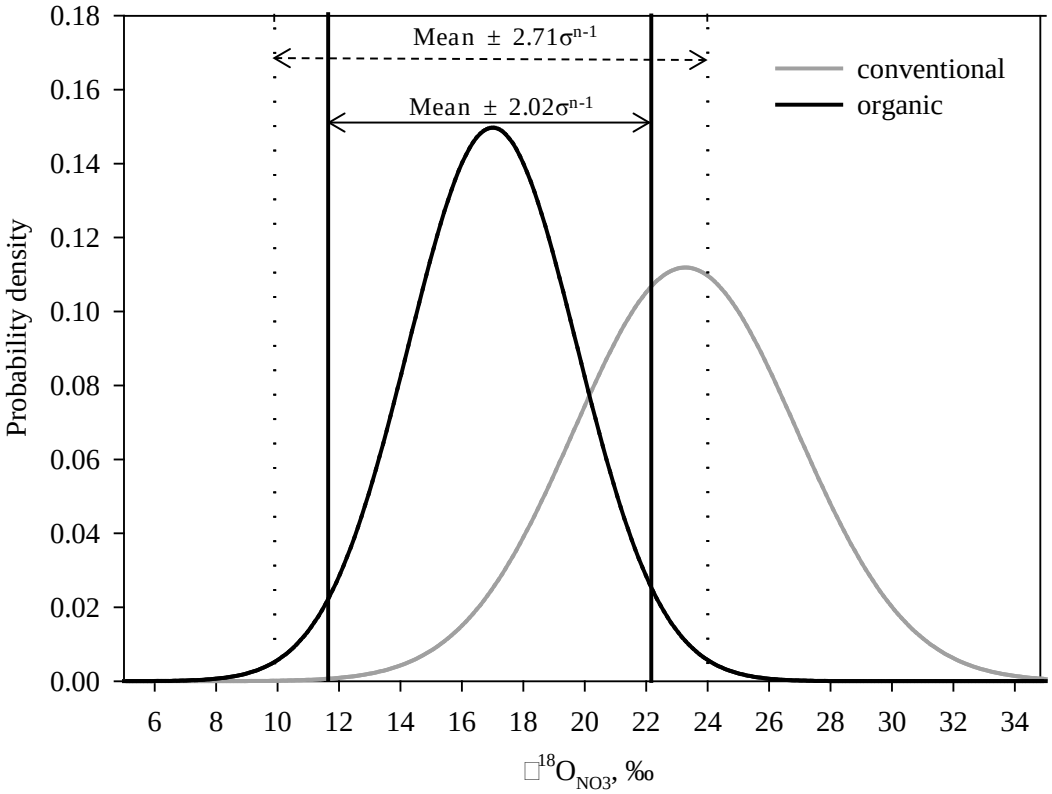

Figure 3.11. Modelled normal distribution curves for the organic and conventional potato populations. The solid and dashed lines show the limits between which 95% and 99% of the organic population would be expected to fall.

Ninety nine percent (p = 0.01) of the modelled organic population would be expected to have $\delta^{18}O_{NO3}$ values in the range between +9.8‰ and +24.0‰ (Table 3.11 and Figure 3.11). A potato with a $\delta^{18}O_{NO3}$ value of >24.0‰ can be described as statistically highly unlikely to be drawn from a population with the same mean as set for organic potatoes analyzed in this study. Of the remaining 1% of the population, 0.5% would be expected to fall in each of the distributions tails suggesting that one out of 200 organic samples could be expected to have $\delta^{18}O_{NO3}$ values > 24.0‰.

Conventional potato population overlaps with organic population (Figure 3.11). Ninety five percent (p = 0.05) of the modelled conventional population would be expected to have $\delta^{18}O_{NO3}$ values in a range between +15.6‰ and +30.3‰, while ninety nine percent (p = 0.01) of the modelled conventional population would be expected to have $\delta^{18}O_{NO3}$ values in a range between +13.0‰ and +32.8‰ (Table 3.11). The lower limits of the $\delta^{18}O_{NO3}$ values of conventional potatoes fall in the range of organic samples, however overall less than 50% of conventionally grown potatoes would be likely to have $\delta^{18}O_{NO3}$ values which would be in the range of organic samples (p=0.05).

3.4. Discussion

This study focused on the application of bulk and novel compound-specific stable isotope analyses for the discrimination between conventional and organic field grown and retail vegetables. Firstly, I address here the differences found in the isotopic composition of synthetic and organic fertilisers from the field trials, and then discuss the differences in the total nitrogen content and bulk nitrogen isotope composition of conventional and organic potatoes, faba beans, cabbages, carrots and onions from the field trials. Then I discuss the results of the compound-specific isotope analysis of nitrate and address the differences observed in the oxygen isotope compositions of nitrate from conventional and organic potatoes. Further, the capability of the compound-specific oxygen isotope analysis of nitrate for the differentiation between organic and conventional cultivation is discussed with respect to retail potato and tomato samples.

3.4.1. Differences in the stable isotope composition of synthetic and organic fertilisers used in the field trials

Synthetic fertilisers and animal manures used in the field trials exhibit significant differences in their isotopic composition. The $\delta^{15}N_{bulk}$ values of synthetic NPK fertilisers are significantly lower ($p<0.05$) than those of animal manures (mean $\delta^{15}N_{bulk}$ = -0.3‰ and +5.8‰, respectively) (Table 3.1). Similarly, the $\delta^{15}N_{NO3}$ values of NPK fertilisers are significantly lower ($p<0.05$) compared to $\delta^{15}N_{NO3}$ values of animal manures (mean $\delta^{15}N_{NO3}$ = +2.2‰ and +8.8‰, respectively). In contrast, the $\delta^{18}O_{NO3}$ values of synthetic NPK fertilisers are significantly higher ($p<0.05$) than those of manures (mean $\delta^{18}O_{NO3}$ = +23.8‰ and +15.9‰, respectively) (Table 3.1). Thus, analysed fertilisers can be well differentiated on the basis of their nitrogen and oxygen isotope values – fertiliser samples with $\delta^{18}O_{NO3}$ values below +20‰ and $\delta^{15}N_{bulk}$ values above +2‰ are of organic origin, whereas fertilisers with $\delta^{18}O_{NO3}$ values higher than +20‰ and $\delta^{15}N_{bulk}$ values lower than +2‰ have synthetic origin (Figure 3.2).

The nitrogen isotope composition of bulk fertiliser samples as well as the nitrogen and oxygen isotope composition of nitrate from the analysed fertilisers reflect nitrogen and oxygen isotope compositions of the sources of these two groups

of fertilisers. NPK fertilisers can contain several nitrogen compounds (e.g. synthetic ammonia, ammonium, nitrate, urea) and are produced from air in the Haber-Bosch process. Thus, the sources of nitrogen and oxygen atoms in synthetic NPK fertilisers are atmospheric nitrogen ($\delta^{15}N$ = 0‰, Mariotti 1983) and oxygen ($\delta^{18}O$ = +23.5‰, Kroopnick and Craig 1972), respectively, and therefore the $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of the synthetic NPK fertilisers from the present study are close to the $\delta^{15}N$ and $\delta^{18}O$ of air (Table 3.1). In contrast, sources of nitrogen and oxygen atoms in animal manures include a wide range of compounds, such as amino acids, ammonia, ammonium, hydroxylamine, nitrous and nitric oxide, nitrous and nitric acid, nitrite and nitrate ions (United States Department of Agriculture, Natural Resources Conservation Service 2007). This results in more ^{15}N-enriched and more ^{18}O-depleted values of manures compared to those of synthetic fertilisers (Table 3.1).

Stable isotope values of synthetic NPK fertilisers and animal manures from the present study are in agreement with the fertiliser values reported previously (Wassenaar 1995, Kendall 1998, Nakano et al. 2003, Curt et al. 2004, Vitoria et al. 2004, Bateman and Kelly 2007, Rapisarda et al. 2010). Vitoria et al. (2004) performed the multi-element stable isotope analysis of various synthetic fertilisers and reported that the $\delta^{15}N_{bulk}$ values of NPK fertilisers (n=13) were in the range between -1.7‰ and +1.8‰, with the mean $\delta^{15}N_{bulk}$ being -0.1‰, which is close to the mean $\delta^{15}N_{bulk}$ value of NPK fertilisers from the present study (-0.3‰). Vitoria et al. (2004) also performed nitrogen and oxygen isotope analysis of nitrate from synthetic fertilisers, and reported that $\delta^{15}N_{NO3}$ values of NPK fertilisers (n=9) ranged from -1.6 to +2.2‰ (mean $\delta^{15}N_{NO3}$ = 0.7‰), while $\delta^{18}O_{NO3}$ values of synthetic fertilisers ranged from +19.2 to 24.2‰, which agrees with the present findings.

Another study of a wide range of fertilisers, including both synthetic and organic fertilisers, sourced in the UK was performed by Bateman and Kelly (2007). NPK fertilisers (n=6) from their study had $\delta^{15}N_{bulk}$ values in the range from -0.7‰ to +1.9‰ (mean $\delta^{15}N_{bulk}$ = +0.5‰), whereas $\delta^{15}N_{bulk}$ values of farmyard manures (n=8) were in the much higher range from +3.5 to +16.2‰ (mean $\delta^{15}N_{bulk}$ = +8.8‰). The $\delta^{15}N_{bulk}$ values of animal manures from the present study are in the range from +2.6 to +12.5‰, and therefore agree with the nitrogen isotope data from the study by Bateman and Kelly (2007).

Thus, synthetic NPK fertilisers and animal manures have distinctive $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values, which reflect the difference in the isotope composition of the sources of these fertilisers.

3.4.2. Differences in the total nitrogen content and bulk nitrogen isotope composition of organic and conventional vegetables from the field trials

The total nitrogen (TN) values of conventionally grown cabbage, carrots and onions are higher compared to those from organic systems, however the difference between conventional and two organic systems is significant ($p<0.05$) for carrot samples only. There is a little overlap in TN values of carrots from three agricultural systems (Figure 3.5). Most of the carrot samples which have TN value below 0.8% are of organic origin whereas samples with TN values above 0.8% are conventional, and thus, in carrots TN content contributes to the discrimination between conventional and organic systems.

The difference in the TN values of vegetable from three agricultural system agrees with the amounts of nitrogen fertiliser (kg/ha) applied in each of the systems (Appendix B, Table B2). Conventional vegetables received the highest amount of nitrogen compared to OA or OB system, which is reflected in the higher TN values of conventional vegetable samples. This is in agreement with the findings of extensive reviews by Woese et al. (1997), Bourn and Prescott (2002) and Dangour et al. (2009), who compiled data from a wide range of studies on the chemical composition of organic and conventional food products and reported that conventionally produced crops generally had a significantly higher content of nitrogen compared to organic crops.

From the five analysed vegetable species the highest TN values are in faba beans (5.0-5.1%) (Figure 3.3). Beans accumulate great amount of nitrogen fixing it directly from atmospheric N_2, which therefore is reflected in high nitrogen concentration in their tissues. On the other hand, carrots require low nitrogen inputs and received the lowest amount of nitrogen fertiliser (Appendix B, Table B2), which resulted in the lowest TN values of this vegetable species (0.7-0.9%) (Figure 3.3).

Nitrogen isotope composition of bulk vegetables samples exhibits differences among three agricultural systems, and in all vegetable species the $\delta^{15}N_{bulk}$ values of conventional samples are lower compared to those of organic samples (Figure 3.4).

Significantly lower ($p<0.05$) $\delta^{15}N_{bulk}$ values of vegetable samples from conventional system compared to OA system are observed in three out of five analysed vegetable species: potato, cabbage and onion (Figure 3.5). For each vegetable species plots representing different agricultural systems were located in the same geographical area with the same climate and soil profile, therefore the major factor which could have introduced differences in $\delta^{15}N_{bulk}$ values among the samples from three agricultural systems is the applied fertiliser. Significantly higher $\delta^{15}N_{bulk}$ values of vegetables from OA system compared to conventional system can be attributed to the application of nitrogen in the form of animal manure, which has significantly higher $\delta^{15}N_{bulk}$ values compared to synthetic NPK fertilisers (Table 3.1), as discussed in the previous section.

Significant differences found in the three above vegetable species between conventional system and organic practise with the application of animal manure are in agreement with findings reported previously in several other studies. Camin et al. (2007) determined $\delta^{15}N_{bulk}$ values of the field grown conventional and organic potatoes fertilised with synthetic ammonium and a range of organic fertilisers of animal origin, respectively. The mean $\delta^{15}N_{bulk}$ value of conventionally grown potatoes from their study ($\delta^{15}N_{bulk}$ = +3.4‰) was significantly lower than that of organically cultivated potatoes ($\delta^{15}N_{bulk}$ = +7.2‰). Despite an overlap in the $\delta^{15}N_{bulk}$ values of some conventional and organic samples, authors suggested that potato samples could be successfully discriminated on the basis of $\delta^{15}N_{bulk}$ values.

Results of the multi-element isotope ratio analysis of cabbages, onions, lettuces and Chinese cabbages from a 2-year field experiment were reported by Georgi et al. (2005). In their study vegetables were cultivated in either conventional system with the application of synthetic ammonium nitrate ($\delta^{15}N_{bulk}$ = +0.7‰) or in organic system where nitrogen was supplied in the form of hornmeal ($\delta^{15}N_{bulk}$ = +6.0‰). The $\delta^{15}N_{bulk}$ values of conventional cabbages were reported to be significantly lower compared to those of organic cabbages (mean $\delta^{15}N_{bulk}$ = +6.6‰ and +8.5‰, respectively), however there was an overlap of the $\delta^{15}N_{bulk}$ values of conventional and organic samples, and therefore a full discrimination between two agricultural practises was not possible. Also the $\delta^{15}N_{bulk}$ values of conventional onions in the study by Georgi et al. (2005) were significantly lower than those of organic onions (mean $\delta^{15}N_{bulk}$ = +5.8‰ and +8.0‰, respectively), which agrees with the results of the present study.

Thus, the nitrogen isotope compositions of bulk potato, cabbage and onion samples from the field trials reflect the difference in the nitrogen isotope composition of synthetic and organic fertilisers applied in the field and can be a good discriminator between conventional system where synthetic NPK fertilisers were applied versus organic system with application of animal manure.

On the other hand, difference in the $\delta^{15}N_{bulk}$ values between conventional and OB samples is significant only for one vegetable species – onions (Figure 3.4). As nitrogen in OB system was mainly provided by cover crops which were incorporated into soil in the course of the field trials, the $\delta^{15}N_{bulk}$ values of vegetables in OB system were greatly influenced by the isotopic composition of cover crops. Some of the used cover crops were nitrogen fixing plants: hairy vetch, white clover, red clover. As nitrogen fixation is associated with no or little fractionation, $\delta^{15}N_{bulk}$ values of nitrogen-fixing plants are generally close to the $\delta^{15}N_{bulk}$ values of air nitrogen ($\delta^{15}N = 0.0‰$) (Handley and Raven 1992, Högberg 1997, Bedard-Haughn et al. 2003). Therefore, incorporation of these plants shifted the $\delta^{15}N$ values of soil nitrogen to the more negative values, which have diminished the difference in the $\delta^{15}N_{bulk}$ values between conventional and OB vegetable samples.

The impact of the nitrogen fixation on the bulk nitrogen isotope composition has been also observed in the analysed faba bean samples. Mean $\delta^{15}N_{bulk}$ values of faba beans do not differ significantly among three agricultural systems, and are the lowest among all the analysed vegetable species (Figure 3.4). Faba bean plants mainly rely on the fixation of atmospheric nitrogen, and therefore the $\delta^{15}N_{bulk}$ of faba beans from conventional, OA and OB systems (mean $\delta^{15}N_{bulk}$ = 0.4, 0.6 and 0.6 ‰, respectively) are close to $\delta^{15}N$ of air N_2 (0‰, Mariotti 1983). These results are in agreement with data reported in other studies where stable nitrogen composition of legumes was analysed (Kohl and Shearer 1980, Shearer et al. 1980, Steele et al. 1983, Yoneyama et al. 1986, Okito et al. 2004). For example, the $\delta^{15}N_{bulk}$ values of soybeans were reported to range from -2.6 to +1.9‰ (Kohl and Shearer 1980, Shearer et al. 1980, Steele et al. 1983, Yoneyama et al. 1986), which agrees with the $\delta^{15}N_{bulk}$ values of faba beans from the present study.

No significant differences in the $\delta^{15}N_{bulk}$ values have been observed in carrots from three agricultural systems. The $\delta^{15}N_{bulk}$ values of conventional carrots are lower than those of OA or OB samples, however the difference is not significant ($p>0.05$) and the $\delta^{15}N_{bulk}$ values of the samples from three agricultural systems overlap (Figure

3.4). The effect of the fertiliser type on the nitrogen isotope composition of bulk carrots was analysed in the study by Bateman et al. (2005). Authors reported a significant impact of the fertiliser on the $\delta^{15}N_{bulk}$ of carrots, and indicated significantly higher values of carrots fertilised with chicken manure compared to those fertilised with ammonium nitrate in the controlled greenhouse conditions. Bateman et al. (2005) discuss that the application of synthetic nitrogen fertiliser result in crops with lower $\delta^{15}N_{bulk}$ values under greenhouse conditions where light and temperature regime, type of substrate and irrigation water were kept the same. In the present study vegetables were grown in multiple plots in the field and thus, were subjected to a greater variability of environmental factors. Nitrogen isotope composition cannot be considered as a good discriminator of the agricultural system for carrots from the present field trials. As suggested earlier in this section, total nitrogen content has a significantly higher discriminating potential in respect to conventional and organic carrot samples.

3.4.3. Differences in the stable isotope composition of nitrate from organic and conventional vegetables from the field trials

To evaluate whether stable isotope composition of fertiliser sources from three agricultural systems is reflected better in one compound rather than the whole bulk vegetable matter, the compound-specific stable nitrogen and oxygen isotope analysis of nitrate was performed on the vegetable samples from the field trials. Results suggest that there is a significant improvement in the discrimination between conventional and organic potato samples on the basis of the oxygen isotope composition of nitrate. Conventional potato samples have significantly higher ($p<0.05$) $\delta^{18}O_{NO3}$ values compared to those from two organic systems (Figure 3.6). The $\delta^{18}O_{NO3}$ value of potatoes from conventional system (mean $\delta^{18}O_{NO3}$ = +23.4‰) are close to the $\delta^{18}O$ of air oxygen (+23.5‰, Kroopnick and Craig 1972), whereas the $\delta^{18}O_{NO3}$ values of OA and OB potato samples are lower (mean $\delta^{18}O_{NO3}$ = +18.1‰ and +17.9‰, respectively). This can be explained by two different sources of oxygen atoms in nitrate produced during nitrification in organic soils: air oxygen and soil water oxygen. Out of three oxygen atoms that form NO_3-, one originates from air oxygen while two are derived from soil water (Andersson and Hooper 1983, Kumar et al. 1983, Hollocher 1984, Dispirito and Hooper 1986, Wassenaar 1995, Böhlke et

al. 1997, Kendall 1998, Mayer et al. 2001). The $\delta^{18}O$ value of soil water in a particular location has been reported to reflect the mean $\delta^{18}O$ of annual precipitation in that location (Clark and Fritz 1997, Marshal et al. 2007). The $\delta^{18}O$ values of precipitation water in the field trial locations: Flakkebjerg, Foulum, Jyndevad and Aarslev are -9.5‰, -9.7‰, -9.1‰ and -9.4‰, respectively (Bowen 2012). These values are much lower compared to the $\delta^{18}O$ values of air oxygen (+23.5‰, Kroopnick and Craig 1972) and thus, shift the $\delta^{18}O$ of nitrate produced in organic soils towards more negative values compared to those from conventional system, which is further reflected in the $\delta^{18}O_{NO3}$ values of potatoes.

The canonical discriminant analysis (CDA) of potato samples indicates that out of four predictor variables used (TN, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$), the $\delta^{18}O_{NO3}$ has the greatest discriminative power in the classification of potato samples into three agricultural systems, followed by the $\delta^{15}N_{bulk}$ (Table 3.7). The results of the cross-validated classification show that 86.1% of potato samples are correctly classified when all four analysed parameters are included in the model, which is an improvement of more than 35% compared to the results of classification when only TN and $\delta^{15}N_{bulk}$ are used (Tables 3.5 and 3.6). Thus, compound-specific oxygen isotope analysis of nitrate shows high discriminative potential in the case of conventional and organic potato samples.

In all other vegetable species, $\delta^{18}O_{NO3}$ values did not introduce significant improvement in discrimination between samples from three agricultural systems (Figure 3.7). Cabbage requires the highest nitrogen inputs and received the highest amounts of nitrogen fertiliser (conventional/organic) compared to all other analysed vegetable species. Similarly to potato, the $\delta^{18}O_{NO3}$ values of conventional cabbage samples (mean $\delta^{18}O_{NO3}$ = +21.2‰) are close to the $\delta^{18}O_{NO3}$ of air nitrogen (+23.5‰) whereas the $\delta^{18}O_{NO3}$ values of OA and OB cabbage samples are lower (mean $\delta^{18}O_{NO3}$ = +19.1‰ and +17.8‰, respectively). However, the $\delta^{18}O_{NO3}$ values of cabbage from three agricultural systems overlap, and thus the observed difference among the agricultural systems is not significant enough to discriminate conventional and organic cabbage samples (Figure 3.7). Little differences in the $\delta^{18}O_{NO3}$ values among three agricultural systems are observed in carrot, onion and faba bean samples (Figure 3.6). In the case of faba beans, the lack of the difference in the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values among three agricultural systems can be attributed to the fact that faba beans did not receive external nitrogen fertilisation and solely relied on nitrogen

fixation from air, and thus the difference in the isotope composition of fertilisers could not be observed in beans.

Insignificant difference in the $\delta^{18}O_{NO3}$ values between organic and conventional systems in carrot and onion samples can arise due to the different geographical location of the field plots of these vegetables compared to potatoes. In comparison with potato plots that were located in Foulum, Jundevad and Flakkebjerg, cabbage, carrots and onions were cultivated in a different geographical location – Aarslev. Different soil profile and higher annual temperature in Aarslev (Appendix B, Tables B1 and B2) could have resulted in the ^{18}O-enrichment of nitrate in the organic soils prior to plant uptake. This may be due to the ^{18}O-enrichment of soil water as a result of possibly higher evaporation or due to increased nitrification and denitrification rates in organic soils in Aarslev compared to other experimental locations. Denitrification can introduce significant alterations in the isotopic composition of soil nitrate resulting in the enrichment in heavy isotopes in the remaining soil nitrate pool (Mariotti et al. 1981, Handley and Raven 1992, Högberg 1997, Evans 2001, Mayer et al. 2002, Fukada et al. 2003), and may have had shifted the $\delta^{18}O_{NO3}$ of vegetables in organic systems in Aarslev towards more positive values.

Another factor which can account for lack of significant differences in the $\delta^{18}O_{NO3}$ values of conventional and organic cabbage, carrots and onion samples in comparison with potatoes, is the morphological and physiological difference among these vegetable species. Analyzed vegetables represent different plant families and species, and different plant organs: tubers (potato), leaves and stems (onion, cabbage), roots (carrot) and fruits (beans). Nitrate assimilation introduces a significant nitrogen and oxygen isotope fractionation (Handley and Raven 1992, Yoneyama et al. 1993) and occurs differently in different plant species and different plant organs (Evans et al. 1996, Robinson et al. 1998). In many plant species, nitrate is assimilated in roots as well as leaves, and a continuous translocation of nitrate is observed between these plant organs (Handley and Raven 1992, Evans et al. 1996, Kendall 1998, Robinson et al. 1998, Evans 2001). This constant translocation of the assimilated and unassimilated nitrate may result in the simultaneous presence of different nitrate pools with different isotope compositions in leaves and roots (Robinson et al. 1998). Therefore, the analysis of isotope composition of nitrate extracted from leaves/roots may be less successful in reflecting the isotope

composition of soil nitrate compared to plant organs that do not directly participate in nitrate assimilation and serve mainly as storage organs (e.g. fruits, tubers). Storage organs do not have a continuous flow of nitrate in and out of the organ, and thus can preserve the isotopic signature of the nitrate taken up from soil better than in the case when nitrate is being constantly transported, assimilated and mixed. This may be one of the reasons why the oxygen isotope composition of nitrate showed greater differences between conventional and organic potato samples (tubers), but was not successful enough to discriminate other analysed vegetable species. The analysis of oxygen isotope composition of nitrate in a wider range of vegetable species representing plant storage organs, such as for example tomatoes, peppers, aubergines, cucumbers, sweet potatoes etc., are needed to evaluate the discriminative power of the compound-specific approach in those vegetables species.

3.4.4. Differences in the stable isotope composition of nitrate from organic and conventional retail vegetables

Oxygen isotope composition of nitrate showed high discriminative potential in conventional and organic potato samples from the field trials. The compound-specific approach has been further applied to evaluate its capability for the discrimination between conventional and organic retail potato samples from different geographical locations (UK, Jersey, Israel and Egypt) as well as between conventional and organic retail tomato samples from UK.

The difference in the $\delta^{18}O_{NO3}$ values between conventional and organic samples is significant ($p<0.05$) for both retail potato and tomato samples. Conventional potatoes have significantly higher ($p<0.05$) $\delta^{18}O_{NO3}$ values compared to organic potatoes (mean $\delta^{18}O_{NO3}$ = +22.6‰ and +15.1‰, respectively). Similarly, the $\delta^{18}O_{NO3}$ values of conventional tomatoes (mean $\delta^{18}O_{NO3}$ = +45.3‰) are significantly higher ($p<0.05$) than those of organic tomato samples (mean $\delta^{18}O_{NO3}$ = +27.3‰) (Figure 3.9). Both potatoes and tomatoes are plant organs that do not directly participate in nitrate assimilation and serve mainly as storage organs, therefore the oxygen isotope composition of nitrate from conventional and organic soil has been successfully reflected in the $\delta^{18}O_{NO3}$ values of these two vegetable species.

The CDA of retail potato samples showed a significant correlation of the $\delta^{18}O_{NO3}$ with the discriminant function (Appendix B, Table B17) and resulted in

84.8% of cross-validated potato samples to be correctly classified (Table 3.10). A factor that may have introduced variation in the $\delta^{18}O_{NO3}$ values of retail potato samples is the geographical origin of the samples. Most of the conventional potato samples provided by the Food and Environment Research Agency for the present study came from either UK or Jersey, with only a few samples originating from Egypt and Israel (Appendix B, Table B15). In contrast, none of the organic samples originated from UK, most of the samples came from Israel with four samples being from Egypt. Climatic conditions in Egypt and Israel differ greatly from UK: mean annual temperature in Israel and Egypt is +17.4 °C and +22.0 °C, respectively, compared to +10.4 °C in UK. Also the annual amount of precipitation in UK is more than 20 times higher compared to Egypt: 594 mm and 26 mm, respectively (World Climate and Temperature Information, http://www.climatetemp.info). Stable oxygen composition of precipitation is -6.2‰, -4.4‰ and -7.7‰ for Israel, Egypt and UK, respectively (Bowen 2012). Higher $\delta^{18}O$ values of precipitation in Israel and Egypt, hotter climatic conditions and higher rate of water losses from soils can significantly increase the $\delta^{18}O$ values of soil water in those countries. As two oxygen atoms are taken from soil water during microbial nitrification (Andersson and Hooper 1983, Kumar et al. 1983, Hollocher 1984, Dispirito and Hooper 1986), nitrate which originates in soil with higher $\delta^{18}O$ values of soil water will be more ^{18}O-enriched, and consequently will result in a more ^{18}O-enriched plant nitrate. As organic potato samples from Israel and Egypt were compared to conventional potatoes from UK, the difference in the $\delta^{18}O_{NO3}$ values between conventional and organic agricultural systems may have been diminished due to the ^{18}O-enriched values of nitrate in organic potato samples.

Significant difference between two types of potato samples and high percentage of correctly classified samples suggest that the compound-specific oxygen isotope analysis has a potential in discriminating potato samples from conventional and organic agricultural practises, however may be less successful when applied to samples originating from geographical locations with distinct climatic differences.

The probability densities of the normal distribution curves for the expected entire organic and conventional potato populations generated on the basis of the $\delta^{18}O_{NO3}$ values show that 95% of the modelled organic population (p=0.05) would be expected to have $\delta^{18}O_{NO3}$ values between +11.6‰ and +22.2‰, and 99% of the

modelled organic population (p=0.01) would be expected to have $\delta^{18}O_{NO3}$ values in the range from +9.8‰ and +24.0‰ (Table 3.11, Figure 3.11). This suggests that a potato sample of an unknown origin with a $\delta^{18}O_{NO3}$ value higher than +24.0‰ would be statistically highly unlikely to be drawn from a population with the same mean as set for organic potatoes analysed in this study, and therefore could be considered as conventional.

Setting threshold $\delta^{18}O_{NO3}$ value for the organic potato population would be complicated because $\delta^{18}O_{NO3}$ values of conventional potato population overlap with those of organic population and therefore, the classification of potatoes of the unknown origin as organic/conventional is subject to an uncertainty. The $\delta^{18}O_{NO3}$ values of conventional potato population would likely (p=0.05) fall into a range from +15.6‰ to +30.3‰, and highly likely (p=0.01) – into the range between +13.0‰ and +32.8‰. Thus, a potato sample of an unknown origin that has $\delta^{18}O_{NO3}$ value lower than +13‰ would be highly unlikely to be drawn from a population with the same mean as set for the conventional potatoes analysed in this study and thus, could be considered organic. These findings suggest that due to the overlapping $\delta^{18}O_{NO3}$ values of conventional and organic potato populations, the oxygen isotope approach cannot be considered useful for the authentication of organic potato samples if used alone, however it may serve as a useful supporting evidence for discriminating between organic and conventional potatoes; low $\delta^{18}O_{NO3}$ values (<13‰) are typical of organic samples while high $\delta^{18}O_{NO3}$ values (>24‰) are more typical of conventional samples.

3.5. Conclusions

The present study focused on the application of bulk and compound-specific stable isotope analyses for the discrimination between field grown vegetables from three different agricultural systems: conventional with the application of synthetic NPK fertilisers, organic with the application of animal manure (OA) and organic with the use of cover crops (OB). Differentiation between conventional and two types of organic vegetable samples was performed on the basis of total nitrogen content and the nitrogen composition of bulk vegetable mater as well as, for the first time, using nitrogen and oxygen isotope composition of vegetable-derived nitrate.

Synthetic NPK fertilisers and animal manures used in the present study have distinctive $\delta^{15}N_{bulk,}$ $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values, which reflect the difference in the isotope composition of the sources of these fertilisers. Nitrogen isotope composition of bulk vegetables samples exhibits differences among three agricultural systems, and in all vegetable species the $\delta^{15}N_{bulk}$ values of conventional samples are lower compared to those of samples from two organic systems. The greatest difference in $\delta^{15}N_{bulk}$ values is observed between vegetable samples from conventional and OA system. The $\delta^{15}N_{bulk}$ values of potato, cabbage and onion from OA system (+4.5‰, +7.5‰ and +5.7‰, respectively) are significantly higher ($p<0.05$) compared to the corresponding samples from conventional system (+1.3‰, +2.9‰ and +1.0‰, respectively). In these vegetable species, $\delta^{15}N_{bulk}$ values reflect the difference in the nitrogen isotope composition of the applied organic animal manure and synthetic NPK fertilisers. In contrast, significant difference ($p<0.05$) between vegetable samples from conventional and OB system is observed only in onions and reveals the limitations of the bulk nitrogen isotope analysis for the differentiating between agricultural systems relying on synthetic fertilisation versus application of cover crops.

Total nitrogen content shows significant differences between conventional and both organic systems only in carrots, and for this vegetable species is a better discriminator compared to bulk nitrogen isotope composition.

A novel analytical approach – stable nitrogen and oxygen isotope analysis of vegetable derived nitrate – has introduced a significant improvement (>35%) in the discrimination of potato samples from conventional and organic systems on the basis of oxygen isotope composition of nitrate. The $\delta^{18}O_{NO3}$ values of conventional potato samples (mean $\delta^{18}O_{NO3}$ = +23.4‰) are significantly higher ($p<0.05$) compared to

those of potatoes from OA and OB systems (mean $\delta^{18}O_{NO3}$ = +18.1‰ and +17.9‰, respectively). The canonical discriminant analysis (CDA) of potato samples indicates that the $\delta^{18}O_{NO3}$ has the greatest discriminative power in the classification of potato samples into one of three agricultural systems compared to other variables (TN, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$).

The compound-specific oxygen isotope analysis of nitrate has been further applied to evaluate its capability for the discrimination between conventional and organic retail potato samples from different geographical locations (UK, Jersey, Israel and Egypt) as well as between conventional and organic retail tomato samples from UK. The difference in the $\delta^{18}O_{NO3}$ values between conventional and organic samples is significant ($p<0.05$) in both retail potato and tomato samples. Conventional potatoes have significantly higher ($p<0.05$) $\delta^{18}O_{NO3}$ values compared to organic potatoes (mean $\delta^{18}O_{NO3}$ = +22.6‰ and +15.1‰, respectively). Similarly, the $\delta^{18}O_{NO3}$ values of conventional tomatoes (mean $\delta^{18}O_{NO3}$ = +45.3‰) are significantly higher ($p<0.05$) than those of organic tomato samples (mean $\delta^{18}O_{NO3}$ = +27.3‰).

The CDA of retail potato samples with $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ used as predictor variables resulted in 84.8% of correctly classified samples. Compound-specific oxygen isotope analysis has a potential in discriminating potato samples from conventional and organic agricultural practises, however may be less successful when applied to samples originating from geographical locations with distinct climatic differences.

Based on the generated probability densities of the normal distribution curves for the expected entire organic and conventional potato populations, upper and lower limits for the $\delta^{18}O_{NO3}$ values have been set for the organic and conventional potato populations. 99% of the modelled organic population ($p=0.01$) would be expected to have $\delta^{18}O_{NO3}$ values in the range from +9.8‰ and +24.0‰, while the $\delta^{18}O_{NO3}$ values of conventional potato population would highly likely ($p=0.01$) fall into the range between +13.0‰ and +32.8‰. Due to overlapping $\delta^{18}O_{NO3}$ values of conventional and organic potato populations, the oxygen isotope approach cannot be suggested as useful for authentication of organic potato samples, if used alone, however may serve as a useful supporting evidence for discriminating organic and conventional potatoes; low $\delta^{18}O_{NO3}$ values (<13‰) are typical of organic samples while high $\delta^{18}O_{NO3}$ values (>24‰) are more typical of conventional samples.

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CHAPTER 4

APPLICATION OF MOLECULAR AND STABLE ISOTOPE ANALYSES OF N-ALKANES FOR THE GEOGRAPHIC CLASSIFICATION OF MEDITERRANEAN EXTRA VIRGIN OLIVE OILS

Abstract

Traceability of the geographic origin of virgin olive oils has become an important issue from both commercial and health perspectives. *n*-Alkanes, which are present in the hydrocarbon fraction of olive oil, are components of the cuticular wax fraction of olive fruits, and have been studied to a limited extent.

In this study the molecular and, for the first time, stable carbon and hydrogen isotope compositions of *n*-alkanes have been determined in extra virgin olive oils from eight Mediterranean countries (Italy, France, Croatia, Slovenia, Portugal, Spain, Greece, Morocco) and evaluated for their capability to differentiate oil samples into the Northern and Southern Mediterranean regions.

The most prevalent *n*-alkanes in olive oils are: *n*-C_{23}, *n*-C_{24}, *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31}. However, the distribution of these compounds is different depending on the country of origin, and result in differences in the *n*-alkane average chain length (ACL) values of oils from different locations. Total concentration of *n*-alkanes is significantly higher ($p<0.05$) in olive oils from the Northern compared to Southern Mediterranean region.

Stable carbon and hydrogen isotope compositions of *n*-C_{29} alkane ($\delta^{13}C_{C29}$ and δD_{C29}) in olive oils from eight production countries exhibit significant variation. $\delta^{13}C_{C29}$ and δD_{C29} values are significantly more positive ($p<0.05$) in olive oils from the Southern compared to Northern Mediterranean region and show significant correlations with latitude, the δD of precipitation and the mean temperature during the period of oil accumulation in the olives (August-December). Canonical discriminant analysis (CDA) reveals that δD_{C29} values have the highest discriminative potential for the classification of olive oils into their production region. Overall 93.4% of olive oil samples have been correctly classified. Compound-specific stable isotope analyses of *n*-alkanes can be a useful tool for the

regional classification of olive oils, and may find applications also for the geographical provenancing of other products of plant origin.

4.1. Introduction

4.1.1. Virgin olive oil: main production regions and current situation on the market

Virgin olive oil is obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alterations in the chemical composition of the oil. To be labelled as “virgin” olive must not have undergone any treatment other than washing, decanting, centrifuging, and filtering (Codex Alimentarius Commission 2003). “Extra virgin” is a superior category of olive oil; it is characterised by free acidity (defined as the amount of free fatty acids expressed as grams of oleic acid per 100 g of oil) below 0.8 g/100 g of oil (European Commission 2002, Codex Alimentarius Commission 2003). Virgin olive oil is highly valued among consumers due to its nutritional properties, sensory characteristics and multiple health benefits (Harwood and Aparicio 2000).

At present over 850 million olive trees are grown worldwide (International Olive Oil Council 2000), and more than 1250 olive tree varieties are known (Bartolini 2008). According to the latest statistical data provided by the International Olive Oil Council, 79% of the global production of olive oil takes place in the Mediterranean region with Spain, Italy and Greece being the main oil producers (Figure 4.1). Nevertheless, due to greatly appreciated nutritional and health properties of olive oil, olive cultivation has been increasing in other regions of the world, such as North and South America, and Oceania. The leading oil producers outside the European Union are Tunisia, Syria, Turkey, Morocco and Algeria (International Olive Oil Council 2012).

Olive tree cultivation requires specific climatic conditions, i.e. temperature, humidity, and altitude. Olive oil production in the Mediterranean region is very common because of its climate, which is characterised by relatively mild winters and hot, dry summers. The areas belonging to this climate typically lie between 30° and 45° north and south latitudes (International Olive Oil Council 2000). Olive trees are drought resistant and because of their extensive root system are one of the few crops that can survive on only 200-300 mm of annual rainfall (Fresco 1996). The trees grow slowly, mature only after about ten years, but can easily reach an age of more than a century.

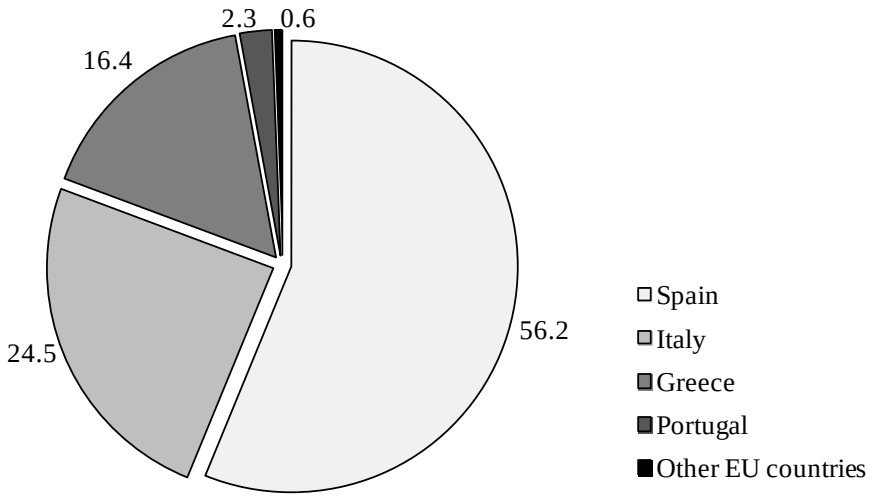

Figure 4.1. Olive oil production (%) in European Union for the period 2005-2012 (International Olive Oil Council 2012).

The highest consumption of olive oils is concentrated mainly in the production regions. Countries of the EU consume more than 65% of olive oil produced in the world, and 80.1% of this amount corresponds to the three leading oil producers – with Italy being the leading consumer, followed by Spain and Greece (Figure 4.2). Outside the EU, countries with high olive oil consumption include the USA, Syria, Turkey, Morocco, Brazil and Australia (International Olive Oil Council 2012).

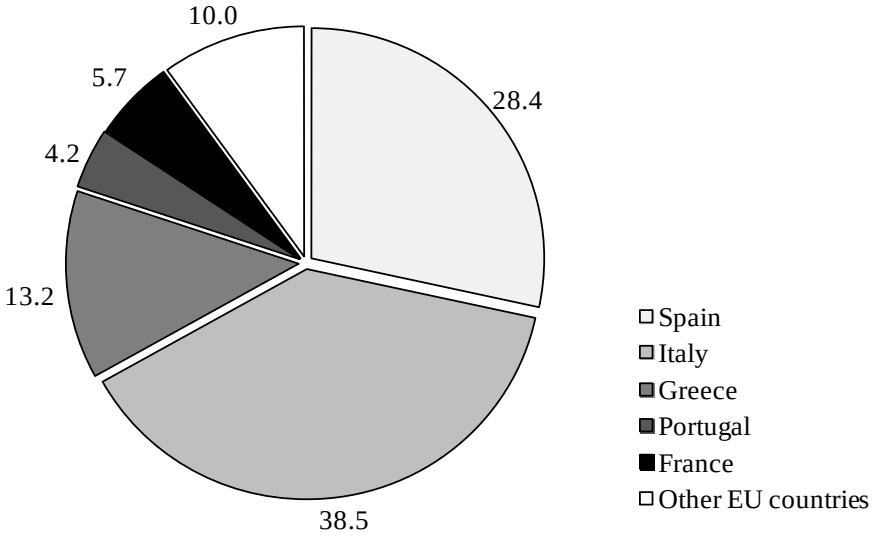

Figure 4.2. Olive oil consumption (%) in European Union for the period 2005-2012 (International Olive Oil Council 2012).

Virgin olive oil is an expensive product, and its price is usually four to five times higher compared with common vegetable oils, such as corn or sunflower oil (European Commission 2003a). This high market price of virgin olive oil is due to its highly valued organoleptic and nutritional properties, and laborious olive cultivation, harvesting and processing practises (Harwood and Aparicio 2000, Lerma-García et al. 2010). The taste and quality of virgin olive oils from different geographical origins varies as a consequence of distinct local agricultural traditions such as olive variety, oil extraction technique, and oil blending practice (European Commission 2003b). To protect the oils of a particular geographical origin, the European Commission has set regulations on the protection of claims relating to geographic indications and designations of origin for agricultural products and foodstuffs (European Commission 2006). Thus, currently virgin olive oils produced in defined oil-producing regions may use a protected designation of origin (PDO) or protected geographical indication (PGI) on their labels. For this reason, geographic origin is an essential element of olive oil authenticity. To protect the market from fraudulent practises and false label claims, a wide range of analytical strategies have recently emerged to confirm olive oil authenticity (Harwood and Aparicio 2000). However, there is still a great need of new reliable methodologies for tracing geographical origin of virgin olive oils.

4.1.2. Molecular composition of virgin olive oil

Composition of virgin olive oil determines its intrinsic quality, oxidative stability, sensory and health properties (Bendini et al. 2007). Olive oil constituents can be divided into two major groups: i) saponifiable fraction and ii) so called “minor components”. Saponifiable fraction mainly consists of triglycerides, free fatty acids and phospholipids, and makes up over 98% of total olive oil composition (Harwood and Aparicio 2000, Lercker and Rodriguez-Estrada 2000). The therapeutic properties of olive oil are often attributed to its high levels of monounsaturated fatty acids with oleic acid (18:1) being the major component (65-85 wt %) (Gunstone1996).

The minor components of virgin olive oil are classified into two types: the unsaponifiable fraction, defined as the fraction extracted with solvents after the saponification of the oil, and the soluble fraction, which includes the phenolic

compounds. The unsaponifiable fraction, which accounts only for about 0.5-2% of the virgin olive oil, is rich in minor components that have antioxidant and anti-inflammatory properties. The major components of the unsaponifiable fraction of olive oil are: hydrocarbons, tocopherols, linear fatty alcohols, di- and triterpenic alcohols, methylsterols, sterols, and polar pigments (chlorophylls and pheophytins) (Boskou 2000, Harwood and Aparicio 2000, Lercker and Rodriguez-Estrada 2000, Kiritsakis and Christie 2000, Owen et al. 2000, Covas et al. 2006).

Hydrocarbons are the main components of the unsaponifiable fraction of olive oil. The major component of the hydrocarbon fraction is squalene, a precursor of other sterols and triterpenoid alcohols (Owen et al. 2000, Lercker and Rodriguez-Estrada 2000, Bortolomeazzi et al. 2001). It has been proposed that the high squalene content of olive oil plays a role in the cancer risk-reducing properties of olive oil (Newmark 1999). Besides squalene, the hydrocarbon fraction of olive oil comprises low quantities of *n*-alkanes, *n*-alkenes, sesquiterpenes and other terpenic hydrocarbons (Lanzon et al. 1994, Guinda et al. 1996, Koprivnjak et al. 2005, Bueno et al. 2005).

Tocopherols present in olive oil contribute to the total pool of vitamins and antioxidants (Covas et al. 2006). Phytosterols are reported to exhibit anti-inflammatory, antibacterial, antifungal, fever reducing activities, and blood cholesterol level reducing properties (Pérez-Jiménez et al. 2007, Temime et al. 2008). Phenolic compounds from olive oils have antioxidant effects and play a key role in preventing oil oxidation which significantly increases storage stability of virgin olive oils (Rahmani and Saari Csallany 1998, Gimeno et al. 2002). Other constituents of the unsaponifiable matter are pigments (carotenoids, chlorophylls, and pheophytins) which impart characteristic colour to olive oil (Minguez-Mosquera et al. 1990).

4.1.3. Previous studies of hydrocarbon fraction of olive oil

Some components of the unsaponifiable fraction of olive oil, such as sterols, tocopherols and terpenic alcohols have been extensively studied due to their importance both for characterization and for assessing the quality of oil. However, the hydrocarbon composition of olive and other edible oils has been studied only to a limited extent.

In natural products hydrocarbons are present in the unsaponifiable fraction in rather small amounts – on average 0.2% of the total lipid content. Olive oil has exceptionally high hydrocarbon concentration (0.5-0.7% of the total lipid fraction) (Bastic et al. 1978, Lanzon et al. 1994, Lercker and Rodriguez-Estrada 2000, Owen et al. 2000, Bortolomeazzi et al. 2001).

One of the first reported studies on hydrocarbon composition of olive oil was done by Capella et al. (1963) who analyzed hydrocarbons in several vegetable oils, and reported the presence of squalene and *n*-alkanes (C_{11} - C_{30}) in olive oils. Authors also mentioned that *n*-alkanes with an odd number of carbon atoms predominated over even numbered carbon chains. Eisner et al. (1965) reported that squalene made up around 80-90% of the hydrocarbon fraction, the rest was composed of a homologous series of normal chain, iso-, anteiso-, and multiply branched hydrocarbons in the range of C_{16}-C_{36}. Bastic et al. (1978) reported the presence of *n*-alkanes, isoprenoidal polyolefins, and squalene in virgin olive oils, with the latter comprising more than 90% of the hydrocarbon fraction. The same authors concluded that the compositions of the hydrocarbon fractions of vegetable oils showed differences which could be used for oil characterization. McGill et al. (1993) studied the composition of *n*-alkanes in retail samples of edible oils. They reported that *n*-alkanes with chain lengths of C_{27}, C_{29} and C_{31} predominated in all of the plant oils except olive oil, where C_{23}, C_{25} and C_{27} were the most significant. Moreover, it was suggested that the *n*-alkane compositions may be useful in characterising different edible oils. In another study the hydrocarbon fraction of virgin olive oil and its changes resulting from refining was analyzed by Lanzon et al. (1994). Authors analyzed numerous Spanish virgin olive oils and detected the series of *n*-alkanes from C_{14} to C_{35}, squalene as well as 6,10-dimethyl-1-undecene, *n*-8-heptadecene and various sesquiterpenes. In refined oils, a notable feature was the absence of the most volatile compounds and the appearance of other hydrocarbons produced during the refining process. Squalene was reported to be the major hydrocarbon component in all oils, both virgin and refined. Guinda et al. (1996) found significant differences in the content of some hydrocarbons from five Spanish olive varieties and suggested that the hydrocarbon fraction could be used to differentiate those oils. Webster et al. (2000) demonstrated that principal component analysis applied to *n*-alkane concentrations could differentiate olive oil from other vegetable oils as well as distinguish olive oils from different geographical regions.

More recently, Koprivnjak et al. (2005) used the distribution of *n*-alkanes for authenticating three Croatian olive varieties. Linear discriminant analysis based on the *n*-alkane components was able to correctly identify olive variety in 97.5% of the oil samples. Bueno et al. (2005) also used discriminant analysis applied to the hydrocarbon fraction of Spanish virgin olive oils and reported that 90% of samples were classified correctly according to their variety. Thus, the composition of the *n*-alkane fraction is essentially unique in virgin olive oils. This suggests that *n*-alkane analysis has the potential be a useful tool in olive oil authenticity studies, particularly when combined with other analytical techniques and statistical analysis.

4.1.4. Factors that impact molecular composition of olive oil

Chemical composition of virgin olive oil can be influenced by a variety of factors which can be classified into five major groups: i) genetic (olive variety), ii) environmental (soil, climate), iii) agronomic (irrigation, fertilisation), iv) cultivation (time of harvesting), and v) technological (fruit storage, extraction procedure) factors (Aparicio and Luna 2002, Baccouri et al. 2008).

Olive variety has been reported to have a major impact on the molecular composition and sensory attributes of olive oil. This is because olive varieties exhibit differences in the amounts and types of chemical compounds present in their fruits (Aparicio et al. 1997, Harwood and Aparicio 2000). For example, Aparicio et al. (1997) analyzed composition (31 non-volatile compounds and 65 volatile compounds) of virgin olive oil samples from four varieties (Arbequina, Coratina, Koroneiki, and Picual), and reported significant differences in chemical components among the analyzed varieties. Taamalli et al. (2010) found significant differences in the molecular composition of olive oils from six Tunisian olive varieties, collected at the same season and processed in the same way. Similarly, inter-varietal differences in respect to chemical composition were reported in other studies (e.g. by Esti et al. 1998, Romani et al. 1999, Pinelli et al. 2003, Dhifi et al. 2004, Cerretani et al. 2006, Arslan and Özcan 2011.

Environmental factors, such as geography and climate of the oil production region, also have a great impact on the molecular composition of olive oil. In the study by Aparicio et al. (1994) 126 samples of virgin olive oil from two different locations with different climatology and altitude (valley < 400 m and mountains >

700 m) were characterized by 53 chemical compounds. Authors reported that sterols, some triterpenic alcohols and hydrocarbons changed systematically with altitude. Significant effect of the geographical location on the molecular composition of olive oil was also reported in other studies (e.g. Mousa et al. 1996, Guerfel et al. 2009, Issaoui et al. 2010, Youssef et al. 2011). For example, oils from high altitudes were reported to have higher chlorophyll content compared with low altitudes, which was explained by a slower maturation rate of olive fruits at higher altitudes (Mousa et al. 1996, Issaoui et al. 2010). The ratio unsaturated/saturated fatty acids was generally greater in oils from high elevations, which was explained by the impact of the temperature, i.e. low temperatures at higher altitudes favour oil unsaturation (Mousa et al. 1996, Issaoui et al. 2010).

Agronomic factors, such as olive orchard irrigation and fertilisation, have been reported to impact chemical composition of olive oil. For example, the concentration of phenolic compounds was found to be significantly lower in oils from irrigated orchards compared to rain fed olive groves (Servili et al. 2004, Gómez-Rico et al. 2007). In addition, time of olive harvesting has been reported to have a significant impact on the content of various chemical compounds (e.g. total phenols, sterols, pigments, fatty acids, volatile compounds) in olive oils, which is due to a continuous change in the molecular composition of olive fruits during maturation (Aparicio and Morales 1998, Gutiérrez et al. 1999, Caponio et al. 2001, Salvador et al. 2001, Youssef et al. 2010). What is more, different oil extraction systems have an impact on the chemical composition of olive oils due to a difference in olive fruit crushing techniques, centrifugation systems, absence or presence of filtration (Angerosa and Di Giovacchino 1996, Ranalli and Angerosa 1996, Di Giovacchino 2000).

The influence of these various geographical, botanical, and production factors is highly unlikely to be the same in different oil production regions, consequently molecular composition contain valuable information for the geographical characterisation of olive oils.

4.1.5. Stable isotope ratio analyses of olive oil

Stable isotope ratio analysis has been introduced as a promising technique for characterising geographical origin of olive oils (Camin et al. 2010a, Camin et al. 2010b). Stable isotopes can represent a fingerprint of the climatic and environmental

conditions in the areas of oil production. Despite the great variety of chemical compounds constituting olive oil, their elemental composition can be reduced principally to three elements: carbon, hydrogen, and oxygen, and therefore stable isotope ratios which are most widely used for olive oil authentication are: $^{13}C/^{12}C$, $^{18}O/^{16}O$ and $^{2}H/^{1}H$.

Stable isotope analyses of olive oils have been mostly performed on bulk oil, however some studies included analysis of individual oil compounds, such as fatty acids (Spangenberg et al. 1998), sterols and aliphatic alcohols (Angerosa et al. 1999) and glycerol (Camin et al. 2010a). The majority of published studies focused on the analysis of stable carbon and oxygen isotope ratios. Spangenberg et al. (1998) characterized olive oils from different EU countries using stable carbon isotope analysis of individual fatty acids. Results allowed discrimination between genuine extra virgin olive oil and other quality olive oils, and also served as a way of assessing whether olive oil was blended with refined olive oils or other vegetable oils. Angerosa et al. (1999) compared olive oils from different Mediterranean countries on the basis of stable carbon and oxygen isotope analysis of bulk oil and some of its fractions (sterols and aliphatic alcohols). The results, obtained by applying statistical analyses, provided information about the differences in climatic conditions in different locations and showed that it was not possible to obtain a clear differentiation of the producing countries with a similar climate. Chiavaro et al. (2011) used a variety of methodologies for the geographical and botanical classification of 53 olive oil samples from Italy and Croatia. Stable carbon and oxygen isotope analysis and two other analytical techniques – triglycerol and fatty acid composition and cooling properties measured by differential scanning calorimetry – were used in their study. The $\delta^{18}O$ values of olive oils from different regions showed significant differences and resulted in a good discrimination of the samples.

Over recent years stable hydrogen isotope analysis has been introduced to olive oil authenticity studies, and has shown high discriminative capabilities. Bontempo et al. (2009) measured stable C, O and H isotopes in Italian oil samples, and reported that olive oils from the Adriatic and Tyrrhenian coasts could be distinguished mainly on the basis of their δD values. Camin et al. (2010a) performed authentication of Italian olive oils using stable isotope ratios of C, O and H as well as elemental composition. Authors reported significant differences among the production years

and in some cases between PDOs of oil from the same Italian regions. Another extensive study on European olive oils was also conducted by Camin et al. (2010b) within the framework of the European TRACE project. $^{2}H/^{1}H$ ratios of olive oils showed promising geographical discrimination capability.

4.1.6. Factors that impact isotopic composition of olive oils

Stable carbon isotope composition of plants is mostly affected by the type of carbon fixation pathway, i.e. C3, C4 and CAM. However, within a single plant species such as olive (*Olea europaea*), variation in carbon isotope values are influenced by genetics (different varieties) as well as by a range of environmental factors. These factors include: irradiance, temperature below or well above the growth optimum, relative humidity and amount of precipitation, atmospheric partial pressure of CO_2, plant age and maturation (Smith and Epstein 1971, O'Leary 1981, Farquhar et al. 1989, Tieszen 1991, Ehleringer and Dawson 1992, Martin and Martin 2003).

Stable oxygen and hydrogen isotope composition of plant organic matter is related to that of the soil water that feeds the plant. The $\delta^{18}O$ and δD values of ground water are strictly related to the isotopic composition of the local precipitation, which in turn depends on several climatic and environmental factors. The major drivers of variation in stable oxygen and hydrogen ratios of precipitation are: atmospheric temperature, altitude, distance from the sea, and the amount of precipitation. The $\delta^{18}O$ and δD values of meteoric water generally show positive correlation with temperature and negative correlation with distance from the sea, elevation and the amount of atmospheric precipitation. In respect to plants, a factor that may significantly affect the oxygen and hydrogen isotope composition of leaves and fruits is evapotranspiration, which increases, for example, in low relative humidity and high temperature conditions (Dansgaard 1964, Yurtsever 1975, Kendall and Caldwell 1998, Marshall et al. 2007).

The impact of various geographic and climatic factors on the isotopic composition of olive oils has been reported previously. Aramendia et al. (2007) measured stable oxygen isotope ratios of Andalusian olive oils and reported that $\delta^{18}O$ values of bulk olive oils showed correlation with latitude. Moreover, authors reported differences in stable carbon isotope composition of oils extracted from different olive

varieties. Iacumin et al. (2009) measured stable oxygen and carbon isotopic values of 150 samples of extra virgin olive oil from eight different Italian regions and from three different production years. Their results showed a strong correlation of the $\delta^{18}O$ values of bulk oil with latitude, mean annual temperature, and mean relative humidity in oil production locations. This allowed discrimination of different years of olive oil production. The $\delta^{13}C$ values of oil showed correlation with latitude only and, consequently were reported to be less suitable for discriminating the geographic origin of oil. Bontempo et al. (2009) measured stable C, O and H isotope values in Italian oil samples, and reported that δD values of olive oils were influenced by the amount of rainfall, along with the average temperature and humidity. Camin et al. (2010b) reported that the stable H, C, and O isotope composition of bulk olive oils from five EU countries correlated with the climatic (mainly temperature) and geographical (mainly latitude and distance from the coast) characteristics of the provenance sites.

The above studies demonstrate that differences in latitude, mean annual temperature and precipitation among oil production locations can result in significantly different isotopic values of olive oils and thus, could be useful for their geographical provenancing.

4.1.7. Rationale for the study

Because of the expanding market of olive oil and great variation in prices, authentication of its geographic origin has become an important issue. Stable isotope analysis of bulk olive oils has been used previously for authentication purposes (e.g. Aramendia et al. 2007, Bontempo et al. 2009, Iacumin et al. 2009, Chiavaro et al. 2011). However, distinct isotopic fractionations during the biosynthesis of various compound classes (e.g. sugars, lipids) in olive plants may lead to significant variations in the bulk isotope values of olives and consequently in olive oil. Compound-specific stable isotope analysis can provide information regarding the isotopic composition of individual compounds, and has recently been applied for assessing the quality and authenticity of olive oils (e.g. Angerosa et al. 1999, Spangenberg and Dionisi 2001, Camin et al. 2010a). An integration of stable isotopic measurements with other analytical techniques has been suggested as a powerful tool

for olive oil authentication, particularly if coupled with multivariate statistical analyses (Camin et al. 2010a, Camin et al. 2010b, Chiavaro et al. 2011).

The chemical composition of extra virgin olive oil is very complex with various minor compounds present in the unsaponifiable fraction. Identifying a specific compound or a group of compounds that could be used for authentication purposes is a labour intensive and time-consuming process. Although hydrocarbons in olive oils have been studied to a limited extent, existing studies suggest that *n*-alkane analysis may be a useful tool for olive oil authentication (Lanzon et al. 1994, Guinda et al. 1996, Webster et al. 2000, Bueno et al. 2005, Koprivnjak et al. 2005).

n-Alkanes, present in olive oil, are components of the cuticular wax of the olive fruits (Bianchi et al. 1992), and are incorporated into oil during extraction. Until now, all studies on *n*-alkanes in olive oils have focused on their molecular composition. To our knowledge, currently there are no published data available on the isotopic composition of *n*-alkanes in olive oils.

The main goal of this study was to investigate whether molecular as well as hydrogen and carbon isotope compositions of *n*-alkanes exhibit differences in extra virgin olive oils from eight Mediterranean countries and could be used for regional differentiation of oils. In order to achieve this goal, the relationship between the molecular and isotopic compositions of olive oil *n*-alkanes and the geographic and climatic factors associated with oil production locations was analysed. Then concentrations and the distribution of *n*-alkanes as well as their isotopic compositions in olive oils from eight Mediterranean countries have been characterised. Further I screened both the molecular and isotopic parameters of *n*-alkanes in olive oils for significant differences between the Northern and Southern Mediterranean regions and evaluated their discriminative potential for the classification of oils into the production region.

4.2. Materials and methods

4.2.1. Sample origin

Samples of extra virgin olive oils (n=50) were obtained from the exhibition of premium quality extra virgin olive oil of the Mediterranean area and its supply chain "MEDOLIVA" (www.medoliva.it) in Arezzo (Tuscany, Italy) in May 2010. Samples were collected by Dr. Nikolai Pedentchouk (University of East Anglia, Norwich, UK). The samples were from seven different Mediterranean countries: Slovenia (n=3), Croatia (n=4), France (n=3), Italy (n=25), Spain (n=6), Portugal (n=5), and Morocco (n=4). Additionally, extra virgin olive oil samples from Greece (n=14) and France (n=12) were obtained from the European TRACE project and kindly provided by Dr. Jurian Hoogewerff (Oritain Global Ltd., Dunedin, New Zealand). All oil samples were stored at room temperature in glass vials in the dark.

Olive oil samples from "MEDOLIVA" were supplied with the following information: production region, particular production location, altitude, and olive variety. Samples from TRACE project were supplied with the following information: latitude, longitude, and olive variety.

For "MEDOLIVA" samples altitude and longitude coordinates were obtained using Google maps (http://maps.google.co.uk/maps). For TRACE samples altitude for each particular location was obtained from GIS maps at http://studio.messlinger.com/map. The δD values of precipitation for all locations were estimated using the Online Isotopes in Precipitation Calculator, Version 2.2. (Bowen 2012). The mean temperature for each location in the oil production year was obtained from the weather history database at http://www.wunderground.com. As oil accumulates in the mesocarp of olives over roughly 20 weeks (Harwood and Aparicio 2000), the mean temperature was calculated for the time period from August to December, as suggested by Bontempo et al. (2009).

Mean geographical parameters and the number of olive oil samples from each country are shown in Table 4.1. The full list of samples from each country, geographical coordinates of locations and olive varieties used for oil production in each location are shown in the Appendix C (Table C2).

Table 4.1. Mean geographical and climatic characteristics of olive oil production locations.

Country	Region (s)	n	Production year	Altitude, m asl	Latitude, dd	Longitude, dd	Temp., °C
Slovenia	Koper, Šmarje	3	2009	197±90	45.5	13.7	15.2
Croatia	Vodnjan, Pula	4	2009	94±42	44.9	13.9	15.2
France	Provence-Alpes-Côte d'Azur	15	2006, 2009	325±170	44.2	5.1	17.0
Italy (North)	Veneto	4	2009	195±0	45.5	11.2	14.4
Italy (Central)	Tuscany, Umbria, Lazio	14	2009	310±83	43.1	11.9	15.3
Italy (South)	Apulia, Sicily	7	2009	314±157	38.4	14.2	19.8
Portugal	Braganca, Ervosa-Trofa, Guarda, Santarem, Beja	5	2009	402±78	40.3	-7.8	16.3
Greece (North)	Central Macedonia	8	2006	240±0	40.1	23.9	16.4
Greece (South)	Peloponnese	6	2006	212±55	37.1	22.6	18.8
Spain	Jaen	6	2009	700±247	37.8	-3.6	16.8
Morocco	Meknes, El Hajeb	4	2009	500±0	33.8	-5.5	18.0

Values are the means and standard deviations (for altitude) for the n number of samples; m asl – meters above the sea level; dd – decimal degrees; Temp. – mean temperature (August-December).

4.2.2. *n*-Alkane extraction

n-Alkanes were isolated from olive oils using column chromatography. Olive oil sample (75 mg) was added to a 4 ml glass vial containing 0.5 ml of hexane. Pentadecane (*n*-C_{15}) (Sigma-Aldrich, Gillingham, UK) was used as an internal standard (concentration 2.50 mg/l) and added to each vial together with oil samples. Then *n*-alkanes from the acquired mixture were separated by the means of column chromatography. Glass Pasteur pipettes (145 x 9 mm) packed with activated silica gel (0.063-0.200 mm, Sigma-Aldrich, Gillingham, UK) were used as chromatography columns. Each olive oil sample was placed on a column, eluted with hexane (5 ml), and the eluent containing *n*-alkanes was collected in a 4 ml glass vial. Due to low *n*-alkane concentration each oil sample was prepared and extracted twice, and then two *n*-alkane eluents were combined, concentrated under a stream of high grade nitrogen down to 0.5 ml, and stored at room temperature until further analysis.

4.2.3. Gas chromatography

Gas chromatography analysis was performed using HP Agilent 7820A Gas Chromatograph (Agilent Technologies Inc., Wilmington, USA) with a flame ionisation detector (FID). Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The GC was equipped with a DB-5 capillary column (30m × 0.32 mm × 0.25 µm) (Agilent Technologies Inc., Santa Clara, USA) and programmed from 50 °C (held for 1 min) at 20 °C/min to 150 °C and further at 8 °C/min to 320 °C, and held for 10 min isothermally. The temperature of the injector and detector was 300 °C and 280 °C, respectively. Samples were injected using a split/splitless injector in a splitless mode. *n*-Alkanes were identified through comparison of elution times with the known *n*-alkane standard – a mixture of 15 *n*-alkanes: hexadecane (*n*-C_{16}), heptadecane (*n*-C_{17}), octadecane (*n*-C_{18}), nonadecane (*n*-C_{19}), eicosane (*n*-C_{20}), heneicosane (*n*-C_{21}), docosane (*n*-C_{22}), tricosane (*n*-C_{23}), tetracosane (*n*-C_{24}), pentacosane (*n*-C_{25}), hexacosane (*n*-C_{26}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}), nonacosane (*n*-C_{29}), and triacontane (*n*-C_{30}) (purchased from Dr. Arndt Schimmelmann; Department of Geological Sciences, Indiana University, Bloomington, USA). Individual *n*-alkane peak areas were calculated using ChemStation software (Agilent Technologies Inc., Wilmington, USA). *n*-Alkane

average chain length (ACL) was calculated using peak area values of individual *n*-alkanes with carbon chains from C_{19} to C_{35} according to the following formula:

$$ACL = \frac{(19 \cdot A_{19}) + (20 \cdot A_{20}) + (21 \cdot A_{21}) + \ldots + (35 \cdot A_{35})}{(A_{19} + A_{20} + A_{21} + \ldots + A_{35})} \quad (4.1.)$$

where A_x corresponds to the peak area of the individual *n*-alkane from the chromatograph trace.

n-Alkane concentration in olive oil samples was calculated on the basis of the known concentration and the peak area of the internal standard (*n*-C_{15}) according to the following formula, assuming that the response factor is equal to 1:

$$[S] = [St] \cdot \frac{A_S}{A_{St}} \quad (4.2.)$$

where [S] is the concentration of the individual *n*-alkane, [St] is the known concentration of the standard, A_S - peak area of the individual *n*-alkane, A_{St} - peak area of the standard.

4.2.4. Gas chromatography-isotope ratio mass spectrometry

Stable carbon and hydrogen isotope analyses of *n*-alkanes from olive oil were performed using Delta V Advantage isotope ratio mass spectrometer (IRMS) interfaced with GC-Isolink Trace Ultra GC Combustion and High temperature conversion systems (Thermo Scientific, Bremen, Germany). Individual *n*-alkanes were separated using a DB-5 capillary column (30m × 0.32 mm × 0.25 µm). Helium was used as a carrier gas at a flow rate of 1.2 ml/min and 1.0 ml/min for carbon and hydrogen isotope analysis, respectively. The GC oven was programmed from 50 °C (held for 1 min) at 20 °C/min to 150 °C and further at 8 °C/min to 320 °C and held for 10 min isothermally. Samples were injected using programmable temperature vaporization (PTV) injector in splitless mode at 280 °C.

Nonacosane (*n*-C_{29}) was chosen for isotopic characterisation of olive oils, because it was present in sufficient concentration for the GC-IRMS analysis in all oil samples (Appendix C, Table C3). Sample concentrations during carbon and hydrogen isotope analyses were adjusted to give the *n*-C_{29} peaks a similar intensity to those of the corresponding reference gas.

Combustion of *n*-alkanes to CO_2 was conducted at 1030 °C. Carbon isotope composition of *n*-C_{29} ($\delta^{13}C_{C29}$) was reported based on duplicate analyses of well-resolved peaks. For the majority of duplicate measurements the absolute difference in $\delta^{13}C_{C29}$ values was < 0.3‰, never exceeding 0.5‰.

Pyrolytic conversion of *n*-alkanes to H_2 was conducted at 1420 °C. The H_3^+ factor was determined daily using H_2 reference gas. Hydrogen isotopic composition of *n*-C_{29} (δD_{C29}) was reported based on duplicate analyses of well-resolved peaks. For the great majority of duplicate measurements the absolute difference in δD_{C29} values was < 5‰, never exceeding 8‰.

Analytical accuracy and precision of GC-IRMS system during stable carbon and hydrogen compound-specific analyses were determined using a standard mixture of 15 *n*-alkanes (isotopic ratios measured off-line by Dr. Arndt Schimmelmann; Department of Geological Sciences, Indiana University, Bloomington, USA and available at http://mypage.iu.edu/~aschimme), which was analyzed between every 10 samples. Carbon and hydrogen isotopic compositions of *n*-C_{29} were expressed relative to VPDB and VSMOW, respectively, based on a reference gases (CO_2, BOC, UK; H_2, >99.995% purity, BOC, UK) adjusted daily using the above mentioned standard mixture of 15 *n*-alkanes. Isodat software Version 3.0 (Thermo Scientific, Bremen, Germany) was used for processing the data of *n*-alkane measurements.

4.2.5. Statistical analyses

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). All data were checked for normality and homogeneity of variances. Data were normally distributed unless stated otherwise. Correlation analysis was performed to evaluate whether the linear relationship exists between any of the analysed molecular and isotopic parameters of olive oil samples ($\delta^{13}C_{C29}$, δD_{C29}, ACL and *n*-alkane concentration) and geographical factors (altitude, latitude, longitude, δD of precipitation). Correlation was tested using the Pearson correlation coefficient (r). The correlation coefficient indicates the strength and the direction (positive/negative) of the linear relationship between two variables. Coefficient values close to +1 or -1 indicate a strong linear relationship; values close to 0 indicate no linear relationship. The correlation was considered significant at $p < 0.05$.

Based on the results of the correlation analysis, the relationship between the δD_{C29} values of olive oils and the δD values of precipitation was studied further using simple linear regression analysis. The simple linear regression model assumes that there is a linear relationship between the dependent variable and the predictor variable, and is described with the following equation:

$$y = \alpha + \beta x + \varepsilon \quad (4.3.)$$

where y is the value of the dependent variable, α represents the y-intercept, β is the slope of the regression, x is the value of the predictor variable, and ε is the error term.

The regression equation describes the relationship between the dependent variable (y) and the predictor variable (x). The intercept (α), is the value of the dependent variable if the value of predictor variable is zero. Slope is a change in the dependent variable when predictor variable increases by one unit (Field 2000). The main goal of the regression analysis was to test how well the δD values of precipitation (predictor variable) can explain the variation in the δD_{C29} values of olive oils (dependent variable) around their mean.

For the regional classification of olive oils on the basis of the analysed molecular and isotopic parameters, oil production locations were divided into two regions, Northern (n=40) and Southern (n=36) Mediterranean, based on the mean values of latitude and the δD of precipitation. Independent sample t-test was applied to study the difference in the δD of precipitation, $\delta^{13}C_{C29}$, δD_{C29}, ACL, concentration of *n*-C_{29}, and the total *n*-alkane concentrations between the two regions. The difference between two groups was considered significant at $p < 0.05$.

Stepwise canonical discriminant analysis (CDA) (for more detailed description of the analysis see Chapter 3, section 3.2.8.1) was applied to olive oil samples with $\delta^{13}C_{C29}$, δD_{C29}, ACL and total *n*-alkane concentration used as predictor variables. The main objective of the CDA was to perform regional classification of oil samples based on the values of the predictor variables and to determine which predictor variable(s) contributed to the most of the intergroup differences.

4.3. Results

4.3.1. The relationship between geographical factors and molecular and isotopic compositions of *n*-alkanes in extra virgin olive oils

Pearson coefficient (r) and the significance of correlation between isotopic and molecular parameters of extra virgin olive oils and geographical factors are presented in Table 4.2. Stable carbon isotope values of *n*-C_{29} show one of the highest numbers of significant correlations with other parameters. The $\delta^{13}C_{C29}$ values of olive oils positively correlate with δD_{C29} (r = 0.39), altitude (r = 0.25), the mean temperature during August-December (r = 0.24) and the δD of precipitation (r = 0.29), while show negative correlation with latitude (r = -0.35), concentration of *n*-C_{29} alkane (r = -0.32) and total *n*-alkane concentration (r = -0.29). Despite multiple significant correlations between $\delta^{13}C_{C29}$ and other parameters, r values are not high (r<0.50): the highest r value (r = 0.39) is found between $\delta^{13}C_{C29}$ and δD_{C29}.

The δD_{C29} values of olive oils positively correlate with the δD of precipitation (r = 0.73), $\delta^{13}C_{C29}$ (r = 0.39) and the mean temperature during August-December (r = 0.47), whereas they negatively correlate with latitude (r = -0.76), the concentration of *n*-C_{29} (r = -0.41) and of total *n*-alkanes (r = -0.34). The highest r value (r = -0.76) is found between δD_{C29} and latitude.

n-Alkane average chain length (ACL) shows positive correlation with altitude (r = 0.34) and the concentration of *n*-C_{29} (r = 0.44) and is negatively correlated with longitude (r = -0.46). All three correlation coefficient values are not high (r < 0.50).

The concentration of *n*-C_{29} alkane is positively correlated with latitude (r = 0.30), ACL (r = 0.44) and total *n*-alkane concentration (r = 0.83), while it has negative correlation with $\delta^{13}C_{C29}$ (r = -0.32) and δD_{C29} (r = -0.41). Correlation between the concentrations of other individual *n*-alkanes (*n*-C_{20} - *n*-C_{35}) and geographical parameters (latitude, longitude and altitude) is shown in Appendix C (Table C1). Positive correlation is found between a number of individual *n*-alkanes (*n*-C_{24}, *n*-C_{25}, *n*-C_{27}, *n*-C_{30}, *n*-C_{32}) and latitude, while negative correlation is observed between some *n*-alkanes (*n*-C_{23}, *n*-C_{24}, *n*-C_{25}) and altitude.

Finally, the concentration of total *n*-alkanes is negatively correlated with δD_{C29} (r = -0.34) and $\delta^{13}C_{C29}$ (r = -0.29), however, does not show significant correlation with any of the geographical parameters.

Table 4.2. Pearson coefficient (r) and the significance (sig.) of correlation between the isotopic and molecular parameters of extra virgin olive oils and geographical factors.

		altitude	latitude	longitude	temperature	$\delta D_{precipitation}$	$\delta^{13}C_{C29}$	δD_{C29}	ACL	Total *n*-alkane conc.	*n*-C_{29} alkane conc.
$\delta^{13}C_{C29}$	r	**0.25***	**-0.35****	-0.13	**0.24***	**0.29***	1.00	**0.39****	0.08	**-0.29***	**-0.32****
	sig.	0.03	0.00	0.27	0.04	0.01		0.00	0.49	0.01	0.01
δD_{C29}	r	0.10	**-0.76****	0.15	**0.47****	**0.73****	**0.39****	1.00	-0.17	**-0.34****	**-0.41****
	sig.	0.38	0.00	0.21	0.00	0.00	0.00		0.14	0.00	0.00
ACL	r	**0.34****	0.00	**-0.46****	-0.22	-0.01	0.08	-0.17	1.00	0.06	**0.44****
	sig.	0.00	0.98	0.00	0.51	0.93	0.49	0.14		0.63	0.00
***n*-C_{29} concentration**	r	0.04	**0.30****	-0.22	-0.20	**-0.25***	**-0.32****	**-0.41****	**0.44****	**0.83****	1.00
	sig.	0.77	0.00	0.05	0.08	0.03	0.01	0.00	0.00	0.00	
Total *n*-alkane concentration	r	-0.11	0.21	-0.05	-0.90	-0.13	**-0.29***	**-0.34****	0.06	1.00	**0.83****
	sig.	0.34	0.07	0.66	0.44	0.26	0.01	0.00	0.63		0.00

Values in bold indicate significant correlation. ** – correlation is significant at the 0.01 level (2-tailed); * – correlation is significant at the 0.05 level (2-tailed); ACL – *n*-alkane average chain length; conc. – concentration.

As seen from the correlation analysis only a few correlated parameters have high r values. The relationship with highly significant positive correlation found between the δD_{C29} and δD values of precipitation, has been further analysed using simple linear regression analysis. Results of the regression analysis are presented in Table 4.3.

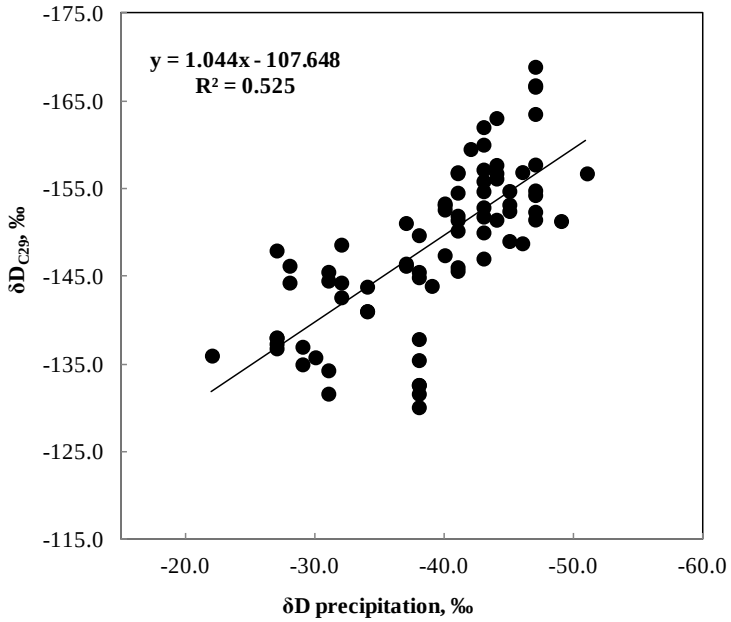

Figure 4.3. δD_{C29} values of olive oil samples as a function of the δD values of precipitation.

There exists a positive relationship between the δD_{C29} values and the δD values of precipitation (Figure 4.3). The coefficient of determination (R^2) for this relationship is 0.53, however, when adjusted to the number of samples, it is equal to 0.52 (Table 4.3). This suggests that 52% of the variation in the δD_{C29} values around their mean can be explained by the regression model.

Table 4.3. The summary of the Linear regression analysis.

	Unstandardized Coefficients		**Sig.**	**95% Confidence Interval for B**	
	B	**Std. Error**		**Lower Bound**	**Upper Bound**
Intercept	-107.65	4.61	0.000	-116.83	-98.46
Slope	1.04	0.12	0.000	0.81	1.27

Model	**R**	**R^2**	**Adjusted R^2**
	0.73	0.53	0.52

The regression equation can be written in the following way:

$$\delta D_{C29} = 1.04 * \delta D_{precipitation} - 107.65 \quad (4.4.)$$

A 1‰ increase in the δD values of precipitation would lead to a 1‰ increase in the δD_{C29} values of olive oils. The 95% confidence interval around the slope parameter suggests that the δD_{C29} can change anywhere from 0.8 to 1.3‰ per 1‰ change in the δD of precipitation (Table 4.3).

4.3.2. Characterisation of olive oils from eight Mediterranean countries on the basis of molecular composition of *n*-alkanes

The mean values of the individual *n*-alkane concentrations, total *n*-alkane amount and *n*-alkane average chain length in extra virgin olive oils from eight Mediterranean countries are shown in Table 4.4. The concentrations of *n*-alkanes and the ACL values of all samples are shown in the Appendix C (Table C3).

Extra virgin olive oil samples from eight prodcution countries can be characterized by the presence of *n*-alkanes with the carbon chains from C_{20} to C_{35}. Both the profile and the concentrations of *n*-alkanes are different between the production countries. Total *n*-alkane concentration in olive oils is in the range from 35.6 to 115.6 mg/kg, and is the highest in oils from Central Italy and the lowest in oils from Northern Greece (Table 4.4).

All olive oil samples exhibit a distinct predominance of odd-numbered *n*-alkanes. The average amount of odd *n*-alkanes constitutes from 73.6% to 82.9% of the total *n*-alkane amount (Table 4.4).

Figure 4.4 shows the concentrations of *n*-alkanes in the analyzed olive oil samples. The most prevalent *n*-alkanes in the analysed olive oils are *n*-C_{23}, *n*-C_{24}, *n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31}. The *n*-alkanes profile in oils differs depending on the country of origin. Olive oils from Italy (Northern, Central and Southern), Morocco, Greece and Croatia are characterized by the high amounts of short-chain *n*-alkanes (*n*-C_{23}, *n*-C_{24}, *n*-C_{25}), while olive oils from Portugal and Spain show the highest concentrations of longer chain *n*-alkanes (*n*-C_{27}, *n*-C_{29}, *n*-C_{31}).

Table 4.4. Mean concentrations of *n*-alkanes in extra virgin olive oils from eight Mediterranean countries.

		***n*-alkane concentration, mg kg^{-1} ± SD**										
	n	***n*C$_{20}$**	***n*C$_{21}$**	***n*C$_{22}$**	***n*C$_{23}$**	***n*C$_{24}$**	***n*C$_{25}$**	***n*C$_{26}$**	***n*C$_{27}$**	***n*C$_{28}$**	***n*C$_{29}$**	***n*C$_{30}$**
Slovenia	3	0.0±0.0	0.6±0.3	0.7±0.4	5.7±0.7	5.4±0.7	11.2±1.2	2.1±0.4	10.9±1.4	1.7±0.5	12.1±3.1	2.8±2.3
Croatia	4	0.1±0.2	0.4±0.3	1.3±0.3	15.0±4.3	12.9±3.1	21.4±4.9	3.2±0.7	10.5±2.5	2.1±0.4	12.9±3.1	1.4±0.5
France	15	1.7±1.8	0.8±0.5	1.6±1.3	9.6±6.6	6.2±4.6	16.3±9.9	2.3±1.3	10.1±5.4	1.9±1.1	9.8±5.8	0.9±0.7
Italy, North	4	0.0±0.0	0.2±0.4	0.7±0.5	12.1±6.9	11.7±7.0	19.5±8.7	2.9±0.9	12.5±1.7	1.7±0.2	11.1±1.3	1.0±0.1
Italy, Central	14	0.0±0.0	0.6±0.3	1.0±0.6	15.0±8.9	11.8±6.9	22.4±10.2	3.2±1.4	14.3±6.0	2.2±0.7	15.8±5.2	1.9±0.7
Italy, South	7	0.0±0.0	0.8±0.9	1.2±1.0	14.2±10.9	10.2±8.2	19.2±12.9	3.1±2.2	10.3±4.5	1.8±0.9	13.9±6.3	1.7±0.9
Portugal	5	0.0±0.0	0.4±0.2	0.5±0.2	4.1±5.7	3.8±3.8	11.3±6.5	2.0±0.7	11.3±4.8	2.4±0.9	14.8±6.4	2.2±0.9
Greece, North	8	1.5±0.7	1.5±0.4	1.0±0.4	3.6±0.8	2.4±0.6	7.4±2.0	2.4±0.9	6.3±1.4	1.3±0.4	5.5±2.1	0.7±0.4
Greece, South	6	0.5±0.3	2.5±3.4	1.4±0.7	18.2±7.5	9.6±3.2	14.5±3.8	2.0±0.6	7.1±3.0	1.7±1.4	6.1±3.7	0.3±0.5
Spain	6	0.0±0.0	0.2±0.3	0.2±0.3	1.2±0.6	0.9±0.4	5.1±2.2	1.7±0.7	6.0±2.0	1.4±0.9	8.4±3.6	1.4±0.5
Morocco	4	2.0±2.5	10.1±10.9	3.0±3.0	13.6±9.2	4.0±1.8	12.0±2.1	2.0±0.3	9.9±1.0	4.1±6.1	8.0±2.1	0.9±0.2

n – number of samples; SD – standard deviation for n number of samples

Table 4.4. Continued.

	n	*n*-alkane concentration, mg kg^{-1} ± SD					Total *n*-alkanes, mg kg^{-1} ± SD	Odd - numbered *n*-alkanes, mg kg^{-1} ± SD	Odd - numbered *n*-alkanes, % of total	ACL
		*n*C$_{31}$	*n*C$_{32}$	*n*C$_{33}$	*n*C$_{34}$	*n*C$_{35}$				
Slovenia	3	5.1±3.9	1.4±0.9	2.0±1.8	0.4±0.8	0.5±0.9	62.7±7.8	48.1±9.0	76.8	27.1±0.5
Croatia	4	8.4±3.3	1.1±0.4	4.4±2.0	0.5±0.3	1.3±0.8	96.7±25.5	74.2±20.2	76.7	26.5±0.3
France	15	3.6±3.3	0.5±0.5	1.2±0.8	0.2±0.3	0.3±0.6	66.8±39.0	51.6±30.1	77.2	26.0±0.4
Italy, North	4	5.3±0.6	1.3±1.5	1.3±0.8	0.0±0.0	0.0±0.0	81.3±26.6	61.9±17.8	76.2	26.3±0.6
Italy, Central	14	13.5±6.1	1.7±1.0	8.5±6.3	1.0±1.1	2.9±2.9	115.6±45.2	92.7±34.9	80.2	27.3±0.8
Italy, South	7	9.7±6.0	1.4±1.1	5.8±4.0	0.8±0.6	2.2±1.9	96.2±58.1	76.2±44.0	79.2	27.2±0.5
Portugal	5	10.8±5.3	1.5±0.6	6.2±2.7	0.9±0.4	1.8±0.9	73.9±27.9	60.7±23.1	82.1	28.4±0.3
Greece, North	8	1.9±1.3	0.1±0.2	0.0±0.1	0.0±0.0	0.0±0.0	35.6±9.9	26.2±7.0	73.6	25.8±0.3
Greece, South	6	2.7±3.0	0.2±0.4	1.0±1.5	0.1±0.3	0.1±0.1	67.8±12.7	52.0±11.3	76.7	25.1±0.9
Spain	6	7.6±3.6	1.0±0.3	3.8±1.7	0.4±0.3	1.0±0.5	40.1±17.3	33.2±14.2	82.9	28.7±0.2
Morocco	4	4.7±2.6	0.6±0.5	2.8±2.4	0.1±0.3	0.2±0.2	78.0±23.2	61.4±17.8	78.7	25.7±1.7

n – number of samples; SD – standard deviation for n number of samples; ACL – *n*-alkane average chain length

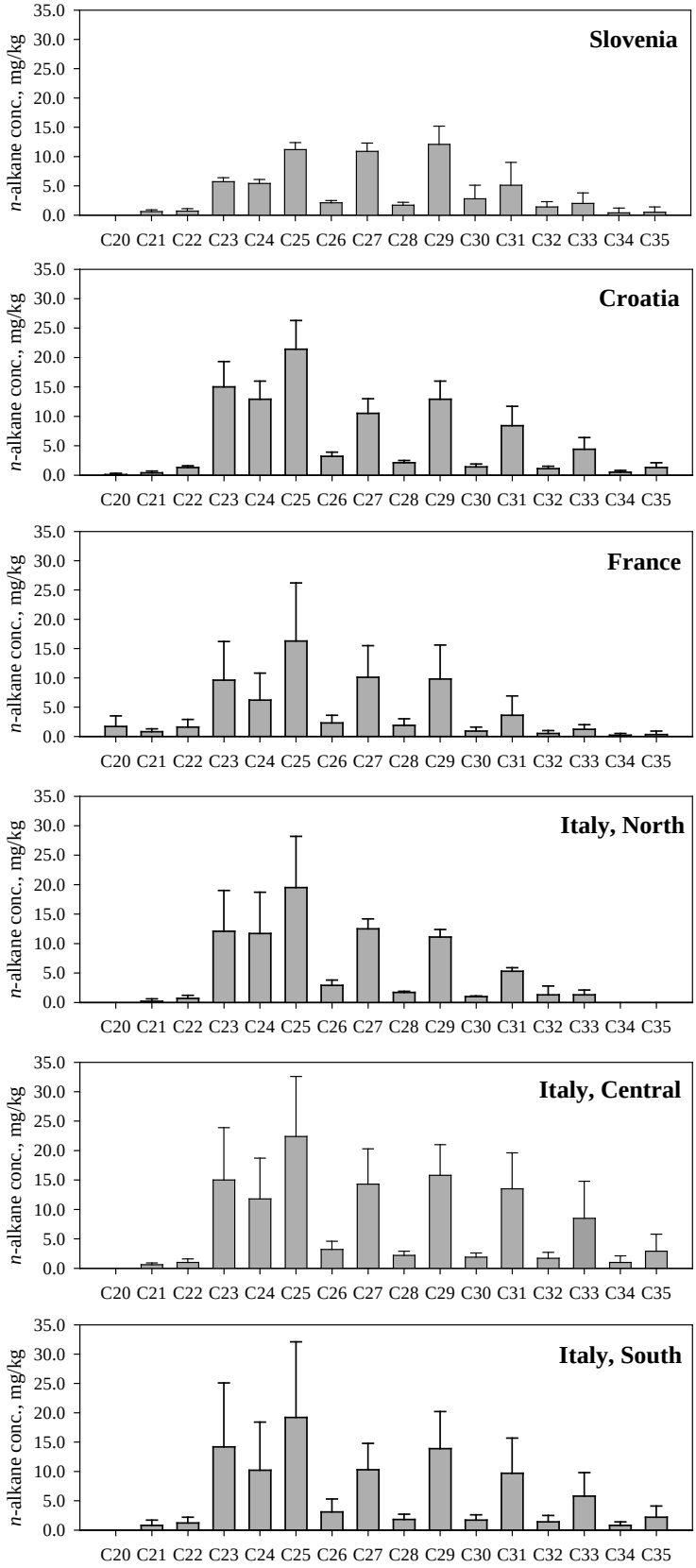

Figure 4.4. Concentrations of *n*-alkanes in extra virgin olive oils from the Mediterranean countries.

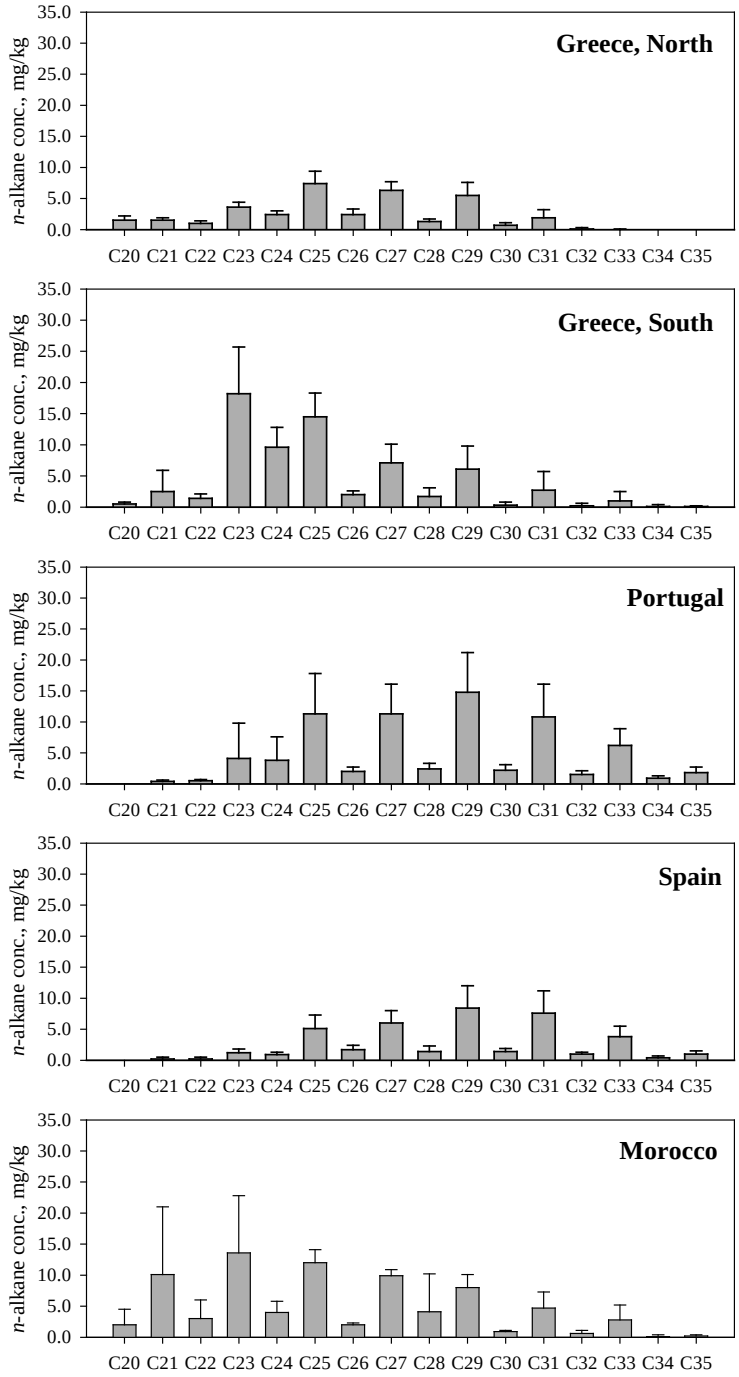

Figure 4.4. Continued.

The values of *n*-alkane average chain length (ACL) are also different in oils from different countries (Table 4.4). The lowest ACL values are found in oils from Morocco, Southern and Northern Greece (25.7, 25.1 and 25.8 ACL units, respectively), whereas the highest ACL values are in oil samples from Spain and Portugal (28.7 and 28.4 ACL units, respectively).

4.3.3. Characterisation of olive oils from eight Mediterranean countries on the basis of hydrogen and carbon isotope compositions of *n*-alkanes

The mean of $\delta^{13}C_{C29}$ and δD_{C29} values of extra virgin olive oils from eight Mediterranean countries as well as the δD values of precipitation in oil production locations are shown in Table 4.5. Isotopic values of the individual oil samples are included in the Appendix C (Table C2).

Table 4.5. The mean isotopic values of *n*-C_{29} in extra virgin olive oils from eight Mediterranean countries.

Country	n	$\delta D_{precipitation}$, ‰	$\delta^{13}C_{C29}$, ‰	δD_{C29}, ‰
Slovenia	3	-48±1	-33.1±0.2	-152±1
Croatia	4	-45±1	-32.2±0.6	-155±2
France	15	-45±3	-32.7±0.5	-154±4
Italy, North	4	-47±0	-33.7±0.2	-166±2
Italy, Central	14	-42±1	-33.1±0.7	-154±4
Italy, South	7	-33±6	-32.8±0.5	-148±6
Portugal	5	-36±4	-31.9±0.5	-144±6
Greece, North	8	-38±0	-32.2±0.6	-137±7
Greece, South	6	-31±2	-32.1±0.4	-137±4
Spain	6	-36±4	-31.7±0.1	-146±1
Morocco	4	-27±0	-32.8±0.4	-138±1

The values represent the mean and standard deviations for n number of samples.

Hydrogen isotope composition of precipitation differs by more than 20‰ among the oil production locations. The lowest δD values of precipitation are in Slovenia and Northern Italy (-48±1‰ and -47±0‰, respectively), whereas the highest $\delta D_{precipitation}$ values are in Morocco and Southern Greece (-27±0‰ and -31±2‰, respectively).

Stable hydrogen isotope composition of *n*-C_{29} alkane in the extra virgin olive oils from eight Mediterranean countries is also subject to a significant variability (Figure 4.5 A). The total range of variation in the δD_{C29} values of oils is as large as 29‰. Olive oils with the lowest δD_{C29} values are from Northern Italy (mean δD_{C29} = -166±2‰), whereas oils with the highest values originate from Morocco, Southern

and Northern Greece (mean δD_{C29} = -138±1‰, -137±4‰ and -137±7‰, respectively).

In respect to carbon isotope composition, the total range of variation in the $\delta^{13}C_{C29}$ values of oils is 2‰. The lowest $\delta^{13}C_{C29}$ values are in olive oils from Northern and Central Italy and Slovenia (mean $\delta^{13}C_{C29}$ = -33.7±0.2‰, -33.1±0.7‰ and -33.1±0.2‰, respectively), whereas the highest values are in oils from Portugal and Spain (mean $\delta^{13}C_{C29}$ = -31.9±0.5‰ and -31.7±0.1‰, respectively).

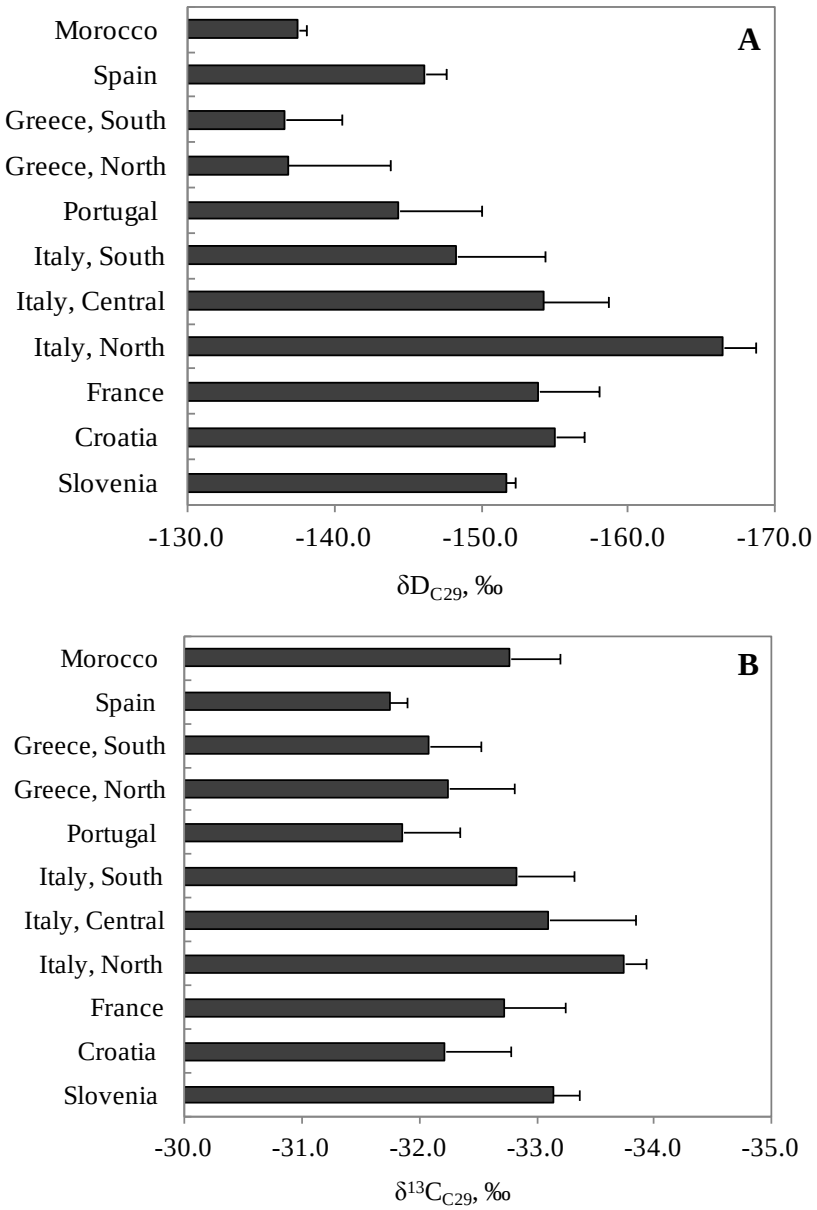

Figure 4.5. The mean δD_{C29} (A) and $\delta^{13}C_{C29}$ (B) values of extra virgin olive oils from eight Mediterranean countries. The error bars represent standard deviations for a given number of samples (see table 4.5) from each country.

4.3.4. Differentiation between olive oils from Northern and Southern Mediterranean regions on the basis of molecular and isotopic compositions of *n*-alkanes

Eight oil production countries were grouped in two regions: Northern and Southern Mediterranean based on the values of latitude and the δD of precipitation. Comparative analysis of the molecular and isotopic compositions of olive oils from both regions was performed. Additionally, the ability of the measured molecular and isotopic compositions of *n*-alkanes to discriminate oil samples into one of two regions was evaluated using canonical discriminant analysis (CDA). Table 4.6 presents oil production countries from Northern and Southern region, as well as the mean values of latitude, $\delta D_{precipitation}$ and temperature (August-December) of two regions.

Table 4.6. Oil production countries, mean latitude, $\delta D_{precipitation}$ and temperature values of the Northern and Southern Mediterranean regions.

Region	**Northern**	**Southern**
Country	Slovenia Croatia Italy (North) Italy (Central) France	Greece Italy (South) Spain Portugal Morocco
Latitude, dd	44.1±1.0	38.2±2.2
$\delta D_{precipitation}$, ‰	-44±3	-34±5
Temperature (August-December), °C	+15.4±1.0	+17.7±1.4
Total number of samples	40	36

The values represent the mean ± standard deviations for the given number of samples; dd – decimal degrees

The mean values of the molecular and isotopic compositions of olive oils from Northern and Southern Mediterranean region are shown in Table 4.7. Significant difference ($p<0.05$) in both $\delta^{13}C_{C29}$ and δD_{C29} values of olive oils is observed between the two regions. Both isotopic parameters show significantly more negative ($p<0.05$) values in olive oils from the Northern compared to the Southern region. The mean $\delta^{13}C_{C29}$ value of olive oils from the Northern Mediterranean is by 0.6‰ lower than that from the Southern region (Table 4.7). The difference in the δD_{C29} of olive oils between the two regions is 13‰.

The concentration of *n*-C_{29} alkane as well as the total *n*-alkane concentration are significantly higher ($p<0.05$) in olive oils from the Northern compared to the Southern Mediterranean (Table 4.7). The *n*-alkane ACL is the only parameter that does not show significant difference ($p>0.05$) between the two production regions, with the values of oils from the North and South being 26.6±0.8 and 26.7±1.5 ACL units, respectively.

Table 4.7. Isotopic and molecular compositions of *n*-alkanes in olive oils from the Northern and Southern Mediterranean.

	North				South			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
$\delta^{13}C_{C29}$, ‰	-32.9*	0.7	-34.2	-31.7	-32.3*	0.6	-33.4	-31.4
δD_{C29}, ‰	-155*	5	-169	-146	-142*	7	-157	-130
ACL	26.6	0.8	25.2	28.6	26.7	1.5	23.8	28.9
***n*-C_{29}, mg kg^{-1}**	12.6*	5.3	3.1	24.9	8.4*	6.2	1.2	24.6
Total *n*-alkane conc., mg kg^{-1}	89.6*	42.9	22.2	226.7	59.6*	40.0	9.2	165.7

* - difference in the mean values between two regions is significant ($p<0.05$); SD - standard deviation, conc. – concentration.

Canonical discriminant analysis (CDA) has been performed on olive oil samples from two Mediterranean regions with $\delta^{13}C_{C29}$, δD_{C29}, ACL and total *n*-alkane concentration used as predictor variables to test whether the measured parameters could successfully classify the oil samples into one of the two production regions. Tables 4.8 and 4.9 show the results of the tests of equality of group means and the structure matrix of the discriminative CDA model, respectively. One discriminant function has been created in the model. Out of four entered predictor variables, δD_{C29} has the highest significant discriminative potential (Table 4.8), and its correlation coefficient with the discriminant Function is 0.870 (Table 4.9). The second significant predictor variable is $\delta^{13}C_{C29}$ (Tables 4.8 and 4.9). Total *n*-alkane concentration has not been used by the CDA model, while ACL does not significantly contribute to the discrimination (Table 4.8).

Table 4.8. The results of the tests of equality of group means from the CDA of olive oils from the Northern and Southern Mediterranean.

Predictor variable	Wilks' Lambda	F	Significance
$\delta^{13}C_{C29}$	0.769	22.263	0.000
δD_{C29}	0.445	92.435	0.000
ACL	0.996	0.329	0.568

Table 4.9. The structure matrix of the model from the CDA of olive oils from the Northern and Southern Mediterranean.

Predictor variable	Function
δD_{C29}	0.870
$\delta^{13}C_{C29}$	0.427
ACL	0.052

Variables ordered by absolute size of correlation within function

Classification results of CDA with the isotopic and molecular parameters of olive oils used as predictor variables are shown in Table 4.10. Overall 93.4% of cross-validated olive oil samples are correctly classified. Olive oil samples from the Northern Mediterranean region are classified correctly in 95.0% of all cases with 5.0% of samples being misclassified as oils from the Southern region. Olive oil samples from the Southern Mediterranean are classified correctly in 91.7% of all cases with 8.3% samples misclassified as oils from the Northern region.

Table 4.10. Classification results of the CDA of olive oils from the Northern and Southern Mediterranean with δD_{C29}, $\delta^{13}C_{C29}$, ACL used as predictor variables.

		Region	Predicted Group Membership		Total
			Northern	Southern	
Cross-validated	Count	Northern	38	2	40
		Southern	3	33	36
	%	Northern	95.0	5.0	100.0
		Southern	8.3	91.7	100.0

93.4% of cross-validated grouped cases correctly classified

4.4. Discussion

The main objective of this study has been to characterise molecular as well as hydrogen and carbon isotope compositions of *n*-alkanes in extra virgin olive oils from eight Mediterranean countries and then to evaluate which molecular and/or stable isotope parameters provide the most discriminative potential in terms of oil classification into Northern and Southern Mediterranean region. Firstly, I discuss the main differences found in the molecular composition of *n*-alkanes from Mediterranean olive oils, then address the differences in the isotopic composition of *n*-alkanes in the analysed olive oils and finally, discuss the discriminative potential of the molecular and isotopic compositions of *n*-alkanes in terms of regional oil discrimination.

4.4.1. Differences in the molecular composition of *n*-alkanes in extra virgin olive oils from the Mediterranean

n-Alkanes in olive oils from eight Mediterranean countries are in a range from C_{20} to C_{35}, and the most prevalent in olive oils are odd-numbered homologues: *n*-C_{23}, *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31}. However, even-numbered *n*-alkane *n*-C_{24} is also present in high amounts in most oil samples. Different relative proportions of *n*-alkanes in olive oils from different countries result in different *n*-alkane average chain length (ACL) values of oils. The lowest ACL values are found in oils from Morocco, Southern and Northern Greece (25.7, 25.1 and 25.8 ACL units, respectively), which have high amounts of *n*-C_{23}, *n*-C_{24} and *n*-C_{25}, whereas the highest ACL values are in oil samples from Spain and Portugal (28.7±0.2 and 28.4±0.3 ACL units, respectively), which have the highest concentrations of longer chain *n*-alkanes: *n*-C_{29} and *n*-C_{31} (Table 4.4).

The range of *n*-alkanes in olive oils from this study is in agreement with the *n*-alkane range that was previously reported for olive oils. Several studies analysed *n*-alkanes in Spanish virgin olive oils and reported the presence of *n*-alkanes from C_{12} to C_{35} (Lanzon et al. 1994, Guinda et al. 1996, Bueno et al. 2005). The samples of Croatian virgin olive oils contained *n*-alkanes with carbon chains from C_{21} to C_{35} (Koprivnjak et al. 2005). The presence of *n*-alkanes in a range from C_{15} to C_{35} was found in extra virgin olive oils from Italy, Greece and Spain (Webster et al. 2000,

Bortolomeazzi et al. 2001). All the above mentioned studies reported the predominance of odd-numbered *n*-alkanes in olive oils, which agrees with the results of the present study. High amounts of even-numbered *n*-alkane *n*-C_{24} is a unique pattern in olive oils from Italy, Croatia, Greece and Morocco and may arise during oil processing as well due to differences in olive varieties used for oil production in the above countries. The presence of high amounts of *n*-C_{24} has also been reported in earlier studies (Webster et al. 2000, Bortolomeazzi et al. 2001, Koprivnjak et al. 2005).

The distribution of *n*-alkanes in Greek, Italian and Spanish olive oils from the present study is in accordance with that from the study by Webster et al. (2000). Authors reported that olive oils from Greece were characterized by the predominance of *n*-C_{23}, *n*-C_{24} and *n*-C_{25}, olive oils from Italy had a greater amount of *n*-C_{27}, whereas Spanish olive oils, similarly to the present findings, were characterized by the predominance of longer chain *n*-alkanes (*n*-C_{27}, *n*-C_{29}, *n*-C_{31}). The discussion of the possible reasons for the differences in the *n*-alkane distribution in oils from different countries is absent in their study, Nevertheless, from the overall discussion it may be concluded that these differences are mainly attributed to the different olive varieties used for oil production in different countries rather than to climatic or geographical differences.

Total *n*-alkane concentration in extra virgin olive oils from the present study also varies with the production country (Table 4.4) and is found to be significantly higher ($p<0.05$) in oils from the Northern compared to the Southern Mediterranean region (Table 4.7). It is difficult to explain the observed differences in the amount of *n*-alkanes in oils from different production locations on the basis of geographical and climatic variation among the countries. On the one hand, olive oil *n*-alkanes are the components of the cuticular wax of olive fruits (Bianchi et al. 1992a, Bianchi et al. 1992b), and as reported previously, the amount of cuticular waxes in plants can be significantly influenced by climatic conditions (Kunst and Samuels 2003, Jetter et al. 2006). Several studies reported that high temperatures and limited water availability can result in higher wax concentration per leaf surface area (Premachandra et al. 1992, Bondada et al. 1996, Rommerskirchen et al. 2006), which would reduce the epidermal conductance of water vapour, thereby decreasing plant water loss in the water-limiting environment (Premachandra et al. 1992). This suggests that difference in the mean temperatures during olive formation period in oil production countries

may influence the amount of *n*-alkanes in oils in a way that olives originating from the drier locations or exhibiting water deficit could produce fruits (and consequently oils) with the higher content of cuticular waxes and *n*-alkanes. However, this has not been observed in the present study. The observation that higher amounts of *n*-alkanes are found in the oils from the Northern part of the Mediterranean is not consistent with the explanation whereby lower ambient temperatures would result in a decrease in *n*-alkane concentrations. What is more, no significant correlations have been found between *n*-alkane concentration and geographic parameters, such as latitude, longitude or altitude. When entered as predictor variables into CDA model, neither total *n*-alkane concentration nor ACL values have contributed significantly to the discrimination of oil samples according to their production region. This suggests that the observed significant differences in the *n*-alkane concentration of olive oils from different Mediterranean regions are subject to variability principally due to factors other than geographical. Such factors can be related to the varietal origin of the oils. Several authors who analysed oils from different olive varieties reported differences in *n*-alkane concentration among different varieties even within the same production area (Guinda et al. 1996, Koprivnjak et al. 2005, Bueno et al. 2005).

A limited amount of other studies have analysed *n*-alkane content in olive oils in respect to their geographic origin. Webster et al. (2000) and Bortolomeazzi et al. (2001) analysed hydrocarbons (including *n*-alkanes) in olive oils from Italy, Spain and Greece. Bortolomeazzi et al. (2001) found that Italian and Spanish olive oils had low amounts of *n*-alkanes (30-65 mg/kg). Similarly, Webster et al. (2000) reported that the lowest *n*-alkane concentration was found in Spanish olive oils (18.6 mg/kg). Neither of these studies explained the observed differences among oils from different countries. Spanish oils from the present study have one of the lowest total *n*-alkane concentrations, and thus the results of both above mentioned studies are in agreement with the present findings.

Thus, significant differences are found in the content and the distribution of *n*-alkanes in olive oils from different production countries. These differences, however, cannot be explained on the basis of primarily geographical or climatic factors, and may arise from differences in varietal origin.

4.4.2. Differences in the isotopic composition of *n*-alkanes in extra virgin olive oils from the Mediterranean

The stable carbon and hydrogen isotope compositions of extra virgin olive oils from eight Mediterranean countries are subject to a significant variability. $\delta^{13}C_{C29}$ values are significantly more positive ($p<0.05$) in olive oil samples from the Southern compared to Northern Mediterranean region (Table 4.7), and show significant discriminative power as a predictor variable in the CDA model (Tables 4.8 and 4.9). The highest $\delta^{13}C_{C29}$ values are found in olive oils from Portugal and Spain (mean $\delta^{13}C_{C29}$ = -31.9±0.5‰ and -31.7±0.1‰, respectively), whereas the lowest values are in oils from Northern and Central Italy and Slovenia (mean $\delta^{13}C_{C29}$ = -33.7±0.2‰, -33.1±0.7‰ and -33.1±0.2‰, respectively) (Figure 4.5B). The total range of variation in $\delta^{13}C_{C29}$ values is 2‰. $\delta^{13}C_{C29}$ values positively correlate with altitude and the mean temperature during August-December, whereas they show negative correlation with latitude (Table 4.2).

Analysed olive oils samples originate in eight countries, which represent most of the Mediterranean region, and therefore the values of geographical parameters (latitude, longitude and altitude) exhibit rather a large variation (Table 4.1). The correlation of $\delta^{13}C_{C29}$ with geographical parameters can arise mainly due to the fact that the $\delta^{13}C_{C29}$ values of plant compounds are influenced by the availability of water, relative humidity and temperature in the growing area. These factors control stomatal aperture and the internal CO_2 concentration in leaves (Smith and Epstein 1971, O'Leary 1981, Farquhar et al. 1989, Tieszen 1991, Ehleringer and Dawson 1992, Martin and Martin 2003) and impact carbon isotope fractionation in olive leaves and subsequently in fruits. As can be seen from Table 4.1 the mean temperatures in the oil production locations are higher in the South of the Mediterranean, and thus can be responsible for the higher $\delta^{13}C_{C29}$ values of olive oils in the southern locations. Correlation factor values between $\delta^{13}C_{C29}$ and the analysed geographical parameters are not very high, which suggests the presence of other factors that impact the variation in the $\delta^{13}C_{C29}$ values of olive oil. For example, the difference among olive varieties used for the oil production in different production locations can also have a strong influence on the $\delta^{13}C$ values of olive oils (Iacumin et al. 2009).

The carbon isotopic composition of olive oils in relation to geographical factors has been discussed previously in several studies. The bulk $\delta^{13}C$ values of

Italian olive oils showed significant negative correlation with latitude (r = -0.68 and -0.50, for two production years) and significant positive correlation with the mean annual temperature of the location (r = 0.38 and 0.31, for two production years) in the study by Iacumin et al. (2009). Similar results were found by Camin et al. (2010b) who measured the isotopic composition of olive oils from five European countries (Italy, France, Spain, Portugal and Greece). Authors reported that the bulk $\delta^{13}C$ values of olive oils negatively correlated with latitude (r = -0.80) and positively correlated with the mean temperature during August-December (r = 0.59), which is in agreement with the results of the present study. The bulk $\delta^{13}C$ values of olive oils from two production years from their study exhibited a total variation of 2.5‰, from -30.9‰ in Northern Italy to -28.4‰ in Southern Greece and Portugal. These findings are in agreement with the results of the present study, and show the impact of geographic and climatic factors on the $\delta^{13}C$ values of olive oils.

The δD_{C29} values of olive oils from eight Mediterranean countries exhibit a much greater variation compared to $\delta^{13}C_{C29}$ values. The total range of variation in the δD_{C29} values of oils is as large as 29‰ (Figure 4.5A). From the four predictor variables entered to the CDA model δD_{C29} values show the most significant discriminative potential (Tables 4.8 and 4.9). The δD_{C29} values of olive oils are significantly more positive in the oil samples from the Southern compared to the Northern Mediterranean region (Table 4.7). Oils with the highest δD_{C29} values are from Southern and Northern Greece and Morocco (mean δD_{C29} = -137±4‰, -137±7‰ and -138±1‰, respectively), whereas oils with the lowest δD_{C29} values originate from Northern Italy (-166±2‰) (Figure 4.5A). These differences in the δD_{C29} of oils among the production countries reflect differences in latitude, the δD of precipitation and the mean temperature during August-December among Southern and Northern oil production locations (Table 4.2). The presence of significant correlation between the δD_{C29} of oils and δD of precipitation reflects a close relationship between the hydrogen isotope composition of *n*-alkanes in olive oils and the hydrogen isotope composition of the source water for the olive trees in the oil production locations. This water is derived from precipitation, which in turn is fractionated during the meteorological cycle of evaporation, condensation and precipitation and is highly impacted by the latitude, altitude and the mean annual temperature, and thus exhibits a significant geographical variation (Dansgaard 1964, Yurtsever 1975, Yuntseover and Gat 1981, Kendall and Caldwell 1998, Marshall et

al. 2007). When ground water is taken up by olive trees, hydrogen and oxygen atoms are incorporated into carbohydrates, and further are used for the lipid synthesis. Although during these biosynthetic processes isotopic fractionations are taking place, the hydrogen signature of meteoric water from a particular location can be still preserved in bulk oil and its individual components (Camin et al. 2010 a,b) such as *n*-alkanes, which has been observed in the present study.

The δD values of precipitation differ by more than 20‰ between some oil production locations from the present study with the lowest δD values of precipitation found in the Northern Mediterranean region and the highest values – in the Southern region (Table 4.7). Based on the results of the regression analysis it can be concluded that the δD of precipitation can explain 52% of the variation in the δD_{C29} values of oils around their mean (Table 4.3). In comparison with the δD of precipitation, olive oils are depleted in ^{2}H on average by as much as 105‰ because of biosynthetic fractionation (Schmidt et al. 2001, Schmidt et al. 2003). This is in agreement with the results from the study by Camin et al. (2010b) who reported that δD values of bulk Mediterranean oils were depleted by about 120‰ compared to the δD of surface waters from each production location.

Stable hydrogen isotope composition of olive oils from different production locations has been analysed in several previous studies. Bontempo et al. (2009) analysed hydrogen and oxygen isotope composition in Italian extra-virgin olive oils from Tyrrhenian and Adriatic coasts and reported negative correlation of both isotopic parameters with the amount of precipitation and relative humidity. Camin et al. (2010b) reported significant differences in the stable hydrogen isotope composition of olive oils from five Mediterranean countries and found that δD values of oils negatively correlated with latitude and distance from the coast, and positively correlated with altitude and the mean temperature during August-December, which agrees with the finding of the present study.

Thus, δD_{C29} and $\delta^{13}C_{C29}$ values of Mediterranean olive oils from this study are significantly affected by the environmental conditions among the oil production locations. This is supported by significant correlations of both isotopic parameters with latitude as well as with the mean temperature during August-December in the oil production locations, and has resulted in successful discrimination between oils from the Northern and Southern Mediterranean regions, as seen from the results of the CDA (Table 4.10). Both δD_{C29} and $\delta^{13}C_{C29}$ values show significant discriminative

potential when entered as predictor variables into the CDA model. δD_{C29} values show the highest discriminative power, and overall 93.4% of olive oil samples have been correctly classified into one of the production regions. The difference in climate across eight Mediterranean countries is not as prominent as it would be among countries from different climatic zones and from a wider latitude range (e.g. Northern Europe versus Southern Europe or Africa). Nevertheless, even within restricted climatic zone of the Mediterranean the magnitude of the correct regional oil classification is high, which suggests that the combination of hydrogen and carbon isotopic compositions of olive oil *n*-alkanes can be a useful tool for the regional differentiation of oils. Moreover, as *n*-alkanes are the components of the cuticular waxes of higher plants (Kunst and Samuels 2003, Jetter et al. 2006), it can be proposed that analysis of the isotopic composition of *n*-alkanes in other products of plant origin, such as agricultural crops or other vegetable oils, may be also successfully applied for the geographical provenancing of those commodities. Further studies on the application of compound-specific stable hydrogen and carbon isotope analyses of *n*-alkanes for the geographical differentiation of plant products are needed in future to evaluate the analytical potential and scope of the approach.

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4.5. Conclusions

The distribution and concentration of *n*-alkanes in extra virgin olive oils from the Mediterranean varies with the production country. Olive oil *n*-alkanes are in a range from C_{20} to C_{35}. The most prevalent homologues in the analyzed olive oils are: *n*-C_{23}, *n*-$C_{24,}$ *n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31}. However, the relative proportions of these compounds are different depending on the country of origin, and result in differences in the ACL values of oils. The lowest ACL values are found in oils from Morocco, Southern and Northern Greece, whereas the highest ACL values are in oil samples from Spain and Portugal, which have high concentrations of longer chain *n*-alkanes *n*-C_{29} and *n*-$C_{31.}$

Total concentration of *n*-alkanes is found to be significantly higher ($p<0.05$) in olive oils from the Northern compared to Southern Mediterranean countries. However, no significant correlations have been found between *n*-alkane concentration and the geographic parameters, such as latitude, longitude or altitude. Therefore, differences in molecular composition of *n*-alkanes in extra virgin olive oils cannot be explained on the basis of primarily geographical or climatic factors, and may arise from differences in varietal origin.

The stable carbon and hydrogen isotope compositions of extra virgin olive oils from eight Mediterranean countries are subject to a significant variability. $\delta^{13}C_{C29}$ values are significantly more positive ($p<0.05$) in olive oil samples from the Southern compared to Northern Mediterranean region. The highest $\delta^{13}C_{C29}$ values are found in olive oils from Portugal and Spain, whereas the lowest values are in oils from Slovenia, Northern and Central Italy. $\delta^{13}C_{C29}$ values of oils positively correlate with altitude and the mean temperature during August-December, while show negative correlation with latitude.

The δD_{C29} values of olive oils from eight Mediterranean countries exhibit a much greater variation compared to $\delta^{13}C_{C29}$ values. The total range of variation in the δD_{C29} values of oils is greater than 29‰. The δD_{C29} values of olive oils are significantly more positive ($p<0.05$) in the oil samples from Southern compared to Northern part of the Mediterranean. Oils with the highest δD_{C29} values are from Greece and Morocco, whereas oils with the lowest δD_{C29} values originate from Northern Italy. These differences in the δD_{C29} of oils between the production countries are supported by a significant correlation found between δD_{C29} values and

latitude, the δD of precipitation and the mean temperature during August-December. The presence of these significant correlations reflects a close relationship between the hydrogen isotope composition of *n*-alkanes in olive oils and the hydrogen isotope composition of source water from the oil production locations. Based on the results of the regression analysis it can be concluded that the δD of precipitation can explain 52% of the variation in the δD_{C29} values of oils around their mean.

Significant differences found in both carbon and hydrogen isotope composition of *n*-C_{29} can be attributed to the differences in climatic conditions among the oil production countries. As predictor variables in the CDA, δD_{C29} values show the most significant discriminative power, followed by $\delta^{13}C_{C29}$ values. Overall 93.4% of olive oil samples have been correctly classified into one of the production regions (Northern or Southern Mediterranean). The combination of hydrogen and carbon isotope compositions of olive oil *n*-alkanes can be a useful tool for the regional differentiation of oils and can find application also for the geographical classification of other plant commodities.

4.6. References

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CHAPTER 5

THE EFFECT OF OLIVE HARVESTING METHODS ON THE MOLECULAR AND ISOTOPIC COMPOSITIONS OF N-ALKANES IN ITALIAN EXTRA VIRGIN OLIVE OILS

Abstract

The price of extra virgin olive oil is affected by the olive harvesting technique used. Olives can be harvested by the means of various mechanical tools or picked manually. It has been observed during olive harvesting that mechanical techniques result in a great amount of leaves collected together with olives.

In the present study molecular and compound-specific carbon isotope analyses of *n*-alkanes were applied to extra virgin olive oils produced during seven consequent years in Ciggiano (Tuscany, Italy) using either manual or mechanical olive harvesting techniques. The study evaluated whether molecular and carbon isotope compositions of *n*-alkanes in olive oils are affected by the type of harvesting technique used for the oil production. Additionally, molecular and, for the first time, stable carbon isotope composition of *n*-alkanes have been analysed in olives and olive leaves from three varieties (Frantoio, Leccino and Moraiolo) used for the oil production.

Olive oils produced from mechanically harvested olives and containing higher amounts of leaf material have significantly higher ($p<0.05$) amounts of long-chain *n*-alkanes (*n*-C_{29}-*n*-C_{35}) and significantly higher ($p<0.05$) values of *n*-alkane average chain length (ACL) than oils obtained from the hand-picked olives, which can be explained by significant differences ($p<0.05$) found in *n*-alkane distribution in olives compared to olive leaves. Olive leaves are characterized by the predominance of long-chain *n*-alkanes (*n*-C_{29}, *n*-C_{31}, *n*-C_{33} and *n*-C_{35}) and significantly higher ($p<0.05$) ACL values compared to olives, which successfully differentiates clean olive oils from those with a high leaf content.

Compound-specific stable carbon isotope analysis of *n*-alkanes does not show significant differences between oils produced using two different olive harvesting methods, but does indicate differences in the carbon isotope compositions among three olive varieties.

5.1. Introduction

5.1.1. Olive harvesting techniques used for the production of olive oil

Olive oil is highly prized for its unique sensory characteristics, nutritional properties and manifold health benefits. Despite high market prices, worldwide olive oil consumption has been steadily increasing over the recent years (International Olive Oil Council 2012). Extra virgin olive oil is the highest grade of olive oil and is obtained from olives using solely mechanical or other physical means (Codex Alimentarius Commission 2003). The main processing steps needed to obtain olive oil after harvesting include: leaf removal and washing, olive crushing, malaxation of the olive mash, extraction of the olive oil and oil centrifugation. On average, one kg of olive oil is produced from processing of five kg of olives (Kapellakis et al. 2008).

Olive harvesting time and technique is very important from the perspective of the quantity and quality of the oil obtained. The price of extra virgin olive oil can be greatly impacted by the olive harvesting method. The efficiency of harvesting and the percent of fruit removed from the total crop of the tree impacts the total value of the processed oil (Ferguson 2006, Ferguson et al. 2010). The choice of the olive harvesting method depends on the size and shape of the olive trees, season of harvesting, orchard cultivation methods, climatic factors, and often harvesting traditions of a particular area (Di Giovacchino 2000).

Olive harvesting techniques have gradually developed over the centuries. Early harvesting methods simply relied on collecting the fruits from the ground at the end of the growing season when the mature olives abscised naturally. Olive oil produced from such olives was not always of the highest quality, as being on the ground olives sometimes became infected, infested or gradually started to degrade. Thus, harvesting fully mature olives from the ground for table or oil processing was abandoned and replaced with manual harvesting of olives from the tree, a method still extensively used worldwide (Ferguson et al. 2010).

During manual olive harvesting fruits are hand-picked directly from the tree. Olives are stripped with a downward motion and placed into baskets, bags or boxes. Olives picked in this way typically show very little damage and are relatively free from foreign matter (soil, leaves, and twigs). Hand reach can be extended with hand held wooden or metal toothed devices resembling coarse combs or rakes, used with the same downward motion (Di Giovacchino 2000, Ferguson 2010). The main

limitations of manual olive harvesting include difficulty in reaching the upper part of the tree, the amount of labour involved and, consequently high cost (Rural Industries Research and Development Corporation 2008).

Mechanical olive harvesting has been developed since the 1940s and now is the most popular harvesting approach. Some of the commonly used mechanical techniques are limb shaking devices, orbital and multidirectional trunk shakers, inertia shakers, double or single sided picking head mechanisms, and straddle type harvesters adapted from bush and vine harvesters (Di Giovacchino 2000, Ferguson 2010). The tree canopy and trunk must be well adapted for olive collection with mechanical devices to reduce damage to the tree. Many new olive orchards are being planted all around the world with trees being specially spaced and pruned to facilitate mechanical harvesting (Ferguson 2006, Ferguson et al. 2010).

Mechanical olive harvesting can speed up harvesting, however, in comparison with hand-picking, mechanical techniques can bruise the fruit, which would result in the increased acidity of the oil (Ferguson et al. 2010). Moreover, shakers and other mechanical devices can sometimes cause an excessive leaf fall and result in a great amount of leaves collected together with olives, which can affect the quality of the oil.

5.1.2. The presence of leaf material in virgin olive oils

If leaves are processed together with olives during olive oil production, the quality and the organoleptic properties of oil will be altered. The presence of leaves in virgin olive oil can be a result of mechanical olive harvesting and will lead to a more intensive green colour and will enhance the so called “green-leaf” or “grass” taste, which may not be agreeable with some consumers (Di Giovacchino et al. 2002). These characteristics will depend on the amount of leaves collected with olives and left after olive washing and leaf removal operations, as well as on the efficiency and the intensity of the extraction system (Di Giovacchino 2000, Di Giovacchino et al. 2002).

Olive oil is extracted from olives by either pressure or centrifugation methods. In a pressure extraction system, olives are crushed with a stone mill. Many stone mills do not carry out leaf removal and washing operations. Stone mills have a non-destructive action and generally break the leaves only into a few large fragments,

which does not significantly affect organoleptic characteristics of the oil. In contrast, when centrifugation methods are used, olives are ground by metallic crushers. In this case, olive washing and the leaf removal step are essential for the safety of the machinery, because leaves could cause mechanical problems associated with the flow of olive paste. Crushers reduce leaves to many small fragments. This can release a large quantity of chemical compounds from leaves, which can add an excessive leafy flavour to the oil. Such oil, particularly if obtained from not fully mature olives, has a rather aggressive fruity and unpleasant taste. Therefore, the presence of leaves can worsen the organoleptic characteristics of oil (Di Giovacchino et al. 1996, Di Giovacchino 2000, Di Giovacchino et al. 2002).

Leaf admixture was reported to increase the content of several chemical compounds in olive oils, namely chlorophyll pigment, responsible for the green colour, and *trans*-2-hexenal (Di Giovacchino et al. 1996). *Trans*-2-hexenal, *cis*-3-hexenal and the corresponding hexenols were reported to be responsible for the "fresh-cut grass" aroma of olive oil (Olías et al. 1993). The presence of an excessive leaf material in oil can also alter its composition of the wax fraction components. Both olive leaves and olives are covered with a waxy cuticle, and the composition of several compound classes including *n*-alkanes, alcohols, aldehydes, fatty acids and alkyl esters, is different between olives and leaves (Bianchi et al. 1992a, Bianchi et al. 1992b).

Interestingly, during recent years, several studies on "gourmet olive oils" that combine the beneficial properties of olive oil with those of other plant-leaf material rich in antioxidants have been published (Gambacorta et al. 2007, Ayadi et al. 2009, Baiano et al. 2009, Nenadis et al. 2010). Such speciality oils are prepared by adding the plant leaf material or plant extracts directly to the oil matrix or by addition to olive fruits before pressing. Several studies have described how olive leaves or certain compounds extracted from olive leaves were added to refined olive oils or other vegetable oils to increase the amount of antioxidants and to protect the oil from oxidation (Paiva-Martins et al. 2007, Salta et al. 2007, Nenadis et al. 2010).

5.1.3. Rationale for the study

There is a big variation in prices of extra virgin olive oil on the market. Factors that impact the price of olive oil include olive oil grade, production location, as well

as olive harvesting method. High quality olive oil produced from manually harvested olives is more expensive, which can lead to fraudulent activities. Thus, the development of analytical tools for the verification of olive fruit harvesting methods is needed.

Mechanical harvesting can result in a significant amount of leaves collected and processed together with olive fruits. One class of compounds that may be affected by the presence of leaves in olive oil are hydrocarbons, which are present in the epicuticular wax fraction of both olive leaves and olives (Bianchi et al. 1992a, Bianchi et al. 1992b, Koprivnjak et al. 2005, Sakouhi et al. 2011). The composition of hydrocarbons in edible oils has been studied to a limited extent. Several studies have been published on the *n*-alkane analysis of virgin olive oils and reported differences in the *n*-alkane composition among different olive varieties (Guinda et al. 1996, Bueno et al. 2005, Koprivnjak et al. 2005) and oil production countries (Webster et al. 2000, Bortolomeazzi et al. 2001). No studies, however, focused on the application of molecular hydrocarbon analysis of olive oils in respect to the olive harvesting techniques used for olive oil production.

Stable carbon isotope analyses of *n*-alkanes has been widely used to study plant biosynthetic processes, source inputs, and paleoenvironmental conditions (e.g. Hayes et al. 1989, Hayes 1993, Chikaraishi et al. 2003, Bi et al. 2005, Vogts et al. 2009). However, to our knowledge, no published data on the composition of *n*-alkanes in olive oils, olives or olive leaves is currently available.

In the present study I have analysed molecular and stable carbon isotope composition of *n*-alkanes in extra virgin olive oils produced during seven consequent years in Ciggiano (Tuscany, Italy) from either manually or mechanically collected olives. Molecular and, for the first time, compound-specific stable carbon isotope analyses of *n*-alkanes in olives and olive leaves from three olive varieties (Frantoio, Leccino and Moraiolo), used for oil production, were also performed. The main objective of the study was to evaluate whether olive oils produced with two different olive harvesting techniques can be differentiated on the basis of their molecular (distribution and concentration) and/or stable carbon isotope composition of *n*-alkanes.

5.2. Materials and methods

5.2.1. Sample origin

Eight samples of extra virgin olive oil were obtained from a private olive mill in Ciggiano, Italy (Tuscany region: 43°37' N, 11°71' E) during the period from 2004 to 2010. A map of the location is shown in Figure D1 (Appendix D). Olive oil samples were kindly provided by Dr. Dimitri Abbado (University of Siena, Department of Earth Sciences, Siena, Italy).

During seven oil-production years two different olive harvesting techniques were used. From 2004 to 2006 olives were harvested manually, while from 2007 to 2009 mechanical olive harvesting was applied. In 2010, olives were harvested both manually and mechanically; equal amounts of olives from each variety were collected using both harvesting techniques and then were processed separately, which resulted in two types of extra virgin olive oil in that year. Photos of manual and mechanical olive harvesting are attached in Appendix D (Figure D2 A-B). Each year oils were produced as blends of three olive varieties: Frantoio, Moraiolo, and Leccino. Olives from each variety were harvested every year during the same time – between the end of October and the beginning of December. The mean temperatures in Ciggiano area for each production year was obtained from the weather history database at http://www.wunderground.com. As oil accumulates in the mesocarp of olives over roughly 20 weeks (Harwood and Aparicio 2000), the mean temperature was calculated for the time period from August to December, as suggested by Bontempo et al. (2009). Temperature data are attached in the Appendix D (Table D1). Obtained olive oil samples (10 ml) were stored in glass vials at room temperature in the dark.

Additionally, in November 2010 samples of olive leaves and olives were collected from three olive varieties that were used for the production of the extra virgin olive oils. Samples collection was performed by Dr. Dimitri Abbado (University of Siena, Siena, Italy) and Dr. Nikolai Pedentchouk (University of East Anglia, Norwich, UK). For each olive variety samples were taken from three individual trees, resulting in nine samples of leaves (c. 50 leaves from an individual tree) and nine samples of olives (c. 15 olives from an individual tree). Leaf and olive samples were stored in paper envelopes at room temperature.

5.2.2. *n*-Alkane extraction

n-Alkanes were isolated from olive oils using column chromatography. A sample of olive oil (75 mg) was added to a 4 ml glass vial containing 0.5 ml of hexane. Pentadecane (*n*-C_{15}) (Sigma-Aldrich, Gillingham, UK) was used as an internal standard (concentration 2.50 mg/l) and added to the vial together with oil sample. The hydrocarbon fraction was separated from olive oil by the means of column chromatography. Glass Pasteur pipettes (145 x 9 mm) packed with activated silica gel (0.063-0.200 mm, Sigma-Aldrich, Gillingham, UK) were used as chromatography columns. The olive oil mixture was placed on the top of the silica gel column, eluted with hexane (5 ml), and the eluent containing *n*-alkanes was collected in a 4 ml glass vial. Due to a low *n*-alkane concentration, each oil sample was prepared and extracted twice, and then two *n*-alkane eluents were combined, concentrated under a stream of high grade nitrogen down to 0.5 ml, and stored at room temperature. Each extraction was performed in duplicate.

For the extraction of *n*-alkanes from olive leaves and olives the following procedure was used. Firstly, total lipid extracts were obtained from the olive samples (c. 15 whole olives per sample) and leaf samples (c. 20 whole leaves per sample) by sonication in hexane (15 min × 2). Then the extracts were concentrated to 1 ml using TurboVap II Concentration Evaporator Workstation (Zymark Corp., Hopkinton, MA, USA). The *n*-alkane fraction was separated by the means of column chromatography. Glass Pasteur pipettes (145 x 9 mm) packed with activated silica gel (Sigma-Aldrich, Gillingham, UK) were used as chromatography columns. 500 μl of total lipid extract was placed on the top of the column, eluted with hexane (5 ml), and the eluent containing *n*-alkanes was collected in a 4 ml glass vial. One chromatography column was used per each sample. Eluents were concentrated under a stream of high grade nitrogen to the volume of 0.5 ml, and stored at room temperature.

5.2.3. Gas chromatography

Gas chromatography analysis was performed using HP Agilent 6890 Gas Chromatograph (Agilent Technologies Inc., Wilmington, USA) with a flame ionisation detector (FID). Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The GC was equipped with a DB-5 capillary column (30m × 0.32 mm × 0.25 μm) (Agilent Technologies Inc., Santa Clara, USA) and programmed from 50

°C (held for 1 min) at 20 °C/min to 150 °C and further at 8 °C/min to 320 °C, and held for 10 min isothermally Injector and detector temperatures were 300°C and 280°C, respectively. Samples were injected using a split/splitless injector in a splitless mode. *n*-Alkanes were identified through comparison of elution times with a known *n*-alkane standard – a mixture of 15 *n*-alkanes: hexadecane (*n*-C_{16}), heptadecane (*n*-C_{17}), octadecane (*n*-C_{18}), nonadecane (*n*-C_{19}), eicosane (*n*-C_{20}), heneicosane (*n*-C_{21}), docosane (*n*-C_{22}), tricosane (*n*-C_{23}), tetracosane (*n*-C_{24}), pentacosane (*n*-C_{25}), hexacosane (*n*-C_{26}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}), nonacosane (*n*-C_{29}) and triacontane (*n*-C_{30}) (purchased from Dr. Arndt Schimmelmann; Department of Geological Sciences, Indiana University, Bloomington, USA). Individual *n*-alkane peak areas were calculated using EZChrom software (Agilent Technologies Inc., Wilmington, USA). *n*-Alkane average chain length (ACL) was calculated using peak area values of individual *n*-alkanes from C_{21} to C_{35} according to the following formula:

$$ACL = \frac{(21 \cdot A_{21}) + (22 \cdot A_{22}) + (23 \cdot A_{23}) + \ldots + (35 \cdot A_{35})}{(A_{21} + A_{22} + A_{23} + \ldots + A_{35})} \quad (5.1.)$$

where A_x corresponds to the peak area of the individual *n*-alkane from the chromatograph trace.

n-Alkane concentration in olive oil samples was calculated on the basis of the known concentration and the peak area of the internal standard (*n*-C_{15}) according to the following formula, assuming the response factor is equal to 1:

$$[S] = [St] \cdot \frac{A_S}{A_{St}} \quad (5.2.)$$

where [S] is the concentration of the individual *n*-alkane, [St] is the known concentration of the standard, A_S - peak area of the individual *n*-alkane, A_{St} - peak area of the standard.

5.2.4. Gas chromatography-isotope ratio mass spectrometry

Stable carbon isotope analysis of *n*-alkanes from olive oil was performed using Delta V Advantage isotope ratio mass spectrometer interfaced with GC-Isolink Trace Ultra GC Combustion and High temperature conversion systems (Thermo Scientific, Bremen, Germany). Individual *n*-alkanes were separated using a DB-5 capillary column (30m × 0.32 mm × 0.25 μm). Helium was used as a carrier gas at a flow rate

of 1.2 ml/min. The GC oven was programmed from 50 °C (held for 1 min) at 20 °C/min to 150 °C and further at 8 °C/min to 320 °C and held for 10 min isothermally. Samples were injected using a programmable temperature vaporization (PTV) injector in splitless mode at 280 °C.

Nonacosane (*n*-C_{29}) was chosen for isotopic characterisation of olive oils, olive leaves and olives, because it was the only *n*-alkane present in sufficient concentration for the GC-IRMS analysis in all three types of samples. Samples concentrations during carbon isotope analyses were adjusted to give the *n*-C_{29} peaks a similar intensity to those of the corresponding reference gas.

Combustion of *n*-alkanes to CO_2 was conducted at 1030 °C. Carbon isotope composition of *n*-C_{29} ($\delta^{13}C_{C29}$) was reported based on duplicate analyses of well-resolved peaks. For the majority of duplicate measurements the absolute difference in $\delta^{13}C_{C29}$ values was < 0.3‰, never exceeding 0.5‰.

The analytical accuracy and precision of GC-IRMS system during stable carbon isotope analyses were determined using a standard mixture of 15 *n*-alkanes (isotopic ratios measured off-line by Dr. Arndt Schimmelmann; Department of Geological Sciences, Indiana University, Bloomington, USA and available at http://mypage.iu.edu/~aschimme), which was analyzed between every 10 samples. Carbon isotopic composition of *n*-C_{29} was expressed relative to V-PDB based on in-house CO_2 reference gas (BOC, UK) adjusted daily using the above standard mixture of 15 *n*-alkanes. Isodat software Version 3.0 (Thermo Scientific, Bremen, Germany) was used for processing the data from *n*-alkane measurements.

5.2.5. Statistical analyses

Statistical analyses were performed using SPSS 16.0 package for Windows (SPSS Inc., Chicago, USA). All data were checked for normality and homogeneity of variances. Data were normally distributed unless stated otherwise. Independent sample t-test was applied to study the difference i) between olive oils produced using manual and mechanical olive harvesting techniques based on the mean concentrations of individual *n*-alkanes, the mean concentrations of total and odd-numbered *n*-alkanes, ACL values and $\delta^{13}C_{C29}$ values; ii) between olives and olive leaves based on the ACL values and $\delta^{13}C_{C29}$ values. The difference between two groups was considered significant at $p < 0.05$.

5.3. Results

5.3.1. Molecular and isotopic composition of *n*-alkanes in extra virgin olive oils produced from manually and mechanically harvested olives

The *n*-Alkane profiles of eight extra virgin olive oils from Ciggiano are characterized by the presence of homologues with carbon chains in the range from *n*-C_{21} to *n*-C_{35}. Figure 5.1 shows the average distribution of individual *n*-alkanes in olive oils produced from manually (A) and mechanically (B) harvested olives.

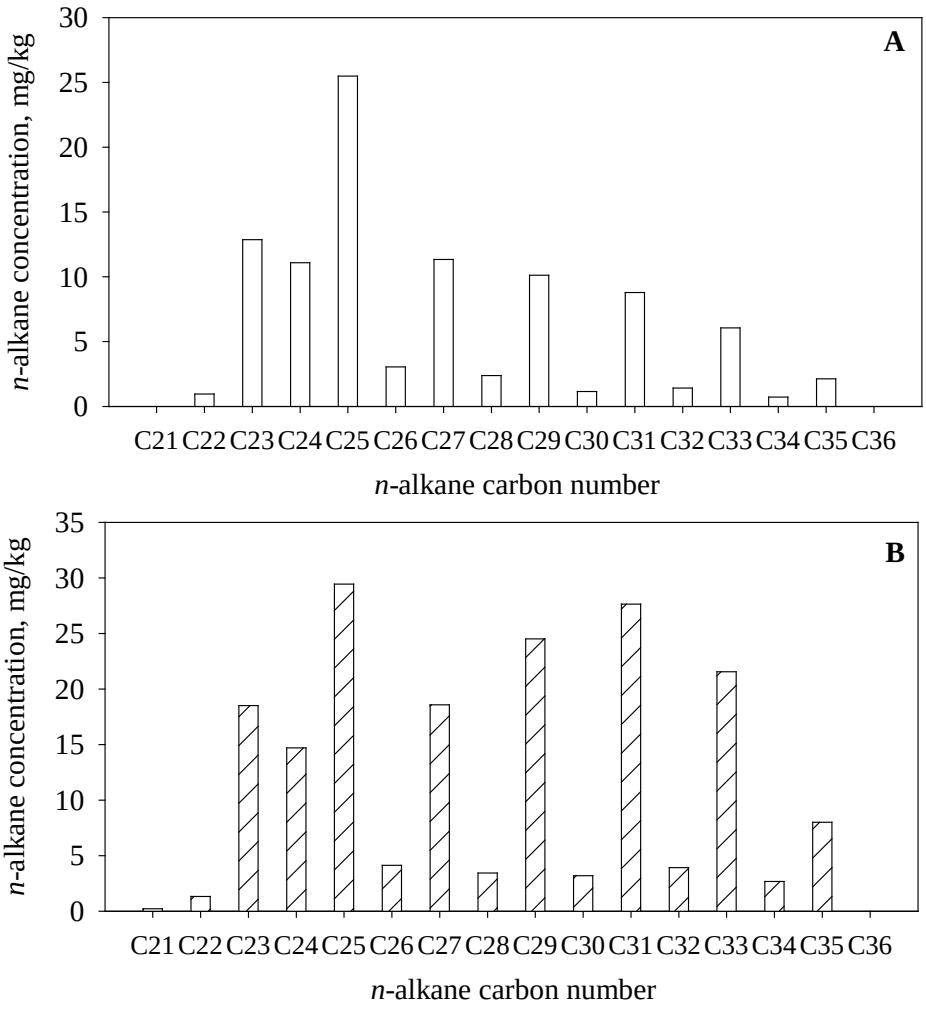

Figure. 5.1. The average *n*-alkane profile of extra virgin olive oils obtained from manually (A) and mechanically (B) harvested olives. Concentrations of individual *n*-alkanes represent the mean values based on four oil samples, as presented in Table 5.1.

Table 5.1. Concentrations of *n*-alkanes (*n*-C_{21} – *n*-C_{35}) in extra virgin olive oils produced from manually and mechanically harvested olives.

Olive harvesting technique	Oil production year	***n*-alkane concentration, mg kg^{-1}**														
		n-C_{21}	*n*-C_{22}	*n*-C_{23}	*n*-C_{24}	*n*-C_{25}	*n*-C_{26}	*n*-C_{27}	*n*-C_{28}	*n*-C_{29}	*n*-C_{30}	*n*-C_{31}	*n*-C_{32}	*n*-C_{33}	*n*-C_{34}	*n*-C_{35}
manual	2004	0.0	0.6	8.1	6.7	20.8	2.6	11.3	2.4	7.8	0.9	5.6	1.1	4.1	0.5	1.5
	2005	0.0	0.6	11.6	11.0	26.7	3.3	12.2	3.7	9.8	1.0	7.4	1.1	4.5	0.5	1.6
	2006	0.0	1.1	14.4	12.9	27.1	3.3	11.0	1.4	11.6	1.3	12.5	1.8	9.1	1.1	3.5
	2010	0.0	1.5	17.5	13.7	27.3	2.9	10.8	1.9	11.4	1.4	9.7	1.6	6.4	0.7	2.0
mechanical	2007	0.0	0.5	8.8	6.7	17.5	2.8	15.3	2.7	22.6	2.9	27.2	3.9	22.9	2.6	7.6
	2008	0.0	0.0	8.4	6.0	13.9	2.2	16.9	2.0	19.6	2.6	24.8	4.0	19.7	2.5	7.4
	2009	0.0	0.0	5.3	4.7	11.0	1.7	8.4	1.4	11.2	1.4	11.9	1.6	7.9	0.8	3.0
	2010	0.9	4.8	51.6	41.4	75.3	9.8	33.6	7.7	44.7	5.9	46.6	6.2	35.6	4.8	13.9
manual	**Mean**	0.0	0.9	12.9	11.1	25.5	3.1	11.3	2.4	10.1a	1.2a	8.8a	1.4a	6.1a	0.7a	2.1a
	SD	0.0	0.4	3.7	2.9	3.6	0.3	0.6	1.6	1.6	0.2	2.8	0.4	2.1	0.3	0.9
mechanical	**Mean**	0.2	1.3	18.5	14.7	29.4	4.1	18.6	3.4	24.5a	3.2a	27.6a	3.9a	21.5a	2.7a	8.0a
	SD	0.4	2.1	20.5	16.5	28.4	3.5	10.0	2.7	13.4	1.8	13.4	1.8	10.6	1.5	4.2

Letters (a) denote significant difference ($p<0.05$) in the mean values between oils produced with two different olive harvesting techniques

Both types of olive oils are characterised by the predominance of *n*-C_{25} (Figure 5.1.). The profile of *n*-alkanes in the range of *n*-C_{22} to *n*-C_{26} is similar in the olive oils obtained with mechanical and those using manual olive harvesting. However, the distribution of *n*-alkanes with longer carbon chains (from *n*-C_{29} to *n*-C_{35}) is different between the two types of oils – the olive oils obtained from mechanically harvested olives have higher amounts of longer chain *n*-alkanes compared to olive oils produced from the hand-picked olive fruits.

The concentrations of individual *n*-alkanes (*n*-C_{21} – *n*-C_{35}) are higher in oils produced from mechanically harvested olives (Table 5.1). Significant difference between two oils types is found in the concentration of *n*-alkanes with carbon chains from *n*-C_{29} to *n*-C_{35} – their concentration is significantly higher ($p<0.05$) in oils produced from mechanically harvested olives (Table 5.1).

Table 5.2. Molecular and stable carbon isotope compositions of *n*-alkanes in extra virgin olive oils produced from manually and mechanically harvested olives.

Olive harvesting technique	Oil production year	Total *n*-alkanes, mg/kg	Odd - numbered *n*-alkanes, mg/kg	Odd - numbered *n*-alkanes, % of total	ACL	$\delta^{13}C_{C29}$, ‰
manual	2004	74.0	59.0	79.8	26.8	-32.2
	2005	95.0	73.8	77.7	26.6	-32.3
	2006	112.4	89.3	79.5	27.2	-31.8
	2010	108.7	85.0	78.2	26.5	-32.4
mechanical	2007	144.0	121.9	84.7	29.1	-32.1
	2008	130.0	110.7	85.2	29.2	-32.6
	2009	70.3	58.7	83.5	28.4	-32.4
	2010	382.9	302.3	79.0	28.0	-32.4
manual	**Mean**	97.5	76.8	78.8a	26.8a	-32.1
	SD	16.4	12.7	1.0	0.3	0.2
mechanical	**Mean**	181.8	148.4	83.1a	28.7a	-32.4
	SD	128.0	98.7	2.9	0.5	0.2

SD – standard deviation based on the four oil samples; Letters (a) denote significant difference ($p<0.05$) in the mean values between oils produced with two different olive harvesting techniques; ACL – *n*-alkane average chain length.

Total *n*-alkane concentrations are higher in oils obtained from mechanically harvested olives compared to oils obtained from manually collected olives (Table 5.2). All oils exhibit distinct odd-numbered *n*-alkane predominance; odd-numbered *n*-alkanes comprise from 78% to 85% of total *n*-alkane concentration and are also present in higher amounts in oils obtained from mechanically harvested olives.

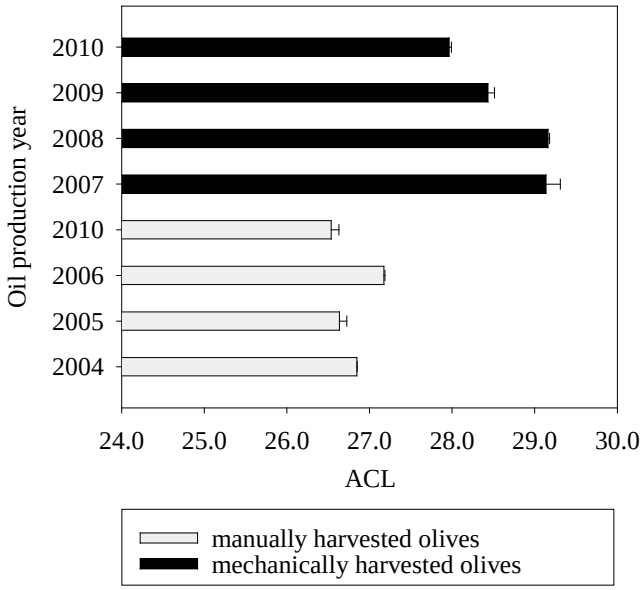

Figure 5.2. *n*-Alkane average chain length (ACL) in extra virgin olive oils obtained from manually and mechanically harvested olives. The error bars represent the absolute difference in ACL values based on duplicate analyses.

The values of *n*-alkane average chain length (ACL) differ significantly ($p<0.05$) between olive oils produced using two different olive harvesting methods (Table 5.2, Figure 5.2). Oils from mechanically harvested olives have significantly ($p<0.05$) longer average chains (ACL = 28.7) than oils from hand-picked olives (ACL = 26.8).

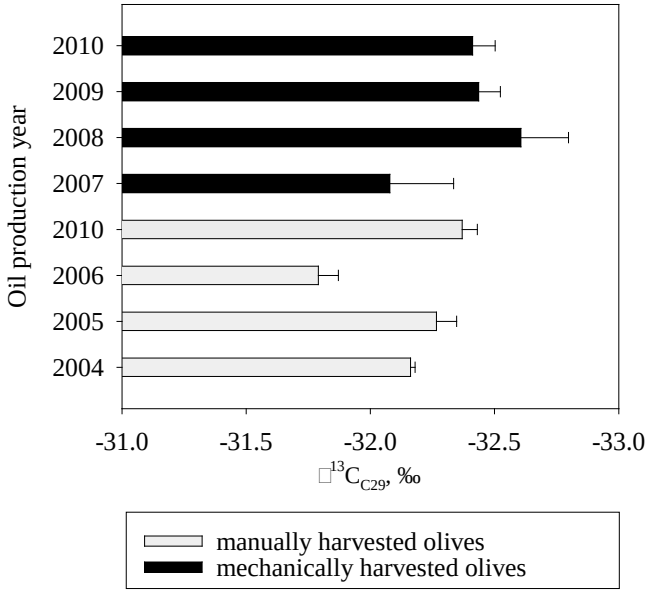

Figure 5.3. Stable carbon isotope composition of *n*-C_{29} in extra virgin olive oils produced from manually and mechanically harvested olives. The error bars represent the absolute difference in $\delta^{13}C_{C29}$ values based on duplicate analyses.

In contrast with molecular composition of *n*-alkanes, the stable carbon isotope composition of *n*-C_{29} does not differ significantly ($p>0.05$) between oils produced with two different olive harvesting techniques (Table 5.2, Figure 5.3). The mean $\delta^{13}C_{C29}$ values are -32.1‰ and -32.4‰ for oils produced from manually and mechanically harvested olives, respectively.

5.3.2. Molecular and isotopic composition of *n*-alkanes in olive leaves and fruits

Olive leaves and olives have significantly different *n*-alkane compositions. Figure 5.4 shows the difference in the *n*-alkane distribution in olives and leaves of Frantoio variety. Similar differences are observed in the distribution of *n*-alkanes in olives and leaves of other two varieties, Leccino and Moraiolo, and thus, are not shown here. Olives are characterized by the predominance of *n*-alkanes with shorter carbon chains that those of leaves. The most prevalent *n*-alkanes in olives are *n*-C_{25}, *n*-C_{27} and *n*-C_{29}, whereas *n*-C_{29}, *n*-C_{31}, *n*-C_{33} and *n*-C_{35} are the most abundant *n*-alkanes in olive leaves. Both olives and leaves show distinct odd-number predominance.

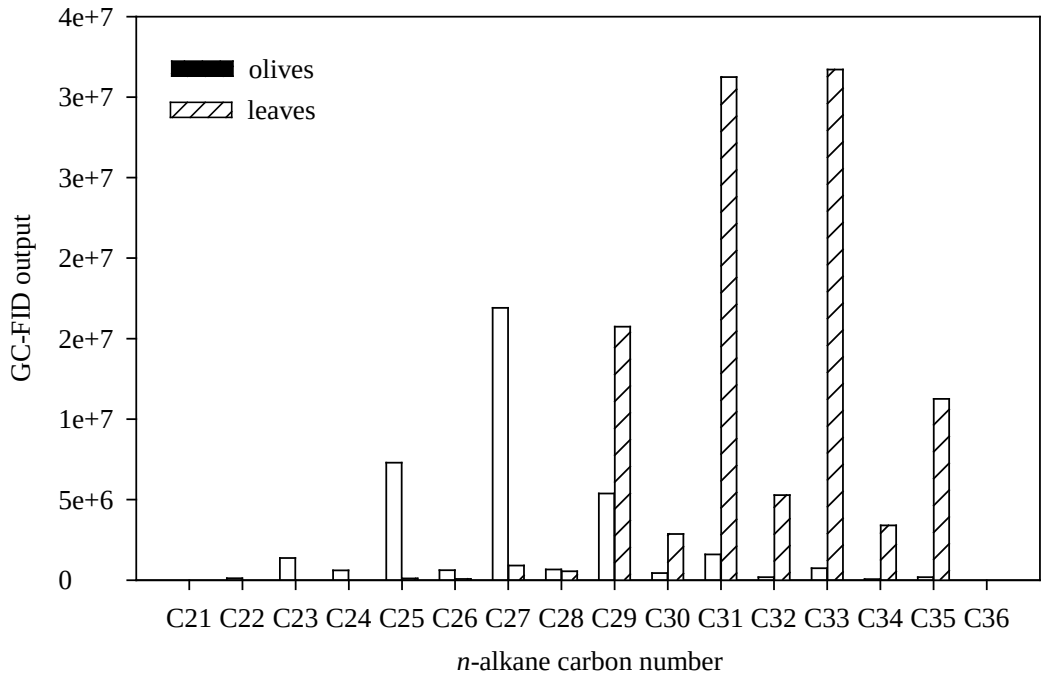

Figure 5.4. The distribution of *n*-alkanes in olives and leaves of Frantoio variety.

The ACL values of olives and olive leaves from the three olive varieties are shown in Table 5.3 and Figure 5.5. Olive leaves have significantly longer ($p<0.05$) *n*-alkane average chain lengths compared to olives (Figure 5.5). The mean ACL values of olives from the three varieties are 27.2±0.3 ACL units, while the mean ACL values of olive leaves are 31.9±0.2 ACL units (Table 5.3). The difference in ACL values among three varieties is insignificant ($p>0.05$) in respect to olives. Leaves of Frantoio variety have significantly lower ($p<0.05$) ACL values compared to leaves of Leccino and Moraiolo.

Table 5.3. The mean ACL and $\delta^{13}C_{C29}$ values of olives and leaves from Frantoio, Leccino and Moraiolo varieties. The values of individual samples from each variety are shown in Table F2, Appendix D.

Olive variety	Number of samples		ACL ± SD_1		$\delta^{13}C_{C29}$, ‰ ± SD_1	
	olives	leaves	olives	leaves	olives	leaves
Frantoio	3	3	27.2±0.5x	31.7±0.1abx	-32.4±0.4ab	-32.1±0.2
Leccino	3	3	27.1±0.2x	32.0±0.1ax	-34.4±0.9ay	-31.8±0.3y
Moraiolo	3	3	27.2±0.1x	32.1±0.1bx	-33.5±0.5by	-31.6±0.2y
Mean ± SD_2	**9**	**9**	**27.2±0.3**x	**31.9±0.2**x	**-33.4±1.1**y	**-31.8±0.3**y

SD_1 – standard deviation based on the values of three replicate samples of each olive variety; SD_2 – standard deviation based on the values of three varieties (n=9). Different letters denote significant difference ($p<0.05$) in the mean values within a row (x,y) or within a column (a,b).

The stable carbon isotope composition of *n*-C_{29} is different between olives and leaves of the three analyzed varieties with $\delta^{13}C_{C29}$ values of leaves being more positive compared to those of olives (Figure 5.6).

The difference in $\delta^{13}C_{C29}$ values between olives and leaves is significant ($p<0.05$) for Leccino and Moraiolo varieties, however is not significant ($p>0.05$) for Frantoio variety (Table 5.3).The mean $\delta^{13}C_{C29}$ value of leaves from the three varieties is -31.8 ± 0.3‰, whereas the mean $\delta^{13}C_{C29}$ of olives is -33.4 ± 0.6‰ (Table 5.3).

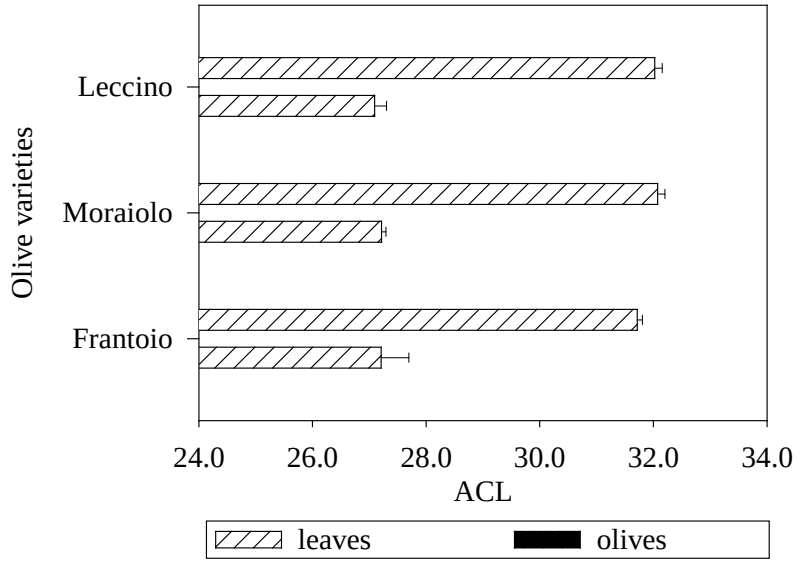

Figure 5.5. The mean ACL values of olives and leaves from Frantoio, Leccino and Moraiolo varieties. The error bars represent standard deviation values based on the three replicate samples from each olive variety.

In contrast with ACL values, stable carbon isotope composition of *n*-C_{C29} in olives is significantly different among three olive varieties. $\delta^{13}C_{C29}$ values of Frantoio olives are significantly more positive ($p<0.05$) than those of the other two varieties (Figure 5.6, Table 5.3). The $\delta^{13}C_{C29}$ values of leaves among the three varieties are not significantly different ($p>0.05$).

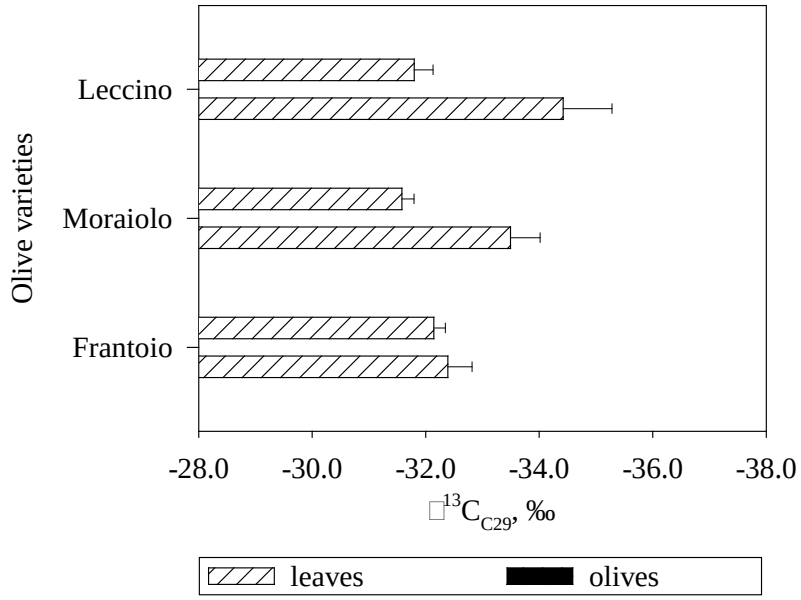

Figure 5.6. The mean $\delta^{13}C_{C29}$ values of olives and leaves from Frantoio, Leccino and Moraiolo varieties. The error bars represent standard deviation values based on the three replicate samples from each olive variety.

5.4. Discussion

The main purpose of this study was to evaluate whether different olive harvesting methods (mechanical/manual) affect the molecular and stable carbon isotope compositions of *n*-alkanes in extra virgin olive oils from Ciggiano, Tuscany, Italy. Potential differences in *n*-alkane profiles between the oils produced using manual and mechanical methods may arise from the incorporation of a significant amount of leaf material during mechanical harvesting of olives. To test the role of this factor, I have determined the distribution and carbon isotope compositions of *n*-alkanes not only from both types of olive oils but also from olives and olive leaves of three olive varieties (Frantoio, Leccino and Moraiolo) used for the oil production in Ciggiano. Firstly, I discuss here the differences observed in the molecular composition of *n*-alkanes between two groups of olive oils as well as between olives and olive leaves. Further, the differences found in the carbon isotope composition of *n*-C_{29} alkane in olive oils, olives and leaves are addressed.

The molecular composition of *n*-alkanes in Ciggiano olive oils exhibits a significant variation depending on the olive harvesting technique. Olive oils produced from mechanically harvested olives have significantly higher ($p<0.05$) amounts of long chain *n*-alkanes (*n*-C_{27} - *n*-C_{35}) and significantly higher ($p<0.05$) ACL values compared to the oils obtained from olives collected manually (Figure 5.2). This difference in the molecular composition of *n*-alkanes in the two types of oils is caused by different amounts of shorter and longer chain *n*-alkane in olives and olive leaves. Olive leaves are dominated by longer chain *n*-alkanes (*n*-C_{29} - C_{35}) and have significantly higher ($p<0.05$) ACL values compared to olives (Figure 5.5). The mean ACL value of leaves is by 4.7 units higher than that of olives.

To better illustrate the impact of leaf material on the molecular composition of olive oils produced using mechanical harvesting technique, I have compared the mean ACL values of olives and olive leaves collected from Frantoio, Leccino and Moraiolo varieties in 2010 with the ACL values of two type of oils which were produced from the above varieties in the same year (Figure 5.7). Olive oil produced from manually harvested olives has the lowest ACL value (26.5 ACL units), which is close to that of olives (27.2 ACL units). The lower ACL of this oil compared to the ACL of olive fruits, from which the oil was obtained, could have resulted from a loss of longer chain *n*-alkanes during olive processing. In contrast, the ACL value of

olive oil produced in 2010 from the same olive fruits, but harvested mechanically, is higher by 1.5 ACL units than that of the oil obtained from manually collected olives (Figure 5.7). Moreover, the ACL of olive oil obtained from mechanically harvested olives is higher by 0.8 ACL values than that of the olives, from which it has been produced. This indicates the presence of external material in this oil, which could have raised the amount of longer chain *n*-alkanes and resulted in a shift of ACL towards the higher values. Because olive leaves have significantly higher ACL values than olives and, as observed in the field, were mixed with olives during mechanical harvesting, this would explain the observed increase in the ACL of olive oil obtained from mechanically harvested olive fruits.

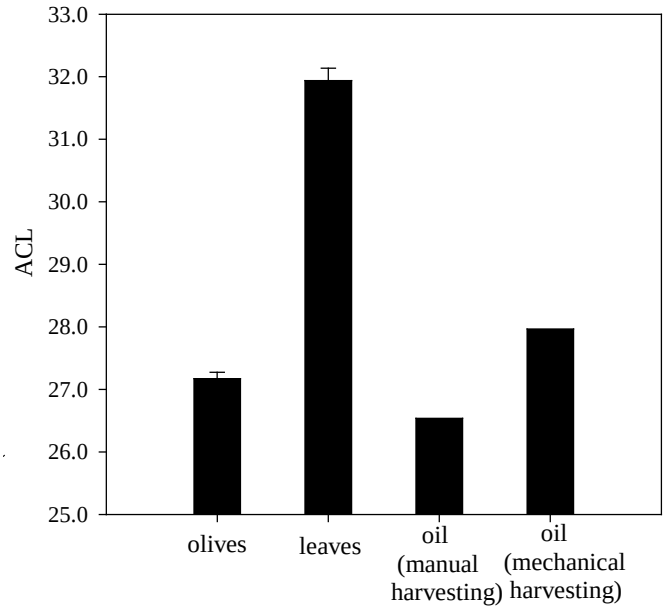

Figure 5.7. The ACL values of extra virgin olive oils produced using two different olive harvesting techniques and the mean (n=9) ACL values of olive leaves and olives from Frantoio, Leccino and Moraiolo varieties. All samples were collected in 2010. For olives and leaves, error bars represent standard deviation values based on nine samples.

The molecular composition of *n*-alkanes of olives and olive leaves from the present study is in accordance with the data reported by Bianchi et al. (1992a), who performed the analysis of epicuticular waxes from olives Italian Coratina variety. Authors reported that *n*-C_{27} alkane was the most prevalent *n*-alkane in the mature Coratina olives (Bianchi et al. 1992a), which has also been observed in olives of the three Italian varieties from the present study. Sakouhi et al. (2011) reported that the most dominant *n*-alkanes in olives of Tunisian Meski variety were *n*-C_{23}, *n*-C_{25}, and *n*-C_{27}. In respect to olive leaves, Bianchi et al. (1992b) analyzed the composition of

olive leaf waxes in two Italian varieties (Coratina and Cipressino) and found that longer chain *n*-alkanes (*n*-C_{29} - *n*-C_{35}) comprised more than 95% of the total *n*-alkanes in olive leaves, which is in agreement with the composition of *n*-alkanes in leaves of olive varieties analysed in the present study. Furthermore, the strong odd-over-even predominance of *n*-alkanes in olives and olive leaves in the present study is in agreement with the results of previous studies on leaf wax composition of different higher plant species (e.g., Eglinton and Hamilton 1967, Reddy et al. 2000, Bi et al. 2005). The pattern is attributed to a loss of one carbon atom from the acyl precursors during the decarbonylation of aldehydes in the process of *n*-alkane biosynthesis (Cheesbrough and Kolattukudy 1984).

With respect to the carbon isotope composition of olive oils, although the $\delta^{13}C$ values of *n*-C_{29} in olive leaves of two varieties (Leccino and Moraiolo) are significantly more positive ($p<0.05$) than those in olives (Figure 5.6), compound-specific stable carbon analysis shows no significant difference ($p>0.05$) in $\delta^{13}C_{C29}$ values between olive oils obtained using two different harvesting techniques (Figure 5.3). This may be explained by the fact that the difference between the $^{13}C/^{12}C$ ratio in leaves and the $^{13}C/^{12}C$ ratio in olives is much smaller compared to the difference in the proportion of shorter versus longer chain *n*-alkanes (as represented by the ACL values) in olives and leaves. The difference in the mean ACL values between olives and leaves is 4.7 ACL units, while the difference in $\delta^{13}C_{C29}$ values is 1.6‰, which apparently is not great enough to shift the $\delta^{13}C_{C29}$ values of the oils produced from mechanically harvested olives towards more positive values. To observe a significant shift in the $\delta^{13}C_{C29}$ values of oils a much greater proportion of leaves has to be present in oil.

Thus, stable carbon isotope composition of *n*-C_{29} is not significantly affected by the presence of leaves in oils, and the variability in the $\delta^{13}C_{C29}$ values of oils from seven production years (Figure 5.3) is likely caused by other factors. These factors may include i) differences in the mean atmospheric temperature during lipid formation period in olives among the production years, and ii) the variability in the proportion of the three varieties harvested each year. The carbon isotope composition of plants is influenced by the stomatal conductance and the plant's intercellular and ambient CO_2 concentration. Factors that impact the $^{13}C/^{12}C$ composition include temperature, relative humidity, amount of precipitation and plant age. Stable carbon isotope ratios have been reported to correlate with plant

water use efficiency, which in turn is dependent on the ambient CO_2 concentration and temperature (Smith and Epstein 1971, O'Leary 1981, Ehleringer and Dawson 1992, Martin and Martin 2003). In the present study, olive oils produced in 2004 and 2006 show more positive $\delta^{13}C_{C29}$ values compared to oils produced in other years (Figure 5.3). This may be related to higher temperatures during oil formation time in olives (from August to December) in these years (Appendix D, Table D1), which may have resulted in lower stomatal conductance in olive trees and smaller discrimination against ^{13}C during photosynthesis, which in turn, could have led to more positive $\delta^{13}C_{C29}$ values of olives and consequently in olive oils in those years.

Moreover, the carbon isotope composition of olive oils from Ciggiano can also be influenced by the difference in the isotopic values among olives from Frantoio, Leccino and Moraiolo varieties (Figure 5.6). Olive oils produced in Ciggiano are blends of these three olive varieties, and the proportion of olives from each variety in the oil has been subject to certain variability every year depending on the yield of each variety. Frantoio olives have significantly more positive ($p<0.05$) $\delta^{13}C_{C29}$ values compared to the olives of other two varieties (Figure 5.6). There is no data available on the relative yields of each variety in the 2004-2010 production years. However, it is possible that the more positive $\delta^{13}C_{C29}$ values of olive oil correspond to those years that were characterized by a relative increase in the proportion of Frantoio in comparison with Leccino or Moraiolo olive harvests.

This is the first time when compound-specific carbon isotope analysis of *n*-alkanes has been applied to olive oils, olives and olive leaves simultaneously. Significant differences in carbon isotope composition of *n*-alkanes in olives among varieties indicate that there is a potential for using compound-specific carbon isotope analysis of leaf waxes for varietal differentiation of olives and olive oils. Furthermore, differences between the *n*-alkane $\delta^{13}C$ values of olives and leaves could be used further to study the physiology and biochemistry of fruit formation in olive trees.

5.5. Conclusions

Mechanical and manual olive harvesting methods result in different physical and chemical characteristics of olive oil. Mechanical olive harvesting in Ciggiano (Tuscany, Italy) generated a significant amount of leaf material, which was incorporated into olive oil during olive production. In this study, I have evaluated whether extra virgin olive oils produced in Ciggiano using manual and mechanical harvesting techniques differ in respect to their molecular and stable carbon isotope compositions of *n*-alkanes.

The presence of leaves has resulted in a significant difference in the molecular composition of *n*-alkanes in olive oils. Olive oils produced from mechanically harvested olives have significantly higher ($p<0.05$) amount of long-chain *n*-alkanes (*n*-C_{29}-*n*-C_{35}) and significantly higher ($p<0.05$) values of *n*-alkane average chain length (ACL = 28.7±0.5) than oils obtained from the hand-picked olives (ACL = 26.8±0.3). This difference resulted from a significant difference in olives and leaves in respect to their molecular composition of *n*-alkanes. Olive leaves are characterized by the predominance of long-chain *n*-alkanes (*n*-C_{29}, *n*-C_{31}, *n*-C_{33}, *n*-C_{35}) and significantly higher ($p<0.05$) ACL values (ACL = 31.9±0.1) compared to olives (ACL = 27.2±0.3). Thus, the molecular composition of *n*-alkanes reflects the high presence of leaf material in olive oils and may be useful for distinguishing clean oils from those with excess leaf content.

A large amount of leaf material incorporated into olive oils during mechanical olive harvesting has not affected the stable carbon isotope composition of *n*-alkanes in those oils. Although the mean $\delta^{13}C$ values of *n*-alkanes in olives and olive leaves of three varieties are significantly different ($p<0.05$), the $\delta^{13}C$ values of *n*-alkanes in olive oils do not show significant differences between oils produced using two different olive harvesting methods. However, significant differences in carbon isotope composition of *n*-alkanes are found among olive varieties, indicating that there is a potential for using compound-specific carbon isotope analysis of *n*-alkanes for the differentiation of the varietal origin of olives and olive oils.

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CHAPTER 6

THE IMPACT OF OLIVE RIPENING STAGE AND VARIETY ON THE MOLECULAR AND STABLE ISOTOPE COMPOSITIONS OF N-ALKANES IN OLIVE LEAVES AND FRUITS

Abstract

Olive tree is one of the most important and widespread fruit trees in the Mediterranean region. Cuticular waxes coat olive leaves and fruits and, being a good physical barrier, play a significant role in olive resistance against drought and other environmental stresses. Finding specific markers for differentiating olive varieties can be highly important for the verification of varietal origin of olive oils.

The present study investigated how the distribution of *n*-alkanes in olives and leaves of three Italian varieties (Frantoio, Leccino and Moraiolo) change during different stages of olive fruit maturity: from July until full olive maturation in November, and whether the molecular compositions of *n*-alkanes differ in three varieties. Furthermore, the differences among three varieties were evaluated on the basis of carbon isotope compositions of the most dominant *n*-alkanes in olives and leaves.

The composition of *n*-alkanes in olives of the three varieties changes greatly throughout the ripening season. A gradual shift from the longer chain *n*-alkanes towards the shorter chain *n*-alkanes is observed in olives of all varieties from July to November. At the beginning of the ripening season longer chain *n*-alkanes (*n*-C_{27}, *n*-C_{29}, *n*-C_{31} and *n*-C_{33}) are the most prevalent in olive fruits, while by the end of the ripening season the most dominant in olives become shorter chain *n*-alkanes (*n*-$C_{25,}$ *n*-C_{27} and *n*-C_{29}). The *n*-alkane average chain length (ACL) values of olives gradually decrease during the whole season from July to November. In contrast, the distribution of *n*-alkanes in olive leaves does not exhibit any significant changes throughout the season and is dominated by three homologues: *n*-C_{29}, *n*-C_{31} and *n*-$C_{33.}$ The molecular analysis of *n*-alkanes does not reveal significant differences among three varieties in respect to the *n*-alkane distribution in leaves. However, differences are observed among the varieties in respect to the ACL of olives: during September,

October and November Moraiolo variety is characterised by higher ACL compared to Frantoio and Leccino.

Compound-specific carbon isotope analysis of *n*-alkanes reveals differences among three olive varieties. The $\delta^{13}C$ values of the most dominant *n*-alkanes in olive fruits (*n*-$C_{25,}$ *n*-$C_{27,}$ *n*-C_{29} and *n*-C_{31}) are more positive in Frantoio variety compared to Leccino and Moraiolo throughout the whole season, from July to November. There is a potential for using the compound-specific carbon isotope analysis of *n*-alkanes for studying the inter-varietal differences in olives.

6.1. Introduction

6.1.1. The adaptation mechanisms of olive trees to hot Mediterranean climate

The olive *(Olea europaea*) is an emblematic species and one of the most important and widespread fruit trees in the Mediterranean (Loumou and Giourga 2003). Although a considerable amount of olives are processed for direct human consumption, most olive production is destined for the production of olive oil. Extra virgin olive oil is a major component of the Mediterranean diet and is highly valued among the consumers for its nutritional and health properties (Harwood and Aparicio 2000, Conde et al. 2008).

Many recent studies have highlighted a general warming trend for the Mediterranean region in the near future (Brunetti et al. 2004, Founda et al. 2004, Kumar et al. 2005, Brunetti et al. 2006, Seneviratne et al. 2006, Diffenbaugh et al. 2007, Bartolini et al. 2008). Model predictions suggest that the highest summer temperatures in the Mediterranean countries are likely to increase more than the average summer temperatures, whereas the annual number of days of precipitation is very likely to decrease (IPCC 2012). Both of these scenarios would result in an increased risk of summer droughts, which can significantly affect the growth and productivity of olive trees in the region.

Olive trees are adapted to the hot Mediterranean climate and are reported to be resistant to drought (Giorio et al. 1999, Tognetti et al. 2004, Connor and Fereres 2005, Bacelar et al. 2006, Bacelar et al. 2007). The ability of olive trees to cope with water deficit involves morphological, anatomical and physiological adaptations at the leaf level. Olive plant adaptations to the reduced water availability include small leaf size, enhanced sclerophylly, high reflectivity and high density of the foliar tissue, thick cuticle and trichome layers (Bacelar et al. 2004, Bacelar et al. 2006, Connor 2005). Olive trees are capable of maintaining turgor through the osmotic adjustment of cell contents, small cell size, and changes in the cell-wall elasticity (Connor 2005). However, under the prolonged or severe water stress conditions, which may become a frequent occurrence under changing climate, olive plants exhibit limitation of CO_2 assimilation because of stomatal closure and show an overall reduction in photosynthetic activity (Angelopoulos et al. 1996). Additionally, stomata closure enhances the production of reactive oxygen species that increase the risk of oxidative damage in olive tissues, such as the oxidation of cellular lipids and proteins, the

destruction of photosynthetic pigments and the inactivation of photosynthetic enzymes (Yordanov 2000, Moller 2001, Bacelar et al. 2006).

Various olive varieties have been reported to exhibit differences in their water use efficiency, ability for adaptation and fruit production under drought conditions (Chartzoulakis et al. 1999, Bosabalidis and Kofidis 2002, Bacelar et al. 2004, Bacelar et al. 2006, Bacelar et al. 2007). Thus, careful selection of olive varieties with the highest resistance capabilities against drought stress is needed to face the changing Mediterranean climate and to ensure high productivity of the future olive orchards.

6.1.2. Cuticular waxes of olive leaves and fruits: composition and role in resistance to environmental stresses

Among the most important structural and physiological adaptations of olive trees to drought is the presence of a thick cuticular wax layer on the surface of olive leaves and fruits (Bianchi et al. 1992a, Bianchi et al. 1992b, Bianchi 2003). Cuticular waxes are hydrophobic compounds that consist of complex mixtures of long chain fatty acids, hydrocarbons (mainly *n*-alkanes), alcohols, aldehydes, ketones, esters, triterpenes, sterols, and flavonoids (Kunst and Samuels 2003, Jetter et al. 2006, Shepherd and Griffiths 2006). The cuticular wax layer restricts non-stomatal water loss, protects plants against ultraviolet radiation and reduces water retention on the surface of the plant, thus minimizing deposition of dust, pollen and air pollutants. In addition, cuticular waxes play an important role in plant defence against bacterial and fungal pathogens and participate in a variety of plant-insect interactions (Eigenbrode and Espelie 1995, Barnes et al. 1996, Kunst and Samuels 2003, Jetter et al. 2006, Shepherd and Griffiths 2006).

Various environmental factors have been reported to have a significant impact on the composition of cuticular waxes (Kunst and Samuels 2003, Jetter et al. 2006, Shepherd and Griffiths 2006). Stress due to drought can result in higher wax load per leaf surface area (Baker and Procopiou 1980, Johnson et al. 1983, Jefferson et al. 1989, Premachandra et al. 1992, Bondada et al. 1996, Rommerskirchen et al. 2006). Drought-induced increase in the amount of cuticular waxes reduces the epidermal conductance of water vapour, and thereby can decrease plant water loss in the water-limiting environment (Premachandra et al. 1992). Higher irradiation levels result in

an increase in cuticular wax thickness (Baker 1974, Shepherd et al. 1995, Shepherd and Griffiths 2006).

Apart from the influence of the environmental factors, olive variety can have a strong impact on the composition of cuticular waxes. For example, different distributions of hydrocarbons were reported in several Spanish varieties (Arbequina, Cornicabra, Empeltre, Hojiblanca, and Picual) by Guinda et al. (1996). Differences in *n*-alkane profiles of three olive varieties (Leccino, Buza and Bjelica) from Croatia were observed by Koprivnjak et al. (2005), and were applied for the differentiation of extra virgin olive oils from those varieties.

n-Alkanes are a major group of organic compounds in cuticular waxes responsible for the restriction of water loss and reduced wettability of the cuticle (Holloway 1969). The composition of *n*-alkanes from cuticular waxes of olive leaves and fruits, however, has been studied only to a limited extent (Bianchi et al. 1992a, Bianchi et al. 1992b, Sakouhi et al. 2011). Bianchi et al. (1992a) reported that cuticular waxes of olive fruits contained *n*-alkanes, saturated and unsaturated alkyl esters, aldehydes, methyl phenyl esters, triacylglycerols, alcohols, fatty acids, and pentacyclic triterpenols and triterpenoid acids (Bianchi et al. 1992a). Authors compared green and black olives of the same variety, and reported differences in their chemical compositions depending on the ripening stage. Cuticular waxes from green olives collected at the earlier stages of ripening season contained significantly higher amounts of uvaol and erythrodiol as well as longer chain *n*-alkanes in comparison with black olives. Waxes of leaves from olive trees contained the ubiquitous classes of *n*-alkanes, alcohols, aldehydes, fatty acids, alkyl esters, methyl phenyl esters (Bianchi et al. 1992b). In respect to *n*-alkanes, olive leaves contained high amounts of odd-number long chain *n*-alkanes: *n*-C_{29}, *n*-C_{31} and *n*-C_{33}. The compositions of triacylglycerols, free fatty acids, aldehydes and alcohols were reported to exhibit differences in two different olive varieties (Bianchi et al. 1992b).

Analysis of chemical composition of olive cuticular waxes is important given expected changes in the Mediterranean climate. Studies addressing the role of varietal and environmental factors on the composition of cuticular waxes will lead to a more informed selection of olive varieties that are better adapted to various environmental stresses. Moreover, studies that address the differences among olive varieties on the basis of their cuticular wax composition can contribute to

differentiation of olive varieties in extra virgin olive oils and thus, can be of interest from the food authentication perspective.

6.1.3. Rationale for the study

Even though *n*-alkanes comprise a significant fraction of plant cuticular waxes and are one of the most abundant lipid compounds biosynthesized by terrestrial plants, their distribution in olive trees has been studied only to a very limited extent (Bianchi et al. 1992a, Bianchi et al. 1992b, Sakouhi et al. 2011). Existing studies have reported different *n*-alkane distributions in olive leaves and fruits of two Italian varieties (Coratina and Cipressino) as well as differences in *n*-alkane distribution among different ripening stages of olives in two Italian (Coratina and Cipressino) and one Tunisian (Meski) varieties (Bianchi et al. 1992a, Bianchi et al. 1992b, Sakouhi et al. 2011). No studies, however, attempt to explain the observed differences.

In our previous study (Chapter 5) a significant difference in the distribution of *n*-alkanes has been found between olives and leaves of three Italian varieties (Frantoio, Leccino and Moraiolo), collected at the end of the ripening season, which allowed discrimination of clean olive oils from those with high leaf content. The present study aims to investigate whether the differences in the molecular composition of *n*-alkanes between olives and olive leaves are observed during different stages of olive fruit development – from the formation of olive fruits in the early summer until their full maturation in the late autumn – and to determine how different stages of olive maturity impact the distribution of *n*-alkanes in olives and leaves. Additionally, this study investigates whether three olive varieties exhibit differences in the distribution of *n*-alkanes at different stages of olive fruit development.

Compound-specific stable isotope analyses of *n*-alkanes in olives may provide important information in respect to varietal differences. Unlike bulk $\delta^{13}C$ signal, which reflects contributions from different compound classes with various $\delta^{13}C$ values, compound-specific $\delta^{13}C$ data provide a more direct link between plant physiology and plant isotopic composition. To our knowledge, there currently no published studies on the carbon isotope composition of *n*-alkanes from either olive leaves or fruits. Therefore, the second major goal of this study has been to perform

the compound-specific carbon isotope analysis of the most dominant *n*-alkanes in olives and olive leaves of three Italian varieties (Frantoio, Leccino and Moraiolo) in order to evaluate the differences among the varieties during different stages of olive fruit development.

6.2. Materials and methods

6.2.1. Sample origin

Samples of olives and current year olive leaves were collected at a private olive grove in Ciggiano, Italy (Tuscany region: 43°37' N, 11°71' E) in 2011. A map of the location is shown in Figure D1 (Appendix D). Olive fruits were collected during five months: from July to November. Changes in olive size and colour from July to November are shown in Figure E2 (Appendix E). Olive leaves were collected during six months: from June to November. Sampling was performed by Dr. Dimitri Abbado (University of Siena, Department of Earth Sciences, Siena, Italy) in June-October and by Dr. Nikolai Pedentchouk (University of East Anglia, Norwich, UK) in November. Every month the samples of olives (5-15 fruits) and leaves (*c*. 40-60 leaves) were collected from the Northern side of the tree on the same day (the first day of the month) from the same olive trees, which were marked with water proof paint (Figure E1, Appendix E). Leaves were collected from the current-year twigs. Samples were collected from three olive varieties: Frantoio, Moraiolo, and Leccino. Three trees of each variety were sampled, resulting in nine samples of olive material and nine samples of leaf material every month. However, due to the small size of olive fruits in July-August, and sometimes insufficient amount of material collected, olive samples from three olive trees of each variety were combined into one sample to ensure sufficient amount of material for both molecular and isotopic analyses. Thus in total, 15 samples of olives (3 varieties x 5 months) and 54 samples of olive leaves (3 varieties x 3 trees x 6 months) were obtained. Leaf and olive samples were stored in paper envelopes at room temperature.

6.2.2. *n*-Alkane extraction

Total lipid extracts were obtained from the olive samples (*c*. 10 whole olives) and leaf samples (*c*. 20 whole leaves) by sonication in hexane (15 min ×2) and then concentrated to 1 ml using TurboVap II Concentration Evaporator Workstation (Zymark Corp., Hopkinton, MA, USA). *n*-Alkanes were separated from the total lipid extract using column chromatography. Glass Pasteur pipettes (145 x 9 mm) packed with activated silica gel (0.063-0.200 mm, Sigma-Aldrich, Gillingham, UK) were used as chromatography columns. The total lipid extract (500 µl) was placed on the top of the column, eluted with hexane (5 ml), and the eluent containing *n*-alkanes

was collected in a 4 ml glass vial. One chromatography column was used per each sample. Eluents were concentrated under a stream of high grade nitrogen down to 0.5 ml, and stored at room temperature until further analysis.

6.2.3. Gas chromatography

Gas chromatography analysis was performed using HP Agilent 7820A Gas Chromatograph (Agilent Technologies Inc., Wilmington, USA) with a flame ionisation detector (FID). Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The GC was equipped with a DB-5 capillary column (30m × 0.32 mm × 0.25 μm) (Agilent Technologies Inc., Santa Clara, USA) and programmed from 50 °C (held for 1 min) at 20 °C/min to 150 °C and further at 8 °C/min to 320 °C, and held for 10 min isothermally. The temperature of the injector and detector was 300 °C and 280 °C, respectively. Samples were injected using a split/splitless injector in a splitless mode. *n*-Alkanes were identified through comparison of elution times with the known *n*-alkane standard – a mixture of 15 *n*-alkanes: hexadecane (*n*-C_{16}), heptadecane (*n*-C_{17}), octadecane (*n*-C_{18}), nonadecane (*n*-C_{19}), eicosane (*n*-C_{20}), heneicosane (*n*-C_{21}), docosane (*n*-C_{22}), tricosane (*n*-C_{23}), tetracosane (*n*-C_{24}), pentacosane (*n*-C_{25}), hexacosane (*n*-C_{26}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}), nonacosane (*n*-C_{29}), and triacontane (*n*-C_{30}) (purchased from Dr. Arndt Schimmelmann; Department of Geological Sciences, Indiana University, Bloomington, USA). Individual *n*-alkane peak areas were calculated using ChemStation software (Agilent Technologies Inc., Wilmington, USA). *n*-Alkane average chain length (ACL) was calculated using peak area values of individual *n*-alkanes from C_{19} to C_{35} according to the following formula:

$$ACL = \frac{(19 \cdot A_{19}) + (20 \cdot A_{20}) + (21 \cdot A_{21}) + \ldots + (35 \cdot A_{35})}{(A_{19} + A_{20} + A_{21} + \ldots + A_{35})} \quad (6.1.)$$

where A_x corresponds to the peak area of an individual *n*-alkane from the gas chromatography trace.

6.2.4. Gas chromatography-isotope ratio mass spectrometry

Stable carbon isotope analysis of *n*-alkanes from olives and olive leaves was performed using a Delta V Advantage isotope ratio mass spectrometer interfaced

with GC-Isolink Trace Ultra GC Combustion and High temperature conversion systems (Thermo Scientific, Bremen, Germany). Individual *n*-alkanes were separated using a DB-5 capillary column (30m × 0.32 mm × 0.25 μm). Helium was used as a carrier gas at a flow rate of 1.2 ml/min. The GC oven was programmed from 50 °C (held for 1 min) at 20 °C/min to 150 °C and further at 8 °C/min to 320 °C and held for 10 min isothermally. Samples were injected using a programmable temperature vaporization (PTV) injector in splitless mode at 280 °C.

Combustion of *n*-alkanes to CO_2 was conducted at 1030 °C. Stable carbon isotope compositions of the most prevalent *n*-alkanes from olives (*n*-C_{25}, *n*-C_{27}, *n*-C_{29}, and *n*-C_{31}) and olive leaves (*n*-C_{29}, *n*-C_{31}, and *n*-C_{33}) were analysed. Carbon isotope values of *n*-alkanes were reported based on duplicate analyses of well-resolved peaks. For the majority of duplicate measurements the absolute difference in $\delta^{13}C$ values was < 0.4‰, never exceeding 0.6‰.

The analytical accuracy and precision of GC-IRMS system during stable carbon isotope analyses were determined using a standard mixture of 15 *n*-alkanes (isotopic ratios measured off-line by Dr. Arndt Schimmelmann; Department of Geological Sciences, Indiana University, Bloomington, USA and available at http://mypage.iu.edu/~aschimme), which was analyzed between every 10 samples. Carbon isotope compositions of *n*-alkanes were expressed relative to VPDB based on in-house CO_2 reference gas (BOC, UK) adjusted daily using the above mentioned standard mixture of 15 *n*-alkanes. Isodat software Version 3.0 (Thermo Scientific, Bremen, Germany) was used for processing the data of *n*-alkane measurements.

6.2.5. Statistical analyses

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). All data were checked for normality and homogeneity of variances. Data were normally distributed unless stated otherwise. One way analysis of variance (ANOVA) was applied to study the difference in i) the ACL values of olive leaves from individual olive varieties over the period of six months (June-November), ii) the mean ACL values of olives and olive leaves from three varieties over the period of five (July-November) and six (June-November) months, respectively, iii) the $\delta^{13}C$ values of *n*-C_{29}, *n*-C_{31}, and *n*-C_{33} alkanes in olive leaves among three olive varieties over the period of six months. Differences in the mean

values were evaluated for significance with Tukey and Games-Howell *post hoc* comparisons; a difference was considered to be significant at $p < 0.05$.

6.3. Results

6.3.1. The distribution of *n*-alkanes in olives and olive leaves during olive ripening season

The composition of *n*-alkanes in olive fruits of three olive varieties, Frantoio, Leccino and Moraiolo, is characterised by the presence of *n*-alkanes in the range from *n*-C_{19} to *n*-C_{34} with a strong predominance of the odd-numbered homologues. The distributions of *n*-alkanes in olives of the three varieties change significantly during different stages of fruit maturity. Figure 6.1 illustrates changes in the *n*-alkane profile of Frantoio olives from July to November. The distribution of *n*-alkanes in olives of Leccino and Moraiolo varieties is similar and is shown in Figures E3 and E4 (Appendix E), respectively.

In July, when olives are very small (< 1cm in length; Figure E2 (A), Appendix E) the most prevalent *n*-alkanes are *n*-C_{29}, *n*-C_{31}, *n*-C_{27} and *n*-C_{33}, in descending order of importance. In August, *n*-C_{29} is still the most dominant *n*-alkane, but the relative amount of *n*-C_{31} decreases and becomes almost equal with *n*-C_{27}, and the amount of *n*-C_{25} starts to increase (Figure 6.1). A similar trend is observed in September when relative amounts of *n*-C_{27} and *n*-C_{25} become even higher. By October, three the most dominant *n*-alkanes in olives are *n*-C_{27}, *n*-C_{29} and *n*-C_{25}, and their relative amounts are almost equal. Finally, in November when olives have reached their maximum size and maturity (Figure E2 (E), Appendix E), *n*-C_{27} alkane becomes the most dominant compound, followed by *n*-C_{25} and *n*-C_{29}. Thus, a gradual shift from longer to shorter chain predominance is observed among *n*-alkanes in olive fruits during the ripening season.

In contrast to olive fruits, the distribution of *n*-alkanes in olive leaves does not vary throughout the ripening season. Similar to olives, *n*-alkane distribution in leaves exhibits little differences among the three varieties, and is shown here only for Frantoio variety (Figure 6.2). The distribution of *n*-alkanes in the leaves of Leccino and Moraiolo varieties is shown in Figures E5 and E6 (Appendix E), respectively. The composition of *n*-alkanes in leaves is in the range from *n*-C_{24} to *n*-C_{35} with a distinctive dominance of odd-numbered *n*-alkanes. During the whole season *n*-alkane profiles of olive leaves are dominated by three homologues: *n*-C_{29}, *n*-C_{31} and *n*-C_{33} (Figure 6.2).

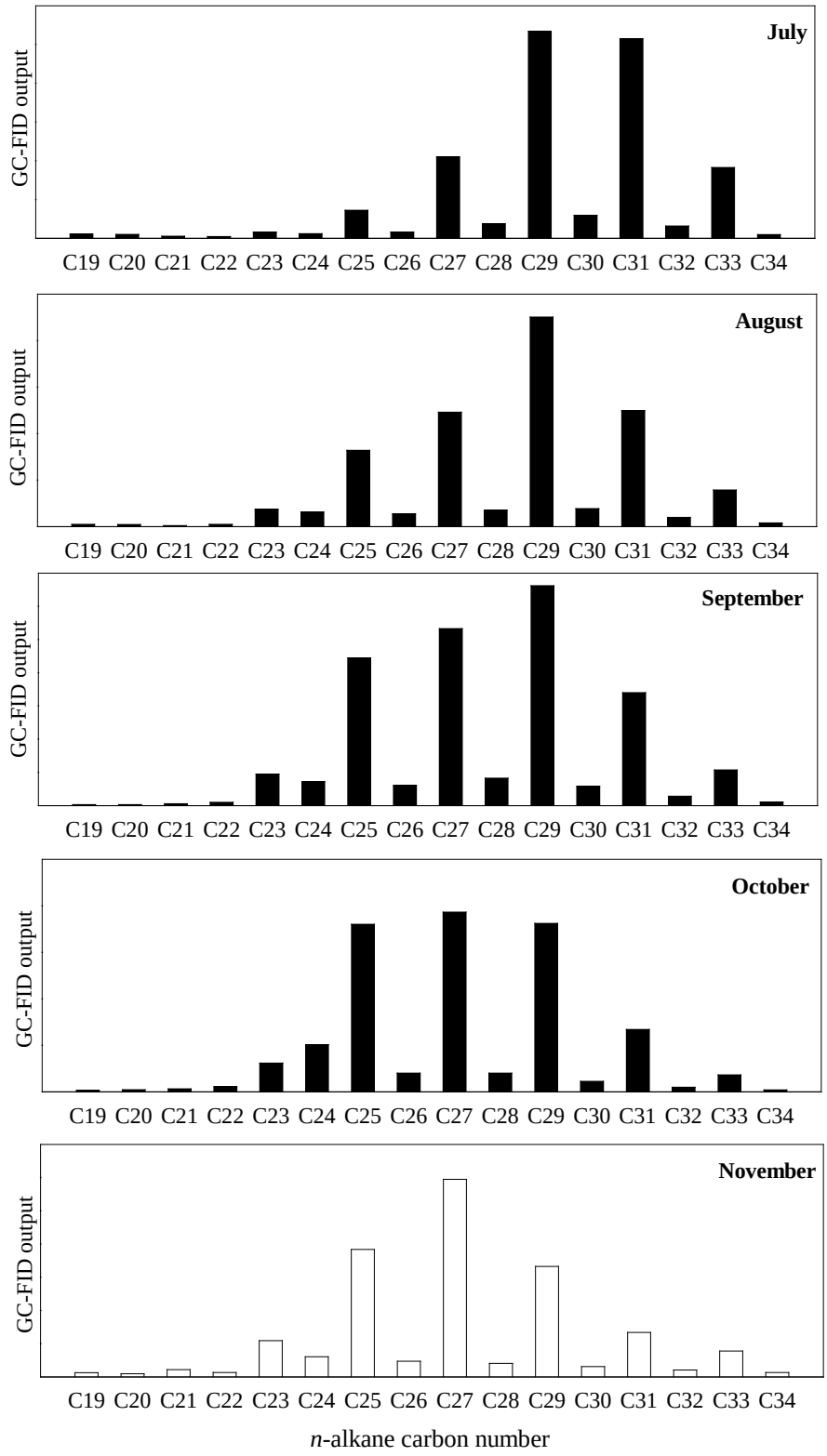

Figure 6.1. The distribution of *n*-alkanes in the olives of Frantoio variety in 2011.

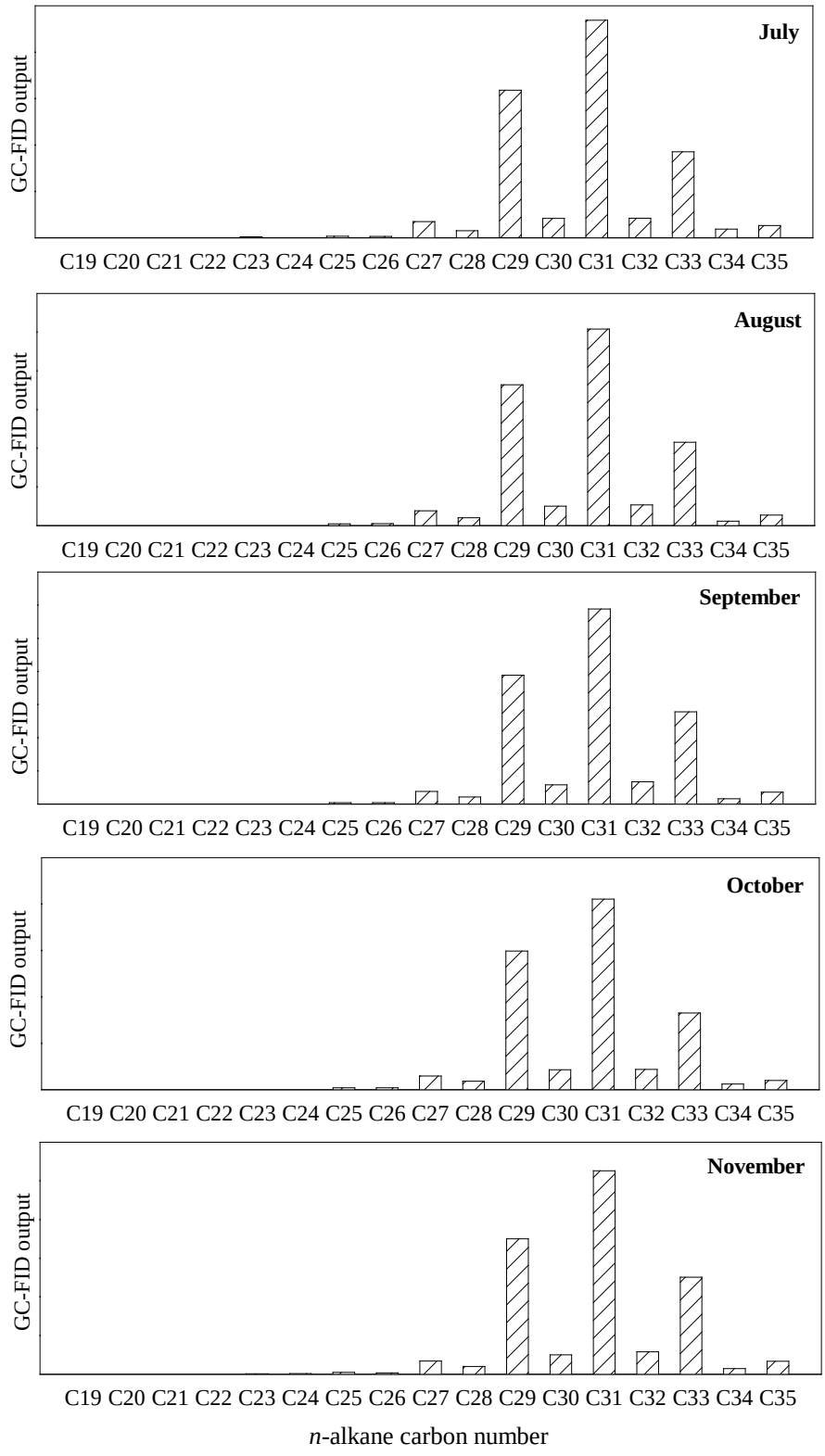

Figure 6.2. The distribution of *n*-alkanes in leaves of Frantoio variety in 2011.

The distribution of *n*-alkanes in olives and olive leaves is reflected in the *n*-alkane average chain length (ACL). The ACL values of olives and olive leaves from individual varieties are shown in Table 6.1 and Table 6.2, respectively. Olive fruits appeared only in July, therefore, olive data are presented for five months: July-November. Leaves, however, were also harvested in June, therefore, the ACL data of leaves are shown for six months. The ACL values of olives differ significantly ($p<0.05$) among five months with an exception of August and September, and October and November, when the difference between the two months is not significant ($p>0.05$).

Table 6.1. The *n*-alkane average chain length (ACL) values of olives from Frantoio, Leccino and Moraiolo varieties in 2011.

	ACL				
Sampling time	**Frantoio**	**Leccino**	**Moraiolo**	**Mean**	**SD**
July	29.4	29.1	29.3	**29.3**	**0.1**
August	28.3	28.6	28.5	**28.5**[a]	**0.2**
September	27.8	27.9	28.4	**28.0**[a]	**0.3**
October	27.1	26.9	27.6	**27.2**[b]	**0.3**
November	26.9	26.8	27.1	**26.9**[b]	**0.2**

SD – standard deviation (n=3). Letters (a,b) denote lack of significant difference ($p>0.05$) in the mean ACL values between two months; difference in the mean ACL values among all other months is significant ($p<0.05$).

The highest ACL values of olives are in July. A gradual decrease in the ACL values is observed throughout the season, resulting in the lowest ACL values of olives in November. The difference in the mean ACL values of olives between July and November is 2.4 ACL units (Figure 6.3).

Table 6.2. The *n*-alkane average chain length (ACL) values of olive leaves from Frantoio, Leccino and Moraiolo varieties in 2011.

	ACL				
Sampling time	**Frantoio (n=3)**	**Leccino (n=3)**	**Moraiolo (n=3)**	**Mean† (n=9)**	**SD (n=9)**
June	30.6*	30.4*	30.9*	**30.6**	**0.2**
July	30.4	30.7	30.8	**30.7**	**0.3**
August	30.6	30.9	30.9	**30.8**	**0.3**
September	30.6	30.7	30.7	**30.7**	**0.3**
October	30.5	30.7	30.8	**30.7**	**0.3**
November	30.8	30.6	30.9	**30.8**	**0.3**

SD – standard deviation. * denotes significant difference ($p<0.05$) in the mean ACL values among three varieties within a month; † – difference in the mean ACL values of three varieties among six months is insignificant ($p>0.05$).

The ACL values of leaves from the three olive varieties do not exhibit significant differences ($p>0.05$) among six months, from June to November (Tables 6.2 and E1, Appendix E). Furthermore, there is no significant difference ($p>0.05$) in the ACL values of leaves among the three varieties during the whole season with the exception of June, when the inter-varietal difference is significant (Table 6.2).

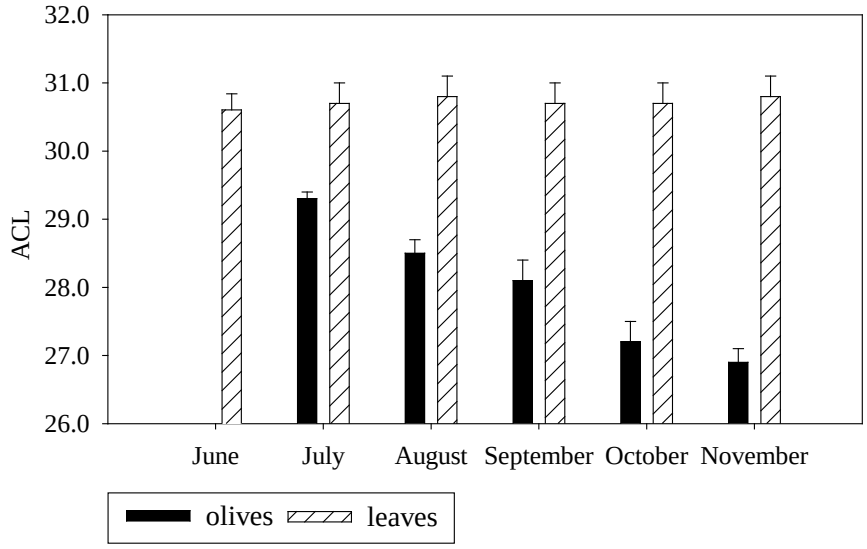

Figure 6.3. The mean ACL values of olives and leaves from Frantoio, Leccino and Moraiolo varieties in 2011. The error bars represent standard deviation based on the number of samples from three varieties collected each month: olives (n=3), leaves (n=9).

During all stages of olive fruit maturity, the ACL values of olives are lower compared to those of leaves (Figure 6.3). This difference is highly significant ($p<0.001$) and becomes greater towards the end of the season due to decreasing ACL values of olives. The difference in the mean ACL values between olives and leaves of the three varieties is 1.3 ACL units in July and becomes as great as 3.9 ACL units by November.

Changes in the ACL of olives from individual varieties are shown in Figure 6.4. The ACL values of Frantoio show a gradual decrease from July to November. The ACL values of Moraiolo olives drop from July until August, are stable between August and September, and then fall and reach the minimum in November. In Leccino, the ACL values gradually fall from July to October, and then change very little between October and November. There are no major differences in the ACL values among three varieties in July and August when fruits have just been formed and start to grow rapidly. However, during the later ripening stages in September-

November, the ACL values of olives from Moraiolo are higher compared to Leccino and Frantoio (Figure 6.4).

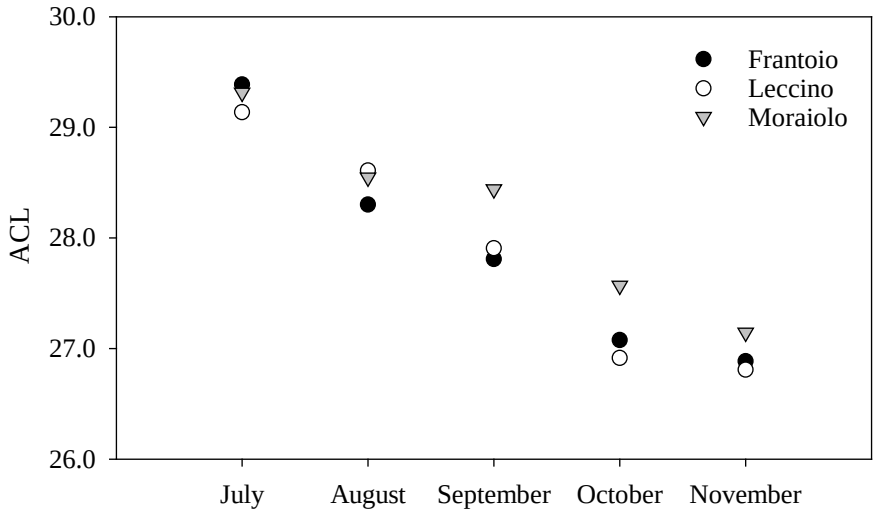

Figure 6.4. Changes in the ACL values of olives from Frantoio, Leccino and Moraiolo varieties in 2011**.**

6.3.2. Stable carbon isotope composition of *n*-alkanes in olives and olive leaves during olive ripening season

The carbon isotope compositions of the most prevalent *n*-alkanes in olives (*n*-C_{25}, *n*-C_{27}, *n*-C_{29}, and *n*-C_{31}) of Frantoio, Leccino and Moraiolo varieties from July to November are shown in Figure 6.5 and Table E3 (Appendix E). Three olive varieties exhibit differences in the carbon isotope compositions of *n*-alkanes. The $\delta^{13}C$ values of *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, and *n*-C_{31} alkanes from olives in all five months are more positive in Frantoio variety compared to Leccino and Moraiolo (Figure 6.5).

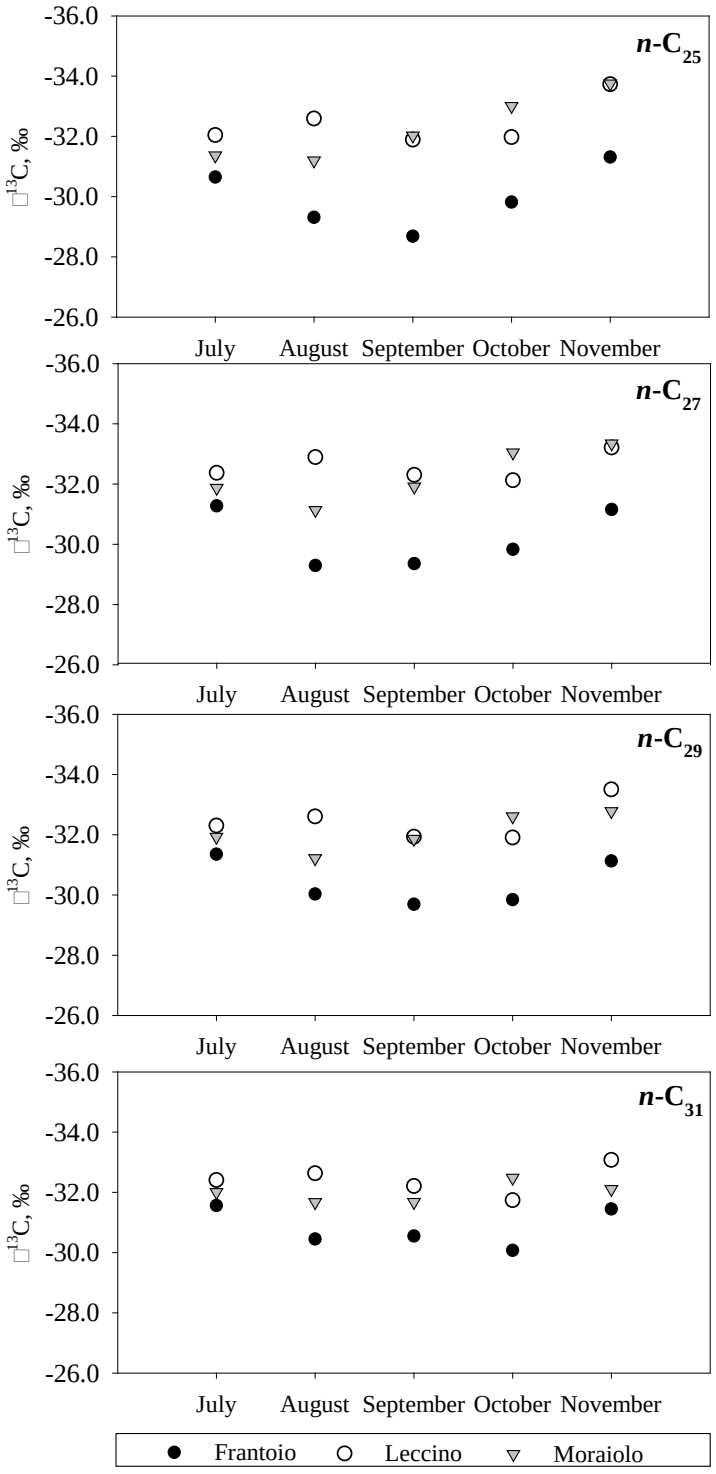

Figure 6.5. The $\delta^{13}C$ values of *n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31} alkanes from olives of Frantoio, Leccino and Moraiolo varieties in 2011.

The mean carbon isotope compositions of the most prevalent *n*-alkanes in olive leaves (*n*-C_{29}, *n*-C_{31} and *n*-C_{33}) of Frantoio, Leccino and Moraiolo varieties from June to November are shown in Figure 6.6 and Table 6.3. The $\delta^{13}C$ values of *n*-C_{29}, *n*-C_{31} and *n*-C_{33} alkanes in olive leaves of individual replicates of three varieties from June to November are shown in Appendix E (Table E2).

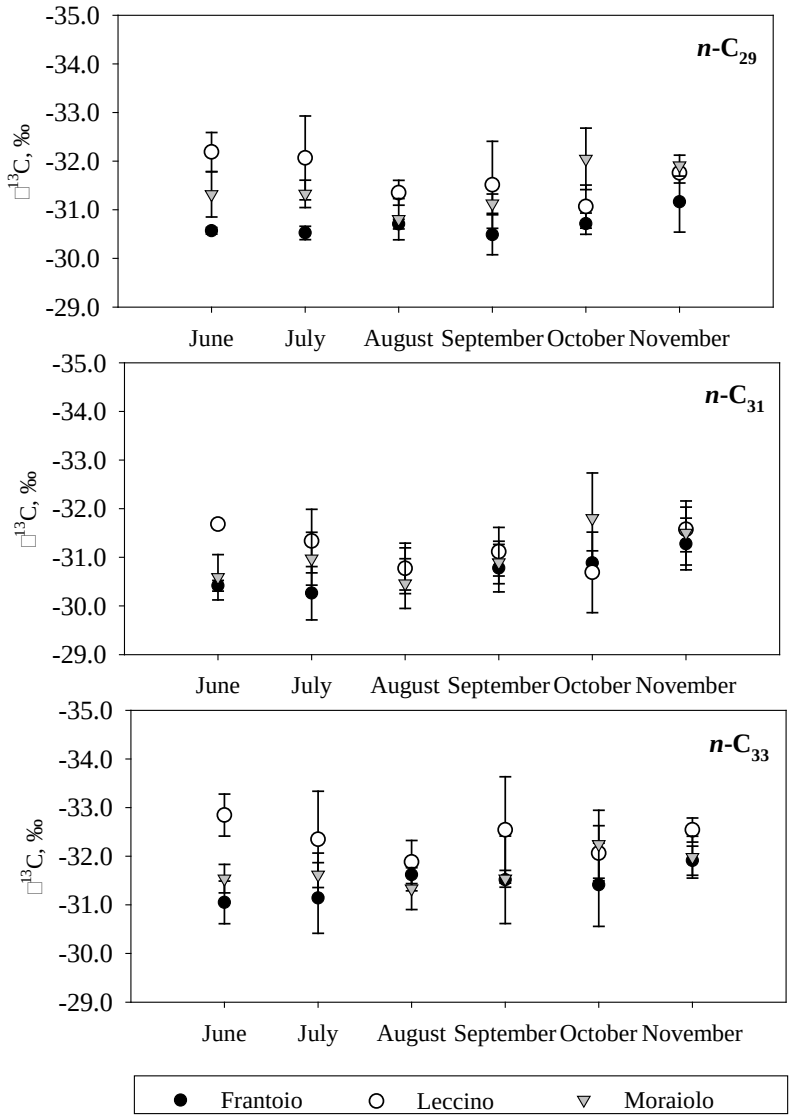

Figure 6.6. The mean $\delta^{13}C$ values of *n*-C_{29}, *n*-C_{31}, and *n*-C_{33} alkanes from olive leaves of Frantoio, Leccino and Moraiolo varieties in 2011. The error bars represent standard deviations based on three replicate samples.

Similarly to olives, the $\delta^{13}C$ values of the analysed *n*-alkanes in leaves are generally more positive in Frantoio variety compared to Leccino and Moraiolo (Table 6.3 and Figure 6.6). The $\delta^{13}C$ values of *n*-C_{29} alkane from Frantoio leaves are

significantly higher ($p<0.05$) in June, July and August compared to Leccino variety, and in October – compared to Moraiolo variety. The $\delta^{13}C$ values of *n*-C_{31} alkane from leaves of Frantoio variety are significantly higher ($p<0.05$) compared to Leccino in June. The $\delta^{13}C$ values of *n*-C_{33} alkane from Frantoio leaves are significantly higher ($p<0.05$) in June compared to Leccino and Moraiolo (Table 6.3). In other months differences among three varieties on the basis of $\delta^{13}C$ values of *n*-C_{31} and *n*-C_{33} alkanes are not significant ($p>0.05$).

Table 6.3. The mean $\delta^{13}C$ values of *n*-C_{29}, *n*-C_{31}, and *n*-C_{33} alkanes from leaves of Frantoio, Leccino and Moraiolo varieties in 2011.

Sampling time	Variety	$\delta^{13}C$, ‰					
		n-C_{29}		*n*-C_{31}		*n*-C_{33}	
		Mean (n=3)	SD	Mean (n=3)	SD	Mean (n=3)	SD
June	Frantoio	-30.6a	0.1	-30.4e	0.1	-31.1f	0.4
	Leccino	-32.2a	0.4	-31.7e	0.0	-32.8fg	0.4
	Moraiolo	-31.3	0.5	-30.6	0.5	-31.5g	0.3
July	Frantoio	-30.5b	0.1	-30.3	0.4	-31.1	0.5
	Leccino	-32.1b	0.9	-31.3	0.7	-32.3	1.0
	Moraiolo	-31.3	0.3	-31.0	0.5	-31.6	0.4
August	Frantoio	-30.7c	0.1	-30.8	0.4	-31.6	0.3
	Leccino	-31.4c	0.3	-30.8	0.5	-31.9	0.4
	Moraiolo	-30.8	0.4	-30.5	0.5	-31.3	0.4
September	Frantoio	-30.5	0.4	-30.8	0.5	-31.5	0.9
	Leccino	-31.5	0.9	-31.1	0.5	-32.5	1.1
	Moraiolo	-31.1	0.2	-30.9	0.4	-31.5	0.2
October	Frantoio	-30.7d	0.2	-30.9	0.2	-31.4	0.9
	Leccino	-31.1	0.4	-30.7	0.8	-32.1	0.6
	Moraiolo	-32.0d	0.6	-31.8	0.9	-32.2	0.7
November	Frantoio	-31.2	0.6	-31.3	0.5	-31.9	0.3
	Leccino	-31.8	0.2	-31.6	0.5	-32.5	0.2
	Moraiolo	-31.9	0.2	-31.5	0.7	-32.0	0.4

SD – standard deviation based on three replicate samples of each variety. Different letters (a-g) denote significant difference ($p<0.05$) in the mean $\delta^{13}C$ values among three varieties.

6.4. Discussion

The present study investigated how different stages of olive maturity, from the formation of olive fruits in the early summer until the full maturation in the late autumn, affect the distribution of *n*-alkanes in olives and leaves of three Italian varieties (Frantoio, Leccino and Moraiolo). Additionally, carbon isotope compositions of the most dominant *n*-alkanes in olives and olive leaves were determined in order to study the differences among three varieties during the growing season. Firstly, I discuss here the observed differences in the molecular composition of *n*-alkanes in olives and olive leaves throughout the season, and then address the differences in the carbon isotope composition of *n*-alkanes in three olive varieties.

6.4.1. Changes in the distribution of *n*-alkanes in leaves and fruits of three olive varieties during olive ripening season

Highly significant difference ($p<0.001$) in the ACL values is found between olives and leaves of Frantoio, Leccino and Moraiolo varieties at the stage of full olive maturity. Leaves have significantly higher ACL values with the difference in the mean ACL values between olives and leaves being 3.9 ACL units at the end of the ripening season (Figure 6.3). This agrees with the finding of the previous study described in Chapter 5. However, depending on the stage of olive fruit maturity, the difference in the ACL between olives and leaves is subject to change. A gradual increase in the difference in ACL between olives and leaves is observed from July to November (Figure 6.3). The reason for this is a gradual decrease in the ACL values of olive fruits from all three varieties throughout the ripening season (Figure 6.4). This decrease in the ACL values of olives reflects a gradual shift from the predominance of longer chain *n*-alkanes (*n*-C_{29} and *n*-C_{31}) towards the shorter chain *n*-alkanes (*n*-C_{25} and *n*-C_{27}) in olives of the three varieties from July to November (Figure 6.1 and Figures E3 and E4, Appendix E). In contrast to olive fruits, olive leaves do not exhibit any significant changes in the ACL values from June to November (Figures 6.3). The distribution of *n*-alkanes in leaves during six months is dominated by three compounds: *n*-C_{29}, *n*-C_{31} and *n*-C_{33}, and does not differ significantly among three varieties (Figure 6.2 and Figures E5 and E6, Appendix E).

The observed shifts in the distribution of *n*-alkanes in olive fruits during the ripening season from predominantly longer chain *n*-alkanes at the beginning of the season to shorter chain *n*-alkanes at the later stages could potentially be explained by a greater physiological need to synthesize longer chain compounds early in the growing season to resist water loss. It has been suggested that longer chain *n*-alkanes can contribute to higher resistance against water loss via cuticle compared to shorter chain *n*-alkanes (Sakouhi et al. 2011). When olives are immature, their cuticular layer is much thinner compared to fully developed mature olives with a thick cuticular wax layer (Proietti et al. 1999). Thus, reducing water loss through a thin cuticle in young olive fruits may be one of the functions of long chain *n*-alkanes in the cuticular layer of olives at the early stages of their development.

Only a few studies analysed *n*-alkane distribution in olives during different stages of maturity. Bianchi et al. (1992a) compared the composition of cuticular waxes in olives of Italian Coratina variety during two ripening stages: green and black. The authors reported that the most dominant *n*-alkanes in both green and black olive fruits were *n*-C_{25}, *n*-C_{27} and *n*-C_{29}. However, green olives were dominated by *n*-C_{29} alkane, while black olives had higher amounts of *n*-C_{27}. These findings agree with the results of the present study. In contrast, Koprivnjak et al. (2005) analysed *n*-alkane composition in olive oils pressed from olives of three Croatian varieties: Leccino, Buza and Bjelica, which were collected at different ripening stages, and did not observe any significant differences in the composition of *n*-alkanes in relation to different periods of harvesting.

The results of the present study do not reveal significant inter-varietal differences in the ACL values of olive leaves during the period from June to November (Table 6.2). Three varieties also do not exhibit a great variation in the ACL of olive fruits at the beginning of the season, in July and August, however during the following months (September, October and November) Moraiolo variety is characterised by higher ACL compared to Frantoio and Leccino (Figure 6.4). This agrees with the results of the above study by Koprivnjak et al. (2005) who reported that the distribution of the majority of *n*-alkane homologues in olive oils from three Croatian varieties (Leccino, Buza and Bjelica) exhibited significant differences depending on the olive variety. Analysis of a greater number of olive varieties in terms of their *n*-alkane distribution is needed to evaluate the capability of *n*-alkane composition to distinguish physiological differences among different olive varieties.

6.4.2. Differences in the stable carbon isotope composition of *n*-alkanes among three olive varieties during the ripening season

Compound-specific carbon isotope analysis of *n*-alkanes from both olive fruits and leaves reveals differences among three varieties. The $\delta^{13}C$ values of the most dominant *n*-alkanes in olives (*n*-C_{25}, *n*-C_{27}, *n*-C_{29}, and *n*-C_{31}) are more positive in Frantoio variety compared to Leccino and Moraiolo during the whole season of olive maturation, from July to November (Figure 6.5). Similarly to olives, the $\delta^{13}C$ values of the prevalent *n*-alkanes in leaves (*n*-C_{29}, *n*-C_{31}, and *n*-C_{33}) are generally more positive in Frantoio variety compared to Leccino and Moraiolo, with the difference being significant mainly for *n*-C_{29} alkane and less significant for *n*-C_{31}, and *n*-C_{33} (Figure 6.6).

As three analysed olive varieties originate from the same grove within a restricted geographical area, the impact of the geographical and climatic factors on the biosynthesis of *n*-alkanes in olives is insignificant, and therefore the observed inter-varietal differences may be mainly attributed to the genetic and physiological factors, which had a different effect on the discrimination against ^{13}C during photosynthesis and further *n*-alkane biosynthesis in different olive varieties. To our knowledge, no studies on the carbon isotope composition of *n*-alkanes in olive leaves, olives or olive oils are available in the present literature, however several previous studies (Bianchi et al. 1993, Royer et al. 1999) performed compound-specific carbon isotope analysis of other chemical compound classes from olives and reported differences among olive varieties. Bianchi et al. (1993) carried out stable carbon isotope analyses of bulk olive oils as well as of some of oil components (sterols, long-chain alcohols and glycerol) from four Italian olive varieties collected at six different stages of ripening during October-January. Even though differences in the $\delta^{13}C$ of bulk oil among the analysed varieties were found insignificant, significant differences in the $\delta^{13}C$ values of certain individual compounds (e.g. sterols) were observed among some of the analysed varieties, however no discussion of the possible reasons for the observed inter-varietal differences was presented in their study. Royer et al. (1999) performed compound specific stable carbon isotope analyses of the most abundant fatty acids in olive oils from the Mediterranean region and reported that significant differences in the $\delta^{13}C$ values of individual fatty acids were found among olive varieties. As in higher plants *n*-alkanes are the products of

the decarboxylation of fatty acids, which in turn are biosynthesized by chain elongation of precursor acetyl-CoA with malonyl-ACP (e.g. Jenks et al. 2002, Shepherd and Griffiths 2006, Samuels et al. 2008), significant differences among olive varieties in respect to their carbon isotope composition of fatty acids reported by Royer et al. (1999) are in accordance with the inter-varietal differences in carbon isotope values of individual *n*-alkanes found in the present study.

Compound-specific carbon isotope analysis of *n*-alkanes may have a potential for differentiating certain olive varieties. Studying inter-varietal differences on the basis of $\delta^{13}C$ composition of *n*-alkanes can contribute to research addressing varietal differences of virgin olive oils. Furthermore, the carbon isotope composition of plant tissues has been shown to positively correlate with season-long WUE in a wide range of plants species (e.g., Farquhar and Richards 1984, Condon et al. 1987, Martin and Thorstenson 1988, Wright et al. 1994, Sun et al. 1996, Seibt et al. 2008, Xu et al. 2009), and therefore, data of the compound-specific carbon isotope analyses of different olive varieties can contribute to studies on olive water use efficiency aimed at selecting drought resistant olive varieties. Analyses of the carbon isotope composition of olive *n*-alkanes from a wider range of varieties are needed to evaluate the scope of application of the compound-specific approach.

6.5. Conclusions

This study investigated whether and how the molecular compositions of *n*-alkanes in olives and leaves of three Italian varieties (Frantoio, Leccino and Moraiolo) change from July until full olive maturation in November, and whether *n*-alkane distributions and carbon isotope ratios can reveal differences among the three varieties.

The distribution of *n*-alkanes in olive fruits of the three varieties is subject to change throughout the whole season. A gradual shift from the longer chain *n*-alkanes towards the shorter chain *n*-alkanes is observed in olives of the three varieties from July to November. At the beginning of the season longer chain *n*-alkanes (*n*-C_{29} and *n*-C_{31}) are the most prevalent in olive fruits, while by the end of the season the most dominant in olives become shorter chain *n*-alkanes (*n*-C_{25} and *n*-C_{27}). Changes in the distribution of *n*-alkanes in olives are reflected in their ACL values that gradually decrease during the whole season from 29.3 ACL units in July to 26.9 ACL units in November. The observed shift can be attributed to a greater physiological need to synthesize longer chain compounds early in the growing season to resist water loss. In contrast, olive leaves do not exhibit any significant changes in the distribution of *n*-alkanes throughout the whole season. *n*-Alkane distribution in leaves of three varieties during six months is dominated by three compounds: *n*-C_{29}, *n*-C_{31} and *n*-$C_{33.}$ Physiological differences among the varieties cannot be seen in the *n*-alkane distribution and the ACL values of olive leaves. However, differences are observed among the varieties in respect to the ACL of olives: during September, October and November Moraiolo variety is characterised by higher ACL compared to Frantoio and Leccino.

The carbon isotope composition of the most dominant *n*-alkanes in olives and leaves differs among the three varieties. The $\delta^{13}C$ values of *n*-alkanes in olive fruits are more positive in Frantoio variety compared to Leccino and Moraiolo throughout the whole season, from July to November. Compound-specific carbon isotope analysis of *n*-alkanes can be a useful tool for studying inter-varietal differences in olives.

6.6. References

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CHAPTER 7

CONCLUSIONS

The dissertation is focused on the application and evaluation of novel compound-specific stable isotope approaches for studying the relationships between environmental conditions and the chemical compositions of plants and plant products. In the first part of this work, the impact of different organic and synthetic fertilisation strategies on the isotopic compositions of greenhouse, field and retail vegetables has been studied using bulk nitrogen isotope analysis and, for the first time, compound-specific nitrogen and oxygen isotope analysis of plant-derived nitrate.

In the second part of this work, the impacts of the geographical location and production technique on the composition of *n*-alkanes in extra virgin olive oils from Mediterranean countries have been studied using molecular and, for the first time, compound-specific carbon and hydrogen isotope analyses. In addition, differences in the molecular and isotopic compositions of *n*-alkanes in olive fruits and leaves of different olive varieties have been examined.

7.1. The effects of synthetic and organic fertilisation regimes on the stable isotope compositions of vegetables

In controlled greenhouse conditions, there is a strong impact of nitrogen isotope composition ($\delta^{15}N_{bulk}$) of fertilisers on the nitrogen isotope composition of bulk lettuce plants. The application of chicken manure has resulted in significantly higher ($p<0.05$) $\delta^{15}N_{bulk}$ values of lettuce plants (mean $\delta^{15}N_{bulk}$ = +7‰) compared to the nitrogen isotope values of lettuces grown with synthetic KNO_3 and urea treatments (mean $\delta^{15}N_{bulk}$ = +3.6 and +3.8‰, respectively). Thus, the difference in the nitrogen isotope compositions of the applied fertilisers is reflected in the nitrogen isotope composition of lettuce plants when factors other than the type of fertiliser (temperature and light regime, substrate type, irrigation water) are constant.

Similarly, in the field conditions, the $\delta^{15}N_{bulk}$ values of organic vegetables fertilised with animal manures are generally higher than those of conventionally grown counterparts fertilised with synthetic NPK fertilisers. The $\delta^{15}N_{bulk}$ values of

potatoes, cabbages and onions grown with animal manures (+4.5, +7.5 and +5.7‰, respectively) are significantly higher ($p<0.05$) compared to those treated with synthetic NPK fertilisers (+1.3, +2.9 and +1.0‰, respectively).

The use of nitrogen-fixing cover crops in the second organic system resulted in less significant differences between organic and conventional crops. Significant difference ($p<0.05$) in the $\delta^{15}N_{bulk}$ values between the organic system with the application of cover crops and conventional system has been observed only in one vegetable species (onions), thus revealing the limitations of the bulk nitrogen isotope analysis in differentiating between agricultural systems with synthetic fertilisation versus the use of nitrogen-fixing crops.

Compound-specific nitrogen and oxygen isotope compositions ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) of plant-derived nitrate, which were used for the first time for the differentiation between conventional and organic fertilisation regimes, allowed distinction of plants grown with synthetic nitrate from those grown with manure in the greenhouse conditions. The $\delta^{15}N_{NO3}$ values of lettuces grown with chicken manure are significantly higher ($p<0.05$) than those treated with synthetic KNO_3 (mean $\delta^{15}N_{NO3}$ = +31.4 and +22.6‰, respectively). In contrast, the $\delta^{18}O_{NO3}$ values of lettuces fertilised with chicken manure are lower compared to those treated with KNO_3 (mean $\delta^{18}O_{NO3}$ = +39.1 and +45.3‰, respectively).

In the field conditions, stable oxygen isotope analysis of vegetable derived nitrate has introduced a significant improvement to the discrimination of potato samples from conventional and organic systems. The $\delta^{18}O_{NO3}$ values of conventional potatoes (mean $\delta^{18}O_{NO3}$ = +23.4‰) are significantly higher ($p<0.05$) compared to those of organic potatoes cultivated with either animal manures or cover crops (mean $\delta^{18}O_{NO3}$ = +18.1 and +17.9‰, respectively), and for this vegetable species has a significantly higher discriminative potential compared to the bulk nitrogen isotope composition. The canonical discriminant analysis (CDA) of potato samples indicates that the $\delta^{18}O_{NO3}$ has the greatest discriminative power in the classification of potato samples into three agricultural systems. Overall, 86.1% of cross-validated potato samples are correctly classified when compound-specific isotope data are used together with bulk nitrogen composition, compared to only 50.0% of correctly classified cases when classification has been performed only on the basis of total nitrogen content and $\delta^{15}N_{bulk}$ values.

The compound-specific oxygen isotope approach has also shown significant differences ($p<0.05$) between conventional and organic retail potato samples from different geographical origins (UK, Jersey, Israel and Egypt) as well as between conventional and organic retail tomatoes from UK. Conventional potatoes have significantly higher ($p<0.05$) $\delta^{18}O_{NO3}$ values compared to organic potatoes (mean $\delta^{18}O_{NO3}$ = +22.6 and +15.1‰, respectively). Similarly, the $\delta^{18}O_{NO3}$ values of conventional tomatoes (mean $\delta^{18}O_{NO3}$ = +45.3‰) are significantly higher ($p<0.05$) than those of organic tomato samples (mean $\delta^{18}O_{NO3}$ = +27.3‰). The CDA of retail potato samples with $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ used as predictor variables resulted in 84.8% of correctly classified cases. Probability densities of the normal distribution curves of $\delta^{18}O_{NO3}$ values for the expected entire organic and conventional potato populations have been generated and suggest that compound-specific oxygen isotope approach can be a useful tool for the discrimination between organic and conventional potatoes when applied to organic samples that typically have low $\delta^{18}O_{NO3}$ values (<13‰) or to conventional samples that typically have high $\delta^{18}O_{NO3}$ values (>24‰).

Both bulk nitrogen isotope composition and the nitrogen and oxygen isotope composition of plant-derived nitrate are strongly affected by the fertiliser application rate. In both synthetic (KNO_3 and urea) and organic (chicken manure) fertiliser treatments, $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of greenhouse lettuces are higher when the fertiliser is applied at the lower rate (50 kg N/kg substrate) compared to the higher application rate (150 kg N/kg substrate), which indicates a positive relationship between the amount of fertiliser and isotope discrimination against ^{15}N and ^{18}O.

An intra-plant isotopic variation has been observed in greenhouse grown lettuces and results in higher $\delta^{15}N_{bulk}$, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of lettuce leaves compared to roots.

This is the first time when $^{18}O/^{16}O$ analysis of nitrate using the denitrifier method has been applied to plant material. The results of greenhouse and field experiments demonstrate that the $\delta^{18}O$ analysis of plant-derived nitrate could be used with mixed success for differentiating plants grown with synthetic nitrate fertilisers and plants cultivated with animal manures. The $\delta^{18}O_{NO3}$ approach has proved to be successful for the discrimination between conventional and organic potatoes and tomatoes, however has been less successful in discrimination between conventional

and organic cabbages, onions, carrots and faba beans. The results of this work suggest that the discriminatory potential of the $\delta^{18}O_{NO3}$ approach could be affected by local climate. The difference between conventional and organic samples will be reduced in hotter climate because of the higher $\delta^{18}O$ values of soil waters and subsequently of organic nitrates in that climatic regime. Further analyses of the oxygen isotope composition of nitrate from a greater number of plant species grown under greenhouse and field conditions and with different conventional and organic fertilisation strategies are needed to establish the scope of application and robustness of $\delta^{18}O_{NO3}$ approach for organic food authentication.

7.2. Differences in the molecular and stable isotope composition of *n*-alkanes in extra virgin olive oils from different geographical and production origins

The distribution and concentration of *n*-alkanes in the Mediterranean extra virgin olive oils exhibits variation depending on the production country. The most prevalent *n*-alkanes in olive oils are odd-numbered homologues: *n*-C_{23}, *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31}, however, the relative proportions of these compounds and the values of *n*-alkane average chain length (ACL) are different depending on the country of origin. The lowest ACL values are found in oils from Morocco, Southern and Northern Greece (25.7, 25.1 and 25.8 ACL units, respectively), whereas the highest ACL values are in oil samples from Spain and Portugal (28.7 and 28.4 ACL units, respectively), which have high concentrations of longer chain *n*-alkanes *n*-C_{29} and *n*-$C_{31.}$ Total concentrations of *n*-alkanes are found to be significantly higher ($p<0.05$) in olive oils from the Northern compared to Southern Mediterranean countries. No significant correlations have been found between geographic parameters (latitude, longitude, altitude, and ambient temperature) and the molecular composition of *n*-alkanes in extra virgin olive oils. The observed differences among production countries in respect to *n*-alkane profiles cannot be explained on the basis of environmental factors, and most likely arise from differences in varietal origin of oils.

The stable carbon and hydrogen isotope compositions of *n*-C_{29} alkane ($\delta^{13}C_{C29}$ and δD_{C29}) in extra virgin olive oils from eight Mediterranean countries exhibit significant variation depending on the production country. The $\delta^{13}C_{C29}$ values are

significantly more positive ($p<0.05$) in olive oil samples from the Southern Mediterranean region compared to the Northern region, and show significant discriminative power as a predictor variable in the CDA. The $\delta^{13}C_{C29}$ values of oils positively correlate with altitude and the mean temperature during August-December, while show negative correlation with latitude.

The δD_{C29} values of olive oils from the Mediterranean countries exhibit a much greater variation compared to $\delta^{13}C_{C29}$ values and show the most significant discriminative potential in the CDA model. A total range of δD_{C29} values of oils from different production countries is as great as 29‰. The δD_{C29} values of olive oils are significantly more positive ($p<0.05$) in the oil samples from Southern compared to Northern Mediterranean countries. The δD_{C29} values of olive oils positively correlate with the δD values of precipitation and the mean temperature during August-December, and show negative correlation with latitude. The presence of these significant correlations indicates a close relationship between the hydrogen isotope composition of *n*-alkanes in olive oils and the hydrogen isotope composition of source water in the oil production locations. Results of the regression analysis suggest that the δD of precipitation can explain 52% of the variation in the δD_{C29} values of oils around their mean.

Compound-specific stable isotope analysis of *n*-alkanes can be a useful tool for the regional differentiation of olive oils. Moreover, as *n*-alkanes are present in relatively high amounts in the cuticular waxes of a variety of higher plants, it can be suggested for the future research that the analysis of the isotopic composition of *n*-alkanes may be also successfully applied for the geographical classification of other products of plant origin, such as agricultural crops or other vegetable oils. Further studies on the application of stable isotope analyses of *n*-alkanes for the authentication of plant products are needed in future.

The distribution of *n*-alkanes in olive oils has shown a potential for the detection of the excessive leaf material in extra virgin olive oils and for the differentiation between olive oils produced in Ciggiano (Tuscany, Italy) using two different olive harvesting methods: manual versus mechanical. Olive oils produced from mechanically harvested olives have significantly higher ($p<0.05$) amount of long-chained *n*-alkanes (*n*-C_{29}-*n*-C_{35}) and significantly higher ($p<0.05$) ACL values than oils obtained from the hand-picked olives. The observed difference in the *n*-alkane profiles between oils from two different olive harvesting techniques is caused

by the presence of excessive leaf material in the oils pressed from mechanically collected olives and by significantly different ($p<0.05$) distributions of *n*-alkanes in olives and leaves. Olive leaves are characterized by the predominance of long-chained *n*-alkanes (*n*-C_{29}, *n*-C_{31}, *n*-C_{33} and *n*-C_{35}) and significantly higher ($p<0.05$) ACL values compared to olives. The analysis of *n*-alkane profiles may be a useful tool for distinguishing clean oils from those with high leaf content, provided that the samples of both olives and leaves from the corresponding varieties, which were used for the oil production, are available.

Results of the present work show significant differences ($p<0.05$) in the distribution of *n*-alkanes in olive fruits of three varieties (Frantoio, Leccino and Moraiolo) depending on the stage of their maturity. In green unripe olives the most prevalent are longer chain *n*-alkanes (*n*-C_{29} and *n*-C_{31}), while by the end of the ripening season the most dominant in olives become shorter chain *n*-alkanes (*n*-C_{25} and *n*-C_{27}). The mean ACL values of olives gradually decrease during the whole olive maturation season from July until November. The observed shift in the profiles of *n*-alkanes in olives throughout the season can be explained by a greater physiological need to synthesize longer chain compounds early in the growing season to resist water loss. In contrast, olive leaves do not exhibit any significant changes ($p>0.05$) in the *n*-alkane distribution and ACL values throughout the season. *n*-Alkane distribution in leaves of three olive varieties during the whole season is dominated by three homologues: *n*-C_{29}, *n*-C_{31} and *n*-C_{33}.

Compound-specific stable carbon isotope analysis of *n*-alkanes does not show significant differences ($p>0.05$) between oils produced using two different olive harvesting methods, however reveals differences among three olive varieties. The $\delta^{13}C$ values of the most dominant *n*-alkanes in olive fruits (*n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31}) are more positive in Frantoio variety compared to Leccino and Moraiolo throughout the whole season, from July to November.

There is a potential for using the compound-specific carbon isotope analysis of *n*-alkanes for studying the inter-varietal differences in olives. Future research on the carbon isotope compositions of *n*-alkanes in olives and olive oils from a greater number of varieties can find application for the differentiation of the varietal origin of olive oils. Furthermore, studies addressing the differences in the carbon isotope composition of *n*-alkanes in different olive varieties can contribute to the research on water use efficiency in olives, which under the scenarios of water scarcity predicted

by climate models for the Mediterranean region, is an important prerequisite for breeding activities aimed at targeting draught resistant olive varieties.

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 2

Figure A1. Lettuce plants at different growth stages: 7 days (A), 30 days (B) and 50 days (C) since the start of the pot experiment.

Table A1. The number of samples collected from each fertiliser treatment during lettuce pot experiment.

Fertiliser applied	Time of sampling, days since the start of the experiemnt	Number of samples collected
Lettuce [a]		
KNO_3 50 mg N/kg substrate	80	5+5
KNO_3 150 mg N/kg substrate	80	5+5
Urea 50 mg N/kg substrate	80	5+5
Urea 150 mg N/kg substrate	80	5+5
Manure 50 mg N/kg substrate	80	5+5
Manure 150 mg N/kg substrate	80	5+5
Substrate		
No fertiliser	0	1
KNO_3 50 mg N/kg substrate	0	1
	80	5
KNO_3 150 mg N/kg substrate	0	1
	80	5
Urea 50 mg N/kg substrate	0	1
	80	5
Urea 150 mg N/kg substrate	0	1
	80	5
Manure 50 mg N/kg substrate	0	1
	80	5
Manure 150 mg N/kg substrate	0	1
	80	5
Fertilisers		
KNO_3	0	1
Urea	0	1
Manure	0	1

[a] – each lettuce sample was split in two – leaves and roots, therefore for each treatment n = 5+5

Table A2. Stable isotope values of substrates from KNO_3, urea and chicken manure treatments.

Substrate samples	Fertiliser treatment	Application rate, mg N/kg substrate	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	KNO_3	50	-2.5	7.0	27.1
2	KNO_3	50	-2.6	4.0	25.0
3	KNO_3	50	-2.7	11.5	32.0
4	KNO_3	50	-2.6	7.6	22.3
5	KNO_3	50	-2.5	8.0	30.5
6	KNO_3	150	-2.2	10.0	35.9
7	KNO_3	150	-2.7	10.3	35.0
8	KNO_3	150	-3.0	19.6	31.3
9	KNO_3	150	-2.6	14.6	37.5
10	KNO_3	150	-2.7	20.3	39.7
11	Urea	50	-2.1	4.5	44.5
12	Urea	50	-2.5	6.1	50.6
13	Urea	50	-2.9	5.1	45.0
14	Urea	50	-2.5	11.3	43.7
15	Urea	50	-2.4	7.5	41.3
16	Urea	150	-2.2	2.3	32.6
17	Urea	150	-3.2	5.3	34.8
18	Urea	150	-2.7	4.9	29.0
19	Urea	150	-2.7	4.3	28.0
20	Urea	150	-2.7	8.9	27.9
21	Manure	50	-2.2	5.9	17.7
22	Manure	50	-2.0	3.2	19.9
23	Manure	50	-2.2	7.9	19.0
24	Manure	50	-2.0	8.0	19.8
25	Manure	50	-2.5	4.0	17.4
26	Manure	150	-1.2	23.1	20.9
27	Manure	150	-1.7	4.2	8.8
28	Manure	150	-1.0	5.1	13.6
29	Manure	150	-1.9	5.9	21.3
30	Manure	150	-1.8	9.0	20.0

Table A3. Stable isotope values of lettuce leaves from KNO_3, urea and chicken manure treatments.

Lettuce plants	Fertiliser treatment	Application rate, mg N/kg substrate	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	KNO_3	50	5.0	21.8	39.4
2	KNO_3	50	4.1	24.9	49.0
3	KNO_3	50	4.7	23.4	45.3
4	KNO_3	50	5.2	23.1	53.5
5	KNO_3	50	4.9	23.1	55.3
6	KNO_3	150	2.0	21.1	41.7
7	KNO_3	150	2.1	21.5	39.0
8	KNO_3	150	3.3	21.9	46.6
9	KNO_3	150	2.6	20.6	39.5
10	KNO_3	150	2.2	24.6	43.0
11	Urea	50	5.7	17.4	46.7
12	Urea	50	7.3	20.3	51.9
13	Urea	50	3.3	25.5	57.2
14	Urea	50	4.0	33.0	50.1
15	Urea	50	6.6	31.4	46.3
16	Urea	150	2.0	22.3	32.4
17	Urea	150	2.2	20.1	38.1
18	Urea	150	2.1	19.9	32.6
19	Urea	150	2.1	20.1	32.6
20	Urea	150	2.7	18.1	46.2
21	Manure	50	8.5	38.9	48.8
22	Manure	50	7.9	35.7	45.4
23	Manure	50	8.3	35.8	35.3
24	Manure	50	7.6	32.9	42.5
25	Manure	50	8.1	33.5	43.0
26	Manure	150	8.1	27.6	29.7
27	Manure	150	5.4	27.7	36.4
28	Manure	150	4.5	26.8	35.9
29	Manure	150	5.6	28.3	38.4
30	Manure	150	6.0	26.4	35.1

Table A4. Stable isotope values of lettuce roots from KNO_3, urea and chicken manure treatments.

Lettuce plants	Fertiliser treatment	Application rate, mg N/kg substrate	$\delta^{15}N_{bulk}$, ‰
1	KNO_3	50	2.6
2	KNO_3	50	1.2
3	KNO_3	50	1.6
4	KNO_3	50	1.1
5	KNO_3	50	1.6
6	KNO_3	150	3.7
7	KNO_3	150	0.7
8	KNO_3	150	0.2
9	KNO_3	150	-2.0
10	KNO_3	150	0.7
11	Urea	50	0.5
12	Urea	50	0.7
13	Urea	50	0.5
14	Urea	50	0.2
15	Urea	50	0.3
16	Urea	150	0.2
17	Urea	150	0.0
18	Urea	150	0.4
19	Urea	150	0.2
20	Urea	150	0.4
21	Manure	50	4.3
22	Manure	50	4.3
23	Manure	50	3.3
24	Manure	50	4.3
25	Manure	50	4.9
26	Manure	150	2.9
27	Manure	150	4.5
28	Manure	150	3.7
29	Manure	150	4.7
30	Manure	150	3.7

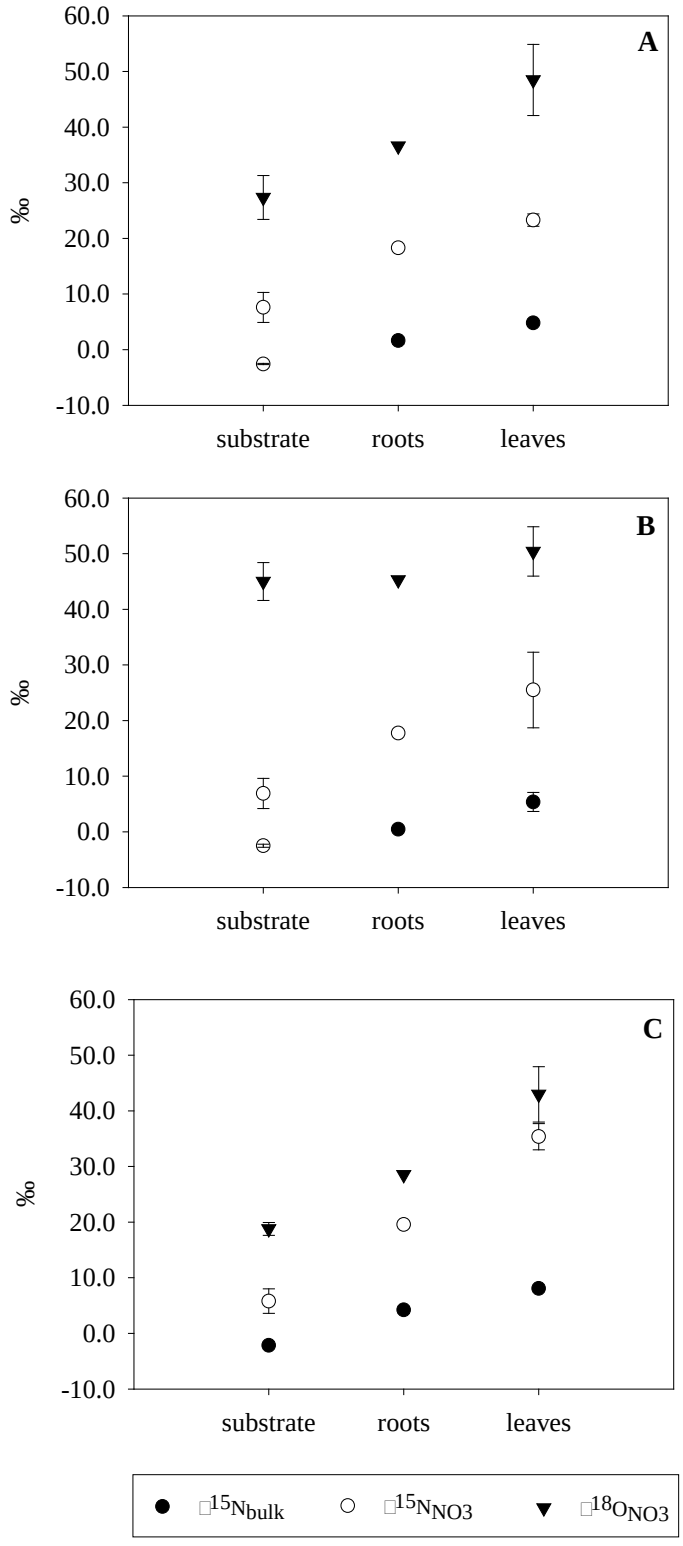

Figure A2. Stable nitrogen and oxygen isotope composition of lettuce leaves, roots and substrates treated with KNO_3 (A), urea (B) and chicken manure (C) at the application rate of 150 mg N/kg substrate. Values are the means of five replicate samples. The error bars represent standard deviations based on five replicate samples.

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 3

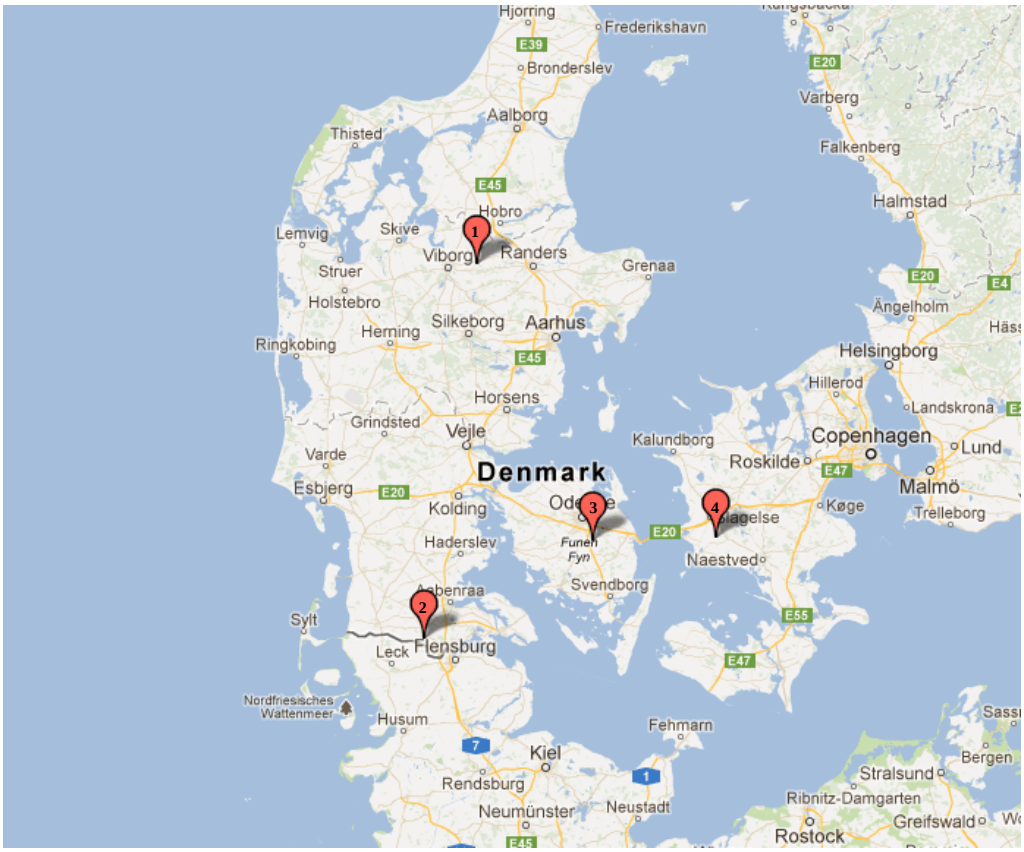

Figure B1. Field trial locations. 1 – Foulum; 2 –Jundevad; 3 – Aarslev; 4 – Flakkebjerg**.** The image adopted from Google maps at http://www.maps.google.co.uk (date accessed: 1 March 2012).

Figure B2. Photographs of the vegetable plots from the field trials. A - onion and carrot plots; B – cabbage plots; C – potato plots.
Photographs were taken by Kristian Holst Laursen (University of Copenhagen, Denmark) in June 2007.

Table B1. Geographical and pedoclimatic conditions at the field trial locations[1].

	Flakkebjerg	Foulum	Jyndevad	Aarslev
Region	Zealand	Central Jutland	South Jutland	Funen
Coordinates	11°23'E, 55°19'N	09°34'E, 56°30'N	09°08'E, 54°54'N	10°27'E, 55°18'N
Plot size (m^2)	169	216	378	120
Soil type	Sandy loam	Loamy sand	Sand	Sandy loam
Clay (%)	16	9	5	15
Silt (%)	12	13	2	27
Sand (%)	70	74	91	25
pH (in 0.01 M $CaCl_2$)[a]	6.5 ± 0.0	5.6 ± 0.1	5.7 ± 0.0	6.2
P (mg/100 g soil)[a]	2.3 ± 0.1	4.4 ± 0.1	4.2 ± 0.1	2.6
K (mg/100 g soil)[a]	9.5 ± 0.2	11.7 ± 0.4	5.4 ± 0.1	12.0
Mg (mg/100 g soil)[a]	4.4 ± 0.1	4.2 ± 0.2	5.3 ± 0.1	4.3
Annual temperature (°C)	7.8	7.3	7.9	9.2
Annual precipitation (mm)	626	704	964	754

[a] : average for plough layer soil samples taken in March across all plots in both growth years and analysed as described in Husted, S; Mikkelsen, B. F.; Jensen, J.; Nielsen, N. E. Elemental fingerprint analysis of barley *(Hordeum vulgare)* using inductively coupled plasma mass spectrometry, isotope-ratio mass spectrometry, and multivariate statistics. *Anal. Bioanal. Chem.* 2004, *378*, 171-182.

[1] Further information can be found in:

(1) Olesen J. E., Askegaard M. and Rasmussen I. A. 2000. Design of an organic farming crop-rotation experiment. *Acta Agr. Scand. B-S P*, 50: 13-21;

(2) Thorup-Kristensen K., Dresboll D. B. and Kristensen H. L. 2012. Crop yield, root growth, and nutrient dynamics in conventional and three organic cropping systems with different levels of external inputs and N re-cycling through fertility building crops. *Europ. J. Agronomy*, 37: 66-82.

Table B2. The types and application rates of fertilisers in three agricultural systems from the field trials (average data for 2007 and 2008)[2]

Vegetable type	Location	Agricultural system		
		C	OA	OB
		N/P/K (Kg/ha)[a]	N/P/K (Kg/ha)[b]	N/P/K (Kg/ha)[c]
Potato	FL	140/50/250	110/15/120	2/0/55
	FO	140/30/175	115/25/145	1/0/90
	JY	140/35/200	125/20/155	1/0/90
Faba bean	FL	0/55/125	0/0/60	0/0/55
	FO	0/25/125	0/0/65	0/0/65
	JY	0/25/125	0/0/30	0/0/65
Cabbage	A	310/45/145	225/15/80	135/10/50
Carrot	A	120/20/60	55/5/20	-/-/-
Onion	A	170/80/65	110/10/40	70/5/25

Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.
Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops.
[a]Applied as inorganic fertilizer.
[b]Applied as animal manure. K was supplemented as vinasse for all crops.
[c]K was applied as vinasse for all crops. Animal manure was also applied to white cabbage and onion.

[2] Further information can be found in:

(1) Soltoft M., Nielsen J., Laursen K. H., Husted S., Halekoh U., Knuthsen P. 2010. Effects of organic and conventional growth systems on the content of flavonoids in onions and phenolic acids in carrots and potatoes. J. Agric. Food Chem., 58: 10323-10329.

(2) Laursen K. H., Schjoerring J. K., Olesen J. E., Askegaard M., Halekoh U., Husted S. 2011. Multielemental fingerprinting as a tool for authentication of oranic wheat, barley, faba bean, and potato. J. Agric. Food Chem., 59: 4385-4396.

Table B3. The type and number of vegetable and fertiliser samples obtained from the field trials.

Sample type	Description	Location	Agricultural system		
			C	OA	OB
			Number of samples		
vegetable	potato	FL, FO, JY	12	12	12
	faba beans	FL, FO, JY	6	6	6
	cabbage	A	6	6	6
	carrot	A	6	6	6
	onion	A	6	6	6
fertiliser	NPK 21-3-10	FL, JY, A	3		
	NPK 14-3-15	FL, FO, JY	3		
	NPK 6-9-25	FL	1		
	Kalkammoniumsalpeter (27% total N)	JY	1		
	NS 24-7	FO	1		
	Animal manure (<5% total N)	FL, FO, JY, A		4	
	Protamylasse (1.7-1.9% total N)	FO		1	

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B4. Stable nitrogen and oxygen isotope composition of fertilisers used in the field trials.

Fertiliser samples	Fertiliser type	Location	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	NPK 21-3-10	FL	0.3	2.3	20.4
2	NPK 21-3-10	JY	0.7	3.1	23.4
3	NPK 21-3-10	A	-0.7	-0.5	23.1
4	NPK 14-3-15	FL	0.0	2.4	23.5
5	NPK 14-3-15	FO	0.4	3.1	22.3
6	NPK 14-3-15	JY	-0.1	3.4	23.7
7	NPK 6-9-25	FL	-0.9	7.7	24.5
8	Kalkammoniumsalpeter (27% total N)	JY	-2.1	-1.5	26.0
9	NS 24-7	FO	-0.8	0.0	27.1
10	Animal manure (<5% total N)	FL	2.6	3.0	10.7
11	Animal manure (<5% total N)	FO	12.5	8.1	19.8
12	Animal manure (<5% total N)	JY	6.8	5.4	11.3
13	Animal manure (<5% total N)	A	4.2	9.0	19.1
14	Protamylasse (1.7-1.9% total N)	FO	2.7	18.9	18.6

Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B5. The amount of potato samples needed for nitrate extraction, nitrate concentration in potato extracts and amounts of extracts needed for the denitrifier method.

Potato sample	Agricul-tural system	Location	Weight of freeze-dried sample, g	Ultrapure water added for extraction, ml	Nitrate concentration in the extract, mg/l	Amount of extract needed for bacteria, ml	Dilution needed, times
1	C	FL	0.5	20.0	4.9	0.5	2.0
2	C	FL	0.5	20.0	5.3	0.5	2.1
3	C	FL	0.5	20.0	6.0	0.5	2.4
4	C	FL	0.5	20.0	4.8	0.5	1.9
5	C	FO	0.5	20.0	8.9	0.5	3.6
6	C	FO	0.5	20.0	5.8	0.5	2.3
7	C	FO	0.5	20.0	4.6	0.5	1.9
8	C	FO	0.5	20.0	4.8	0.5	1.9
9	C	JY	0.5	20.0	5.7	0.5	2.3
10	C	JY	0.5	20.0	5.1	0.5	2.1
11	C	JY	0.5	20.0	3.6	0.5	1.4
12	C	JY	0.5	20.0	4.7	0.5	1.9
13	OA	FL	0.5	20.0	5.1	0.5	2.0
14	OA	FL	0.5	20.0	5.2	0.5	2.1
15	OA	FL	0.5	20.0	4.9	0.5	2.0
16	OA	FL	0.5	20.0	4.9	0.5	2.0
17	OA	FO	0.5	20.0	4.8	0.5	2.0
18	OA	FO	0.5	20.0	5.5	0.5	2.2
19	OA	FO	0.5	20.0	5.1	0.5	2.0
20	OA	FO	0.5	20.0	5.6	0.5	2.3
21	OA	JY	0.5	20.0	4.0	0.5	1.6
22	OA	JY	0.5	20.0	5.9	0.5	2.4
23	OA	JY	0.5	20.0	5.6	0.5	2.2
24	OA	JY	0.5	20.0	4.6	0.5	1.8
25	OB	FL	0.5	20.0	4.4	0.5	1.8
26	OB	FL	0.5	20.0	5.2	0.5	2.1
27	OB	FL	0.5	20.0	4.5	0.5	1.8
28	OB	FL	0.5	20.0	4.1	0.5	1.7
29	OB	FO	0.5	20.0	5.0	0.5	2.0
30	OB	FO	0.5	20.0	5.9	0.5	2.4
31	OB	FO	0.5	20.0	4.5	0.5	1.8
32	OB	FO	0.5	20.0	3.9	0.5	1.6
33	OB	JY	0.5	20.0	5.4	0.5	2.2
34	OB	JY	0.5	20.0	4.5	0.5	1.8
35	OB	JY	0.5	20.0	5.9	0.5	2.4
36	OB	JY	0.5	20.0	4.3	0.5	1.7

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B6. The amount of faba bean samples needed for nitrate extraction, nitrate concentration in faba bean extracts and amounts of extracts needed for the denitrifier method.

Faba bean sample	Agricul-tural system	Location	Weight of freeze-dried sample, g	Ultrapure water added for extraction, ml	Nitrate concentration in the extract, mg/l	Amount of extract needed for bacteria, ml	Dilution needed, times
1	C	FL	1.0	20.0	4.8	0.5	1.9
2	C	FL	1.0	20.0	4.2	0.5	1.7
3	C	FO	1.0	20.0	3.9	0.5	1.6
4	C	FO	1.0	20.0	3.4	0.5	1.4
5	C	JY	1.0	20.0	6.6	0.5	2.6
6	C	JY	1.0	20.0	4.9	0.5	2.0
7	OA	FL	1.0	20.0	5.5	0.5	2.2
8	OA	FL	1.0	20.0	5.4	0.5	2.2
9	OA	FO	1.0	20.0	3.6	0.5	1.4
10	OA	FO	1.0	20.0	5.0	0.5	2.0
11	OA	JY	1.0	20.0	4.0	0.5	1.6
12	OA	JY	1.0	20.0	4.2	0.5	1.7
13	OB	FL	1.0	20.0	3.4	0.5	1.4
14	OB	FL	1.0	20.0	3.1	0.5	1.3
15	OB	FO	1.0	20.0	3.8	0.5	1.5
16	OB	FO	1.0	20.0	5.2	0.5	2.1
17	OB	JY	1.0	20.0	3.9	0.5	1.6
18	OB	JY	1.0	20.0	3.6	0.5	1.5

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B7. The amount of cabbage samples needed for nitrate extraction, nitrate concentration in cabbage extracts and amounts of extracts needed for the denitrifier method.

Cabba-ge sample	Agricul-tural system	Location	Weight of freeze-dried sample, g	Ultrapure water added for extraction, ml	Nitrate concentration in the extract, mg/l	Amount of extract needed for bacteria, ml	Dilution needed, times
1	C	Aarslev	0.5	20.0	65.9	0.2	10.6
2	C	Aarslev	0.5	20.0	91.8	0.2	14.8
3	C	Aarslev	0.5	20.0	71.6	0.2	11.6
4	C	Aarslev	0.5	20.0	25.9	0.2	4.2
5	C	Aarslev	0.5	20.0	37.5	0.2	6.0
6	C	Aarslev	0.5	20.0	24.7	0.2	4.0
7	OA	Aarslev	0.5	20.0	122.3	0.2	19.7
8	OA	Aarslev	0.5	20.0	107.6	0.2	17.4
9	OA	Aarslev	0.5	20.0	75.9	0.2	12.2
10	OA	Aarslev	0.5	20.0	22.0	0.2	3.6
11	OA	Aarslev	0.5	20.0	9.4	0.2	1.5
12	OA	Aarslev	0.5	20.0	32.6	0.2	5.3
13	OB	Aarslev	0.5	20.0	69.4	0.2	11.2
14	OB	Aarslev	0.5	20.0	100.4	0.2	16.2
15	OB	Aarslev	0.5	20.0	94.3	0.2	15.2
16	OB	Aarslev	0.5	20.0	23.8	0.2	3.8
17	OB	Aarslev	0.5	20.0	21.4	0.2	3.4
18	OB	Aarslev	0.5	20.0	21.3	0.2	3.4

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B8. The amount of carrot samples needed for nitrate extraction, nitrate concentration in carrot extracts and amounts of extracts needed for the denitrifier method.

Carrot sample	Agricul-tural system	Location	Weight of freeze-dried sample, g	Ultrapure water added for extraction, ml	Nitrate concentration in the extract, mg/l	Amount of extract needed for bacteria, ml	Dilution needed, times
1	C	Aarslev	1.0	20	12.0	0.6	5.8
2	C	Aarslev	1.0	20	26.3	0.6	12.7
3	C	Aarslev	1.0	20	9.3	0.6	4.5
4	C	Aarslev	1.0	20	12.8	0.6	6.2
5	C	Aarslev	1.0	20	17.0	0.6	8.2
6	C	Aarslev	1.0	20	25.8	0.6	12.5
7	OA	Aarslev	1.0	20	4.1	0.6	2.0
8	OA	Aarslev	1.0	20	6.9	0.6	3.3
9	OA	Aarslev	1.0	20	3.7	0.6	1.8
10	OA	Aarslev	1.0	20	4.0	0.6	1.9
11	OA	Aarslev	1.0	20	6.2	0.6	3.0
12	OA	Aarslev	1.0	20	3.0	0.6	1.4
13	OB	Aarslev	1.0	20	6.0	0.6	2.9
14	OB	Aarslev	1.0	20	18.4	0.6	8.9
15	OB	Aarslev	1.0	20	6.8	0.6	3.3
16	OB	Aarslev	1.0	20	5.8	0.6	2.8
17	OB	Aarslev	1.0	20	7.5	0.6	3.6
18	OB	Aarslev	1.0	20	3.4	0.6	1.6

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B9. The amount of onion samples needed for nitrate extraction, nitrate concentration in onion extracts and amounts of extracts needed for the denitrifier method.

Onion sample	Agricul-tural system	Location	Weight of freeze-dried sample, g	Ultrapure water added for extraction, ml	Nitrate concentration in the extract, mg/l	Amount of extract needed for bacteria, ml	Dilution needed, times
1	C	Aarslev	1.0	20	3.5	0.6	1.7
2	C	Aarslev	1.0	20	6.2	0.6	3.0
3	C	Aarslev	1.0	20	2.7	0.6	1.3
4	C	Aarslev	1.0	20	42.5	0.6	20.6
5	C	Aarslev	1.0	20	30.3	0.6	14.7
6	C	Aarslev	1.0	20	32.7	0.6	15.8
7	OA	Aarslev	1.0	20	2.3	0.6	1.1
8	OA	Aarslev	1.0	20	2.2	0.6	1.1
9	OA	Aarslev	1.0	20	4.1	0.6	2.0
10	OA	Aarslev	1.0	20	21.0	0.6	10.2
11	OA	Aarslev	1.0	20	26.1	0.6	12.6
12	OA	Aarslev	1.0	20	21.8	0.6	10.5
13	OB	Aarslev	1.0	20	3.5	0.6	1.7
14	OB	Aarslev	1.0	20	2.7	0.6	1.3
15	OB	Aarslev	1.0	20	3.8	0.6	1.8
16	OB	Aarslev	1.0	20	28.9	0.6	14.0
17	OB	Aarslev	1.0	20	28.0	0.6	13.6
18	OB	Aarslev	1.0	20	20.0	0.6	9.7

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B10. Total nitrogen content and stable isotope composition of potato samples from the field trials.

Potato samples	Agricultural system	Location	Total N, %	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	C	FL	1.3	3.3	27.0	28.9
2	C	FL	1.4	1.9	22.7	23.9
3	C	FL	1.4	2.5	21.9	24.6
4	C	FL	1.4	1.6	19.7	22.0
5	C	FO	1.8	0.7	17.2	19.9
6	C	FO	1.8	0.8	17.3	21.3
7	C	FO	1.5	0.2	19.2	20.5
8	C	FO	1.5	0.3	18.8	20.6
9	C	JY	1.5	0.9	21.0	26.5
10	C	JY	1.5	0.8	19.2	24.4
11	C	JY	1.1	1.4	20.9	22.8
12	C	JY	1.1	1.4	23.7	25.8
13	OA	FL	1.5	4.8	23.4	17.5
14	OA	FL	1.5	5.3	22.7	15.6
15	OA	FL	1.5	5.9	22.7	17.9
16	OA	FL	1.5	6.1	27.0	20.9
17	OA	FO	1.9	3.1	20.9	14.8
18	OA	FO	2.1	2.8	19.4	13.3
19	OA	FO	1.6	4.4	23.1	18.0
20	OA	FO	1.8	3.9	24.1	18.4
21	OA	JY	1.7	4.5	26.3	22.7
22	OA	JY	1.9	1.9	21.1	17.8
23	OA	JY	1.5	6.2	26.3	20.7
24	OA	JY	1.5	4.4	23.8	19.7
25	OB	FL	1.4	2.5	23.7	19.2
26	OB	FL	1.5	3.9	23.6	17.7
27	OB	FL	1.4	4.2	22.8	18.7
28	OB	FL	1.5	3.8	24.5	20.7
29	OB	FO	1.7	1.2	18.1	15.1
30	OB	FO	1.7	0.6	18.2	15.8
31	OB	FO	1.4	1.9	22.2	18.2
32	OB	FO	1.4	1.0	21.1	17.4
33	OB	JY	1.3	-0.1	18.7	17.8
34	OB	JY	1.4	0.5	19.0	17.9
35	OB	JY	1.3	0.2	22.0	17.8
36	OB	JY	1.5	0.7	19.9	18.5
Average (n=12)	C	FL+ FO+JY	1.5	1.3	20.7	23.4
	OA		1.6	4.5	23.4	18.1
	OB		1.4	1.7	21.2	17.9
Standard deviation	C	FL+ FO+JY	0.2	0.9	2.8	2.8
	OA		0.2	1.3	2.3	2.7
	OB		0.1	1.6	2.3	1.4

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B11. Total nitrogen content and stable isotope composition of faba bean samples from the field trials.

Faba bean samples	Agricultural system	Location	Total N, %	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	C	FL	4.9	0.5	11.4	33.7
2	C	FL	5.2	0.6	10.9	33.2
3	C	FO	5.7	0.8	14.2	23.1
4	C	FO	5.1	0.7	14.4	26.0
5	C	JY	5.0	-0.2	9.7	26.3
6	C	JY	4.8	0.1	11.0	32.5
7	OA	FL	5.2	1.0	11.5	26.8
8	OA	FL	5.0	0.9	13.1	40.8
9	OA	FO	5.1	0.9	11.9	24.2
10	OA	FO	5.0	0.2	11.4	28.3
11	OA	JY	4.9	0.2	9.9	34.5
12	OA	JY	4.9	0.3	8.3	34.3
13	OB	FL	4.9	0.6	11.0	35.7
14	OB	FL	5.1	1.5	15.4	29.8
15	OB	FO	5.4	0.5	14.4	23.3
16	OB	FO	5.4	0.3	13.7	25.0
17	OB	JY	5.0	0.6	7.4	27.6
18	OB	JY	5.0	0.4	6.7	31.3
Average (n=12)	C	FL+ FO+JY	5.1	0.4	11.9	29.1
	OA		5.0	0.6	11.0	31.5
	OB		5.1	0.6	11.5	28.8
Standard deviation	C	FL+ FO+JY	0.3	0.4	1.9	4.5
	OA		0.1	0.4	1.7	6.2
	OB		0.2	0.4	3.7	4.5

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B12. Total nitrogen content and stable isotope composition of cabbage samples from the field trials.

Cabbage samples	Agricultural system	Location	Total N, %	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	C	A	1.9	2.4	20.2	19.3
2	C	A	1.9	6.0	25.0	16.3
3	C	A	2.1	2.0	22.7	19.6
4	C	A	1.7	2.1	27.6	24.5
5	C	A	1.7	2.6	25.4	23.3
6	C	A	1.9	2.1	26.4	24.3
7	OA	A	1.9	8.8	25.2	17.0
8	OA	A	2.0	6.7	22.8	14.0
9	OA	A	2.0	7.7	25.6	16.8
10	OA	A	1.6	8.3	31.5	23.1
11	OA	A	1.6	7.3	32.2	24.1
12	OA	A	1.7	5.9	28.0	19.8
13	OB	A	2.1	4.4	22.5	16.3
14	OB	A	2.1	3.5	18.6	12.3
15	OB	A	2.0	4.4	20.7	14.2
16	OB	A	1.6	4.0	25.4	20.4
17	OB	A	1.6	5.6	29.0	22.2
18	OB	A	1.7	3.6	24.1	21.6
Average (n=12)	C	A	1.9	2.9	24.5	21.2
	OA	A	1.8	7.5	27.6	19.1
	OB	A	1.8	4.2	23.4	17.8
Standard deviation	C	A	0.1	1.5	2.7	3.3
	OA	A	0.2	1.1	3.7	3.9
	OB	A	0.2	0.8	3.7	4.1

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B13. Total nitrogen content and stable isotope composition of carrot samples from the field trials.

Carrot samples	Agricultural system	Location	Total N, %	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	C	A	1.0	2.1	27.6	28.1
2	C	A	1.0	3.9	27.1	25.9
3	C	A	0.8	3.2	28.2	27.1
4	C	A	0.9	2.6	32.7	28.3
5	C	A	0.9	1.3	30.7	26.3
6	C	A	1.1	2.9	33.8	25.8
7	OA	A	0.7	3.2	29.0	26.3
8	OA	A	0.7	3.8	28.8	24.7
9	OA	A	0.7	5.6	28.6	27.4
10	OA	A	0.8	4.3	28.4	26.2
11	OA	A	0.7	7.4	26.1	30.2
12	OA	A	0.7	4.9	28.2	27.0
13	OB	A	0.8	1.6	27.2	25.9
14	OB	A	0.8	5.3	32.4	22.3
15	OB	A	0.8	1.4	38.8	24.6
16	OB	A	0.8	5.4	37.3	30.3
17	OB	A	0.8	4.8	32.7	27.4
18	OB	A	0.8	4.8	34.7	28.1
Average (n=12)	C	A	0.9	2.7	30.0	26.9
	OA	A	0.8	4.5	29.1	26.8
	OB	A	0.8	3.9	33.9	26.4
Standard deviation	C	A	0.1	0.9	2.8	1.1
	OA	A	0.0	1.5	1.1	1.9
	OB	A	0.0	1.8	4.1	2.8

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B14. Total nitrogen content and stable isotope composition of onion samples from the field trials.

Onion samples	Agricultural system	Location	Total N, %	$\delta^{15}N_{bulk}$, ‰	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	C	A	1.2	1.8	32.6	37.5
2	C	A	1.4	1.0	31.7	34.4
3	C	A	1.3	0.9	38.5	43.1
4	C	A	1.7	0.7	39.6	31.8
5	C	A	1.6	0.7	44.0	33.9
6	C	A	1.6	1.2	39.0	33.1
7	OA	A	1.0	3.9	38.6	40.0
8	OA	A	1.1	5.6	37.1	38.7
9	OA	A	1.4	5.1	34.8	35.2
10	OA	A	1.2	7.8	25.6	34.0
11	OA	A	1.6	5.1	26.6	31.1
12	OA	A	1.5	6.8	26.4	32.9
13	OB	A	1.2	2.5	21.4	38.6
14	OB	A	1.3	10.1	31.8	34.1
15	OB	A	1.4	2.0	31.0	33.2
16	OB	A	1.4	5.4	30.7	37.1
17	OB	A	1.4	8.4	27.8	34.1
18	OB	A	1.4	5.0	27.0	37.3
Average (n=12)	C	A	1.5	1.0	37.6	35.6
	OA	A	1.3	5.7	31.5	35.3
	OB	A	1.4	5.6	28.3	35.8
Standard deviation	C	A	0.2	0.4	4.6	4.1
	OA	A	0.2	1.4	6.0	3.4
	OB	A	0.1	3.2	3.9	2.2

Agricultural system: C – conventional, OA – organic with animal manure, OB – organic with cover crops. Location: FL – Flakkebjerg, FO – Foulum, JY – Jyndevad, A – Aarslev.

Table B15. Stable nitrogen and oxygen isotope composition of nitrate from retail potato samples.

Potato samples	Agricultural system	**Variety**	**Country of origin**	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	Conventional	Maris Piper	UK	20.2	20.9
2	Conventional	Maris Piper	UK	26.6	18.3
3	Conventional	Estima	UK	21.7	21.2
4	Conventional	Nicola	Israel	23.5	21.2
5	Conventional	Annabel	UK	14.5	19.5
6	Conventional	Harmony	UK	26.3	29.5
7	Conventional	Hermes	UK	15.5	16.6
8	Conventional	Maris Piper	UK	20.0	23.4
9	Conventional	Maris Piper	UK	19.3	15.5
10	Conventional	Jersey Royal	Jersey	24.2	22.0
11	Conventional	Jersey Royal	Jersey	27.1	28.7
12	Conventional	Jersey Royal	Jersey	19.8	23.9
13	Conventional	Maris Piper	UK	24.1	29.4
14	Conventional	Maris Piper	UK	29.4	27.5
15	Conventional	Maris Piper	UK	25.7	21.5
16	Conventional	unknown	Egypt	13.6	23.3
17	Conventional	unknown	Egypt	16.0	23.4
18	Conventional	unknown	Egypt	16.1	20.7
19	Organic	Nicola	Israel	25.0	16.4
20	Organic	Maris Peer	Israel	24.1	16.5
21	Organic	Vales Emerald	Egypt	12.3	18.1
22	Organic	Maris Peer	Israel	25.1	14.9
23	Organic	Maris Peer	Israel	24.8	16.5
24	Organic	Nicola	Egypt	13.3	20.3
25	Organic	Nicola	Israel	18.9	13.0
26	Organic	Lady Balfour	Israel	21.9	14.0
27	Organic	Maris Peer	Israel	21.6	16.5
28	Organic	Nicola Ware	Israel	19.7	14.3
29	Organic	Nicola	Israel	16.3	9.6
30	Organic	Ditta	Israel	20.5	14.0
31	Organic	Nicola	Israel	21.0	13.5
32	Organic	unknown	Egypt	10.1	14.9
33	Organic	unknown	Egypt	9.7	15.0

Table B16. Stable nitrogen and oxygen isotope composition of nitrate from retail tomato samples.

Tomato samples	Agricultural system	Variety	Country of origin	$\delta^{15}N_{NO3}$, ‰	$\delta^{18}O_{NO3}$, ‰
1	Conventional	De Reuter No. 1	UK	31.6	50.4
2	Conventional	De Reuter No. 2	UK	25.4	45.4
3	Conventional	Jack Hawkins	UK	28.3	32.1
4	Conventional	Standard	UK	33.3	49.3
5	Conventional	Janet	UK	23.8	45.4
6	Conventional	Plum	UK	26.6	48.6
7	Conventional	Beef	UK	24.5	48.1
8	Conventional	Standard	UK	30.7	42.0
9	Conventional	De Reuter No. 1	UK	30.2	45.8
10	Conventional	De Reuter No. 2	UK	23.8	45.9
11	Organic	Caro Rich	UK	38.2	33.6
12	Organic	Pineapple	UK	24.1	24.0
13	Organic	Black Prince	UK	29.7	28.5
14	Organic	Plum	UK	13.1	38.0
15	Organic	Tigerella	UK	33.0	32.5
16	Organic	Marmande	UK	24.3	27.6
17	Organic	De Reuter No. 1	UK	28.1	31.7
18	Organic	De Reuter No. 2	UK	13.4	18.5
19	Organic	G.Delight	UK	10.8	32.3
20	Organic	F1 Durasol	UK	24.3	20.8
21	Organic	G. Delight	UK	32.4	21.0
22	Organic	Diplom	UK	18.0	29.1
23	Organic	unknown	UK	22.7	18.6
24	Organic	G. Delight	UK	19.7	25.4
25	Organic	unknown	UK	33.5	27.9

Table B17. The results of the tests of equality of group means, the discriminant function coefficients, and the structure matrix table from the Canonical Discriminant Analysis of retail potato samples.

Tests of Equality of Group Means

Variable	Wilks' Lambda	F	Sig.
$\delta^{15}N_{NO3}$	.946	1.759	.194
$\delta^{18}O_{NO3}$	.450	37.816	.000

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	1.220	100.0	100.0	.741

Structure Matrix

Variable	Function
	1
$\delta^{18}O_{NO3}$	1.000
$\delta^{15}N_{NO3*}$	.275

Variables ordered by absolute size of correlation within function

* Variable not used in the analysis

Table B18. Critical t values for conventional and organic potato datasets.

	Number of samples	Degrees of freedom (n-1)	Critical t value (p=0.05)	Critical t value (p=0.01)
conventional	30	29	2.045	2.756
organic	39	38	2.024	2.712

Table B19. Summary of the nitrogen isotope composition data of commercial organic and conventional vegetables and fruits.

Crop	Agricultural system	Number of samples	Mean $\delta^{15}N$, ‰	Standard deviation	Reference†
lettuce	organic	49	7.6	4.1	1
	conventional	55	2.9	4.3	
carrot	organic	18	5.1*	3.1*	1,5
	conventional	22	4.1*	2.3*	
potato	organic	45	7.1*	1.9*	2,3,5
	conventional	46	3.2*	1.5*	
tomato	organic	70	8.0*	3.1*	1,3,5
	conventional	55	0.1*	2.1*	
sweet pepper	organic	3	4.3	0.2	5
	conventional	3	3.5	0.7	
aubergine	organic	3	8.5	0.3	3
	conventional	3	4.5	0.3	
cucumber	organic	3	12.3	0.3	3
	conventional	3	2.7	0.3	
zucchini	organic	3	10.6	0.3	3
	conventional	3	2.4	0.3	
pumpkin	organic	3	5.7	0.3	3
	conventional	3	3.5	0.3	
corn	organic	3	4.8	0.3	3
	conventional	3	0.8	0.3	
broccoli	organic	3	12.2	0.3	3
	conventional	3	4.3	0.3	
cauliflower	organic	3	10.7	4.3	5
	conventional	3	5.5	2.7	
garlic	organic	3	4.3	1.9	5
	conventional	3	2.6	2.6	
onion	organic	3	6.8	1.6	5
	conventional	3	5.2	1.8	
leek	organic	4	7.3	3.0	5
	conventional	4	1.9	1.0	
parsley	organic	6	5.4	1.1	5
	conventional	3	4.5	4.5	
chicory	organic	7	6.7*	1.5*	5
	conventional	6	2.2*	1.1*	
orange	organic	42	7.6*	1.5*	4
	conventional	56	5.5*	1.2*	
clementine	organic	49	7.2*	1.7*	4
	conventional	55	6.9*	1.4*	
strawberry	organic	18	3.7*	1.0*	4
	conventional	22	1.5*	1.4*	

* Pooled mean $\delta^{15}N$ value and pooled standard deviation

† References: 1 - Bateman et al. 2007; 2- Camin et al. 2007; 3- Rogers 2008; 4 -Camin et al. 2011; 5- Šturm and Lojen 2011 (full references are included in Chapter 3).

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4

Table C1. Pearson coefficient (r) and significance (sig.) of correlation between the concentration of individual *n*-alkanes in extra virgin olive oils and geographical factors.

***n*-alkane**		**Altitude**	**Latitude**	**Longitude**
n-C_{20}	r	0.046	-0.042	-0.016
	sig.	0.693	0.719	0.890
n-C_{21}	r	0.123	**-0.388****	-0.126
	sig.	0.291	0.001	0.279
n-C_{22}	r	-0.002	-0.090	-0.026
	sig.	0.989	0.437	0.824
n-C_{23}	r	**-0.273***	0.050	0.204
	sig.	0.017	0.670	0.077
n-C_{24}	r	**-0.349****	**0.266***	**0.231***
	sig.	0.002	0.020	0.045
n-C_{25}	r	**-0.277***	**0.301****	0.104
	sig.	0.016	0.008	0.370
n-C_{26}	r	-0.159	0.205	0.174
	sig.	0.171	0.075	0.133
n-C_{27}	r	-0.110	**0.297****	-0.078
	sig.	0.344	0.009	0.503
n-C_{28}	r	0.111	-0.070	-0.181
	sig.	0.340	0.546	0.118
n-C_{29}	r	0.000	**0.281***	-0.138
	sig.	0.997	0.014	0.235
n-C_{30}	r	0.057	**0.228***	-0.179
	sig.	0.622	0.048	0.121
n-C_{31}	r	0.112	0.137	-0.153
	sig.	0.337	0.238	0.187
n-C_{32}	r	0.063	**0.233***	-0.144
	sig.	0.590	0.043	0.216
n-C_{33}	r	0.123	0.085	-0.137
	sig.	0.291	0.467	0.239
n-C_{34}	r	0.142	0.091	-0.096
	sig.	0.222	0.434	0.410
n-C_{35}	r	0.095	0.096	-0.042
	sig.	0.413	0.409	0.717

Values in bold indicate significant correlation. ** – correlation is significant at the 0.01 level (2-tailed); * – correlation is significant at the 0.05 level (2-tailed)

Table C2. Geographical characteristics and isotopic composition of extra virgin olive oils from the Mediterranean.
δD values of precipitation were calculated using the Online Isotopes in Precipitation Calculator, Version 2.2. (Bowen, G. J. 2011, http://www.waterisotopes.org). M asl – meters above the sea level, dd – decimal degrees.

Sample No	Country	Region	Olive variety	Altitude, m asl	Latitude, dd	Longitude, dd	δD of precipitation, ‰	$\delta^{13}C_{C29}$, ‰	δD_{C29}, ‰
1	Slovenia	Koper	Itrana	150	45.5	13.7	-47.0	-33.4	-152
2	Slovenia	Koper	Bianchera istriana, Leccino, Maurino	140	45.5	13.7	-47.0	-33.0	-152
3	Slovenia	Šmarje	Bianchera istriana, Leccino, Frantoio, Maurino	300	45.5	13.7	-49.0	-33.1	-151
4	Croatia	Vodnjan	Buza	130	44.9	13.9	-45.0	-31.8	-152
5	Croatia	Vodnjan	Buza	130	44.9	13.9	-45.0	-31.7	-157
6	Croatia	Vodnjan	Buza	65	44.9	13.9	-44.0	-32.6	-157
7	Croatia	Pula	Buza	50	44.9	13.9	-44.0	-32.8	-156
8	France	Provence-Alpes-Côte d'Azur	unknown	429	44.4	5.3	-47.0	-32.2	-155
9	France	Provence-Alpes-Côte d'Azur	unknown	302	44.4	5.2	-45.0	-32.7	-153
10	France	Provence-Alpes-Côte d'Azur	unknown	200	44.3	5.1	-43.0	-32.5	-147
11	France	Provence-Alpes-Côte d'Azur	unknown	244	44.3	5.1	-44.0	-32.9	-163
12	France	Provence-Alpes-Côte d'Azur	unknown	247	44.3	5.1	-44.0	-32.4	-158
13	France	Provence-Alpes-Côte d'Azur	unknown	437	44.3	5.2	-47.0	-33.5	-158

Table C2. Continued

Sample No	Country	Region	Olive variety	Altitude, m asl	Latitude, dd	Longitude, dd	δD of precipitation, ‰	$\delta^{13}C_{C29}$, ‰	δD_{C29}, ‰
14	France	Provence-Alpes-Côte d'Azur	unknown	437	44.3	5.1	-47.0	-31.9	-154
15	France	Provence-Alpes-Côte d'Azur	unknown	735	44.3	5.3	-51.0	-33.0	-157
16	France	Provence-Alpes-Côte d'Azur	unknown	419	44.3	5.1	-46.0	-33.0	-157
17	France	Provence-Alpes-Côte d'Azur	unknown	431	44.3	5.3	-46.0	-33.1	-149
18	France	Provence-Alpes-Côte d'Azur	unknown	367	44.3	5.2	-45.0	-31.9	-149
19	France	Provence-Alpes-Côte d'Azur	unknown	308	44.2	5.2	-44.0	-32.3	-151
20	France	Languedoc-Roussillon	Calian, Petit ribier, Coratina	66	43.9	4.6	-40.0	-33.7	-153
21	France	Provence-Alpes-Côte d'Azur	Salonenque, Aglandau, Altre	100	43.7	4.8	-40.0	-33.0	-153
22	France	Provence-Alpes-Côte d'Azur	Salonenque, Aglandau, Grossane, Verdale	150	43.7	4.8	-41.0	-32.7	-151
23	Italy	Veneto	Leccino, Perlarola	195	45.5	11.2	-47.0	-33.9	-167
24	Italy	Veneto	Leccino, Perlarola	195	45.5	11.2	-47.0	-33.9	-167
25	Italy	Veneto	Frantoio, Grignano, Perlarola	195	45.5	11.2	-47.0	-33.7	-169
26	Italy	Veneto	Grignano	195	45.5	11.2	-47.0	-33.5	-164
27	Italy	Tuscany	Moraiolo	125	43.8	10.9	-41.0	-33.2	-150
28	Italy	Tuscany	Moraiolo	250	43.7	11.2	-43.0	-33.6	-150
29	Italy	Tuscany	Frantoio	300	43.7	11.3	-43.0	-33.9	-153
30	Italy	Tuscany	Moraiolo	330	43.5	11.9	-43.0	-33.1	-152

Table C2. Continued

Sample No	Country	Region	Olive variety	Altitude, m asl	Latitude, dd	Longitude, dd	δD of precipitation, ‰	$\delta^{13}C_{C29}$, ‰	δD_{C29}, ‰
31	Italy	Tuscany	Frantoio, Moraiolo, Leccino	295	43.4	11.7	-43.0	-33.1	-156
32	Italy	Tuscany	Frantoio, Moraiolo, Leccino	295	43.4	11.7	-43.0	-32.4	-162
33	Italy	Tuscany	Frantoio, Moraiolo, Leccino	295	43.4	11.7	-43.0	-32.4	-160
34	Italy	Tuscany	Frantoio	250	43.0	11.3	-41.0	-33.9	-155
35	Italy	Umbria	Moraiolo	375	43.0	12.7	-43.0	-32.9	-155
36	Italy	Umbria	Moraiolo	350	42.9	12.7	-43.0	-33.0	-157
37	Italy	Umbria	San Felice	350	42.9	12.7	-42.0	-33.9	-160
38	Italy	Lazio	Caninese	350	42.5	12.1	-41.0	-31.8	-152
39	Italy	Lazio	Caninese	270	42.3	12.0	-40.0	-32.0	-153
40	Italy	Lazio	Itrana	500	41.4	13.2	-41.0	-34.2	-146
41	Italy	Apulia	Ogliarola Precoce	450	41.4	15.2	-41.0	-32.1	-157
42	Italy	Apulia	Ogliarola Precoce	450	41.4	15.2	-41.0	-33.0	-157
43	Italy	Sicily	Nocellara del Belice	50	37.6	12.8	-27.0	-33.3	-148
44	Italy	Sicily	Biancolilla	300	37.5	13.3	-31.0	-32.2	-146
45	Italy	Sicily	Nocellara del Belice	150	37.2	13.8	-28.0	-33.1	-144
46	Italy	Sicily	Tonda Iblea	400	37.0	14.7	-32.0	-32.8	-143
47	Italy	Sicily	Tonda Iblea	400	36.9	14.7	-31.0	-33.2	-145
48	Portugal	Braganca	Cobrancosa, Madural, Verdeal transmontana	420	41.8	-6.7	-40.0	-31.5	-147

Table C2. Continued

Sample No	Country	Region	Olive variety	Altitude, m asl	Latitude, dd	Longitude, dd	δD of precipitation, ‰	$\delta^{13}C_{C29}$, ‰	δD_{C29}, ‰
49	Portugal	Ervosa-Trofa	Cobrancosa, Madural, Verdeal transmontana	350	41.3	-8.5	-37.0	-31.5	-151
50	Portugal	Guarda	Verdeal, Cobrancosa, Galega vulgar, Madural	500	41.1	-7.3	-39.0	-32.2	-144
51	Portugal	Santarem	Galega, Cobrancosa, Arbequina	438	39.2	-8.7	-34.0	-31.5	-144
52	Portugal	Beja	Galega	300	38.0	-7.9	-30.0	-32.5	-136
53	Greece	Central Macedonia	Chalkidiki	240	40.2	23.8	-38.0	-32.9	-133
54	Greece	Central Macedonia	Chalkidiki	240	40.2	23.8	-38.0	-33.3	-132
55	Greece	Central Macedonia	Chalkidiki	240	40.1	24.0	-38.0	-32.1	-145
56	Greece	Central Macedonia	Chalkidiki	240	40.1	24.0	-38.0	-31.8	-135
57	Greece	Central Macedonia	Chalkidiki	240	40.1	24.0	-38.0	-32.4	-138
58	Greece	Central Macedonia	Chalkidiki	240	40.0	24.0	-38.0	-31.7	-150
59	Greece	Central Macedonia	Chalkidiki	240	40.0	24.0	-38.0	-31.9	-133
60	Greece	Central Macedonia	Chalkidiki	240	40.0	24.0	-38.0	-31.8	-130
61	Greece	Peloponnese	unknown	250	38.0	22.9	-34.0	-32.4	-141
62	Greece	Peloponnese	unknown	250	38.0	22.9	-34.0	-32.4	-141
63	Greece	Peloponnese	Koroneiki	243	36.7	22.4	-31.0	-32.4	-134
64	Greece	Peloponnese	Koroneiki	243	36.7	22.4	-31.0	-32.2	-132
65	Greece	Peloponnese	Koroneiki	124	36.6	22.5	-29.0	-31.8	-137

Table C2. Continued

Sample No	Country	Region	Olive variety	Altitude, m asl	Latitude, dd	Longitude, dd	δD of precipitation, ‰	$\delta^{13}C_{C29}$, ‰	δD_{C29}, ‰
66	Greece	Peloponnese	Koroneiki	162	36.5	22.4	-29.0	-31.4	-135
67	Spain	Jaen	Picual	1000	38.3	-2.7	-41.0	-31.7	-146
68	Spain	Jaen	Picual	400	37.8	-3.8	-32.0	-32.0	-149
69	Spain	Jaen	Picual	400	37.8	-3.8	-32.0	-31.6	-144
70	Spain	Jaen	Picual	850	37.7	-3.7	-38.0	-31.8	-146
71	Spain	Jaen	Picual	750	37.7	-4.0	-37.0	-31.8	-146
72	Spain	Jaen	Picual	800	37.4	-3.8	-37.0	-31.6	-146
73	Morocco	Meknes	Picholine marocaine	500	33.9	-5.5	-27.0	-32.6	-137
74	Morocco	Meknes	Picholine marocaine	500	33.9	-5.5	-27.0	-33.4	-137
75	Morocco	El Hajeb	Picholine marocaine	500	33.7	-5.4	-27.0	-32.5	-138
76	Morocco	El Hajeb	Picholine marocaine	500	33.7	-5.4	-27.0	-32.5	-138

Table C3. Concentrations of *n*-alkanes in extra virgin olive oils from the Mediterranean. Sample numbers correspond to those from Table E1. ACL – *n*-alkane average chain length.

Sample No	***n*-alkane concentration, mg kg^{-1}**																		
	nC_{20}	nC_{21}	nC_{22}	nC_{23}	nC_{24}	nC_{25}	nC_{26}	nC_{27}	nC_{28}	nC_{29}	nC_{30}	nC_{31}	nC_{32}	nC_{33}	nC_{34}	nC_{35}	**total (n-C_{20}-C_{35})**	**total (odd n-alkanes)**	**ACL**
1	0.0	1.0	1.1	5.0	4.6	10.5	2.4	10.0	2.2	15.7	2.1	8.5	1.1	3.4	1.3	1.5	70.5	55.6	27.7
2	0.0	0.5	0.5	6.3	5.7	10.5	1.7	10.1	1.2	9.9	5.4	0.9	2.4	0.0	0.0	0.0	55.0	38.2	26.7
3	0.0	0.4	0.4	5.9	5.8	12.5	2.1	12.5	1.8	10.8	1.0	5.8	0.8	2.7	0.0	0.0	62.5	50.6	27.0
4	0.3	0.5	1.1	10.1	9.5	17.0	2.9	10.2	2.2	12.5	1.4	6.8	1.0	3.3	0.5	1.0	80.2	61.3	26.7
5	0.0	0.0	1.0	13.1	11.2	17.8	2.5	7.5	1.5	8.8	0.8	4.8	0.7	2.5	0.0	0.5	72.8	55.1	26.1
6	0.0	0.4	1.5	16.6	14.1	23.0	3.4	10.7	2.1	14.2	1.5	9.5	1.1	4.6	0.5	1.3	104.6	80.4	26.5
7	0.0	0.7	1.6	20.0	16.6	27.6	4.1	13.6	2.5	16.2	2.0	12.3	1.7	7.1	0.8	2.4	129.1	99.9	26.7
8	0.9	0.3	0.6	3.6	2.3	5.4	0.8	3.6	0.7	3.1	0.3	0.7	0.0	0.0	0.0	0.0	22.4	16.7	25.5
9	1.7	1.7	2.4	14.1	8.7	25.8	4.9	17.5	2.9	11.3	0.0	1.1	0.0	0.0	0.0	0.0	92.0	71.6	25.4
10	1.9	0.6	1.0	7.1	4.5	10.8	1.5	7.1	1.5	7.0	0.7	2.3	0.3	0.7	0.0	0.0	47.1	35.7	25.8
11	3.3	1.1	2.0	10.3	6.4	16.9	2.2	10.7	2.1	9.9	1.1	3.4	1.1	0.0	0.0	0.0	70.4	52.4	25.7
12	0.4	0.7	1.0	6.4	4.2	11.9	1.7	7.7	1.5	7.0	0.7	1.9	0.0	0.0	0.0	0.0	45.1	35.5	25.9
13	0.4	0.9	0.8	6.4	3.5	13.6	1.6	9.2	1.3	5.9	0.0	0.6	0.0	0.0	0.0	0.0	44.2	36.6	25.6
14	6.3	1.8	5.1	13.9	8.1	26.9	4.7	23.3	5.0	24.9	2.8	8.5	1.3	2.6	0.7	0.7	136.6	102.6	26.4
15	0.0	0.4	0.5	3.2	2.1	5.4	1.1	3.9	0.8	3.6	0.3	0.8	0.0	0.0	0.0	0.0	22.2	17.3	25.9
16	3.4	1.2	2.8	12.6	8.1	19.5	2.6	13.4	2.5	13.2	1.3	4.5	0.7	1.5	0.3	0.4	88.0	66.3	26.0
17	2.1	0.8	1.8	6.1	3.8	12.1	1.7	9.2	1.7	9.2	0.9	3.1	0.4	1.0	0.2	0.2	54.3	41.7	26.2
18	0.3	0.4	0.6	3.0	2.1	6.0	1.2	4.4	0.8	3.6	0.4	0.8	0.0	0.0	0.0	0.0	23.6	18.2	25.9
19	3.4	1.2	2.2	9.6	5.9	17.4	2.5	12.8	2.6	13.2	1.3	4.2	0.6	1.2	0.3	0.3	78.6	59.9	26.1
20	0.0	0.0	0.6	9.2	7.0	14.5	2.1	8.0	1.5	10.2	1.2	6.7	0.6	2.5	0.0	0.5	64.7	51.6	26.7
21	0.0	0.5	1.5	28.5	20.1	41.4	3.5	10.0	1.3	14.8	1.6	11.4	1.5	7.2	0.8	2.2	146.2	115.9	26.2

Table C3. Continued

Sample No	*n*-alkane concentration, mg kg^{-1}																total (*n*-C_{20}-C_{35})	total (odd *n*-alkanes)	ACL
	nC_{20}	nC_{21}	nC_{22}	nC_{23}	nC_{24}	nC_{25}	nC_{26}	nC_{27}	nC_{28}	nC_{29}	nC_{30}	nC_{31}	nC_{32}	nC_{33}	nC_{34}	nC_{35}			
22	0.0	0.7	1.7	25.7	17.5	36.1	4.3	10.6	2.5	12.7	2.0	8.9	1.4	5.1	0.6	1.3	131.2	101.2	26.1
23	0.0	0.8	1.2	19.5	19.2	29.6	3.8	14.8	1.8	12.1	1.1	6.2	2.6	0.7	0.0	0.0	113.3	83.7	25.8
24	0.0	0.0	0.9	16.1	15.8	23.4	3.4	12.2	1.5	9.9	1.0	5.0	0.0	2.0	0.0	0.0	91.0	68.5	25.8
25	0.0	0.0	0.0	4.9	4.3	9.9	1.8	12.1	1.7	10.0	0.9	4.9	0.0	1.9	0.0	0.0	52.5	43.8	27.0
26	0.0	0.0	0.8	7.8	7.4	15.0	2.7	10.7	1.9	12.4	1.1	5.1	2.7	0.6	0.0	0.0	68.2	51.7	26.7
27	0.0	1.0	2.2	35.1	31.5	47.5	7.0	29.0	3.4	24.9	3.1	19.7	2.6	13.7	1.5	4.4	226.7	175.3	26.7
28	0.0	1.0	0.7	12.3	9.4	22.2	3.5	23.9	3.8	24.7	2.8	16.8	1.7	7.6	0.7	2.1	133.1	110.5	27.5
29	0.0	0.5	0.4	6.0	5.1	12.1	2.1	12.3	1.9	13.0	1.5	8.7	0.9	3.5	0.0	1.0	69.0	57.1	27.4
30	0.0	0.4	0.8	13.0	10.2	18.5	2.5	9.8	1.9	15.7	2.0	19.9	2.9	16.0	2.0	6.0	121.5	99.2	28.3
31	0.0	0.0	0.5	8.7	7.8	19.0	2.1	9.5	1.7	11.2	1.4	10.2	1.5	6.4	0.7	2.3	83.2	67.4	27.5
32	0.0	0.3	0.5	9.2	7.2	14.9	2.2	11.1	1.5	9.6	1.1	8.0	1.0	4.7	0.5	1.1	72.9	58.8	27.1
33	0.0	0.3	0.6	9.6	7.7	16.3	2.4	11.3	1.6	10.2	1.2	8.6	1.1	4.9	0.5	1.1	77.5	62.3	27.1
34	0.0	0.0	0.4	6.7	6.2	13.6	2.2	13.2	2.1	15.6	1.8	10.9	1.0	3.9	0.0	1.0	78.7	65.0	27.5
35	0.0	0.7	1.4	17.2	12.4	19.4	3.0	13.6	2.4	18.4	2.6	24.0	3.8	22.4	3.7	9.5	154.5	125.2	28.6
36	0.0	0.6	1.2	16.1	13.5	22.7	3.5	14.7	2.7	19.1	2.5	21.2	3.0	16.5	2.5	7.1	146.9	118.0	28.1
37	0.0	0.7	1.3	14.9	13.6	25.1	4.2	20.1	2.8	21.1	2.6	17.9	2.0	9.8	1.2	3.6	140.7	113.1	27.5
38	0.0	0.8	1.7	22.5	16.3	37.9	5.0	12.1	2.1	14.1	1.8	10.0	0.9	4.4	0.2	0.7	130.5	102.4	26.1
39	0.0	1.0	2.1	30.8	17.9	30.7	3.5	8.5	1.7	10.2	1.2	7.4	0.8	3.4	0.0	0.6	119.8	92.6	25.6
40	0.0	0.4	0.7	7.4	6.2	13.2	2.2	10.5	1.6	12.8	1.4	5.3	0.6	1.7	0.0	0.0	63.9	51.3	26.7
41	0.0	1.2	1.6	21.8	13.1	23.7	2.7	9.7	1.6	17.7	2.2	17.9	2.7	11.9	1.2	3.5	132.6	107.6	27.3
42	0.0	2.6	2.6	21.5	15.2	25.3	4.7	12.0	2.7	16.8	2.8	15.4	2.7	9.4	1.4	2.9	138.0	105.8	27.0
43	0.0	0.0	0.0	1.4	0.9	4.2	0.9	6.1	0.8	6.1	0.6	2.4	0.0	0.9	0.0	0.0	24.2	21.0	27.5
44	0.0	0.2	0.5	5.1	3.6	10.1	1.6	7.8	1.1	9.3	1.0	6.8	0.9	5.2	0.6	1.2	55.2	45.8	27.8
45	0.0	0.0	0.0	1.6	0.9	4.6	0.7	6.8	0.9	8.1	0.9	3.2	0.4	1.2	0.0	0.2	29.5	25.7	27.8

Table C3. Continued

Sample No	*n*-alkane concentration, mg kg^{-1}																		
	nC_{20}	nC_{21}	nC_{22}	nC_{23}	nC_{24}	nC_{25}	nC_{26}	nC_{27}	nC_{28}	nC_{29}	nC_{30}	nC_{31}	nC_{32}	nC_{33}	nC_{34}	nC_{35}	**total (n-C_{20}-C_{35})**	**total (odd *n*-alkanes)**	**ACL**
46	0.0	0.5	1.4	22.5	17.4	30.5	4.3	10.4	2.1	15.7	1.5	9.3	1.0	5.4	1.0	5.4	128.5	99.7	26.6
47	0.0	0.9	2.2	25.7	20.3	36.3	6.5	19.5	3.2	23.6	2.6	13.0	1.8	6.7	1.1	2.3	165.7	128.1	26.6
48	0.0	0.3	0.8	14.0	10.0	20.3	1.7	6.6	2.0	14.8	1.9	10.9	1.6	7.2	1.2	2.3	95.7	76.4	28.3
49	0.0	0.2	0.3	1.1	0.7	3.3	1.0	6.0	1.4	6.8	0.9	3.5	0.5	1.8	0.3	0.6	28.5	23.3	28.2
50	0.0	0.5	0.4	2.2	1.3	8.5	2.1	16.7	3.8	24.6	3.1	16.8	1.8	9.0	1.1	2.9	94.8	81.2	28.9
51	0.0	0.4	0.5	3.0	2.0	9.5	2.1	13.5	2.4	12.5	2.2	8.1	1.6	6.7	1.1	1.7	67.2	55.4	28.4
52	0.0	0.6	0.6	0.0	5.0	15.0	3.0	13.9	2.2	15.2	2.8	14.7	1.9	6.4	0.7	1.5	83.4	67.2	28.3
53	2.4	1.4	1.2	3.0	2.6	9.0	3.4	7.0	1.8	6.0	0.8	1.6	0.4	0.0	0.0	0.0	40.6	28.0	25.8
54	1.6	1.4	1.2	3.2	2.0	7.6	1.8	6.2	1.0	3.6	0.6	0.0	0.0	0.0	0.0	0.0	30.2	22.0	25.3
55	1.0	1.8	0.6	4.6	2.6	10.2	2.8	6.4	1.2	5.0	0.6	1.8	0.0	0.0	0.0	0.0	38.6	29.8	25.7
56	1.4	1.4	1.2	3.4	2.4	5.8	1.8	6.0	1.4	5.8	0.8	2.2	0.0	0.0	0.0	0.0	33.6	24.6	26.0
57	2.6	2.2	1.8	4.8	3.4	8.6	2.8	8.4	1.8	9.2	1.6	4.0	0.4	0.2	0.0	0.0	52.2	37.8	25.9
58	1.2	1.4	0.8	4.0	2.6	7.6	3.6	7.2	1.6	6.8	0.8	3.2	0.0	0.0	0.0	0.0	40.4	30.0	26.2
59	1.0	1.2	0.6	3.2	2.0	6.6	1.8	5.6	1.0	5.0	0.6	2.0	0.0	0.0	0.0	0.0	30.6	23.6	26.0
60	0.6	0.8	0.6	2.6	1.4	4.0	1.2	3.4	0.8	2.4	0.0	0.6	0.0	0.0	0.0	0.0	18.4	14.0	25.4
61	0.4	9.4	0.0	4.8	3.6	9.8	1.5	5.5	4.5	8.3	0.8	5.9	0.8	3.2	0.7	0.0	59.1	46.8	26.2
62	0.0	0.6	1.1	16.1	9.4	20.7	3.1	13.0	1.6	12.7	1.1	6.9	0.6	2.5	0.0	0.3	89.5	72.6	26.2
63	0.6	1.3	1.8	21.2	10.7	13.5	1.5	5.6	0.8	3.6	0.0	0.0	0.0	0.0	0.0	0.0	60.7	45.2	24.4
64	0.7	0.9	1.7	23.7	12.1	15.3	2.0	4.8	1.1	3.6	0.0	0.9	0.0	0.0	0.0	0.0	67.0	49.3	24.5
65	0.7	1.5	2.0	25.6	12.5	15.7	2.1	7.4	1.2	4.9	0.0	1.6	0.0	0.0	0.0	0.0	75.1	56.7	24.6
66	0.7	1.2	1.5	17.8	9.2	11.9	1.7	6.3	0.9	3.4	0.0	0.7	0.0	0.0	0.0	0.0	55.3	41.4	24.6
67	0.0	0.4	0.3	1.4	1.1	6.7	2.3	7.5	2.1	10.3	2.0	9.8	1.5	5.2	0.8	1.4	52.8	42.7	28.3
68	0.0	0.0	0.0	0.6	0.6	3.6	1.1	4.9	0.0	6.9	1.0	6.1	0.7	3.1	0.0	0.5	29.2	25.8	28.9
69	0.0	0.2	0.2	1.1	0.5	4.3	1.2	4.7	1.0	5.8	1.0	5.0	0.8	2.4	0.3	0.5	28.9	24.0	28.5

Table C3. Continued

Sample No	*n*-alkane concentration, mg kg^{-1}																		
	nC_{20}	nC_{21}	nC_{22}	nC_{23}	nC_{24}	nC_{25}	nC_{26}	nC_{27}	nC_{28}	nC_{29}	nC_{30}	nC_{31}	nC_{32}	nC_{33}	nC_{34}	nC_{35}	**total (*n*-C_{20}-C_{35})**	**total (odd *n*-alkanes)**	**ACL**
70	0.0	0.0	0.0	1.1	1.0	3.7	1.4	4.7	1.5	5.9	1.2	5.1	0.8	2.8	0.3	0.8	30.2	24.1	28.7
71	0.0	0.0	0.0	0.7	0.5	3.4	1.1	4.8	1.2	6.5	1.0	5.6	0.8	2.7	0.4	0.9	29.6	24.6	28.9
72	0.0	0.6	0.7	2.3	1.6	8.7	2.8	9.4	2.4	14.8	2.1	13.9	1.3	6.7	0.7	1.6	69.8	58.2	28.7
73	0.4	2.2	0.9	7.0	3.9	11.4	1.8	9.5	13.1	10.5	1.1	8.6	1.3	6.4	0.5	0.5	79.2	56.1	27.5
74	5.6	25.5	7.3	25.7	6.1	14.7	2.4	10.0	0.0	6.1	0.7	2.9	0.0	1.5	0.0	0.0	108.6	86.4	23.8
75	0.3	2.7	0.8	5.9	1.8	9.6	2.0	11.1	1.9	9.0	0.9	3.9	0.5	1.8	0.0	0.3	52.8	44.5	26.6
76	1.5	10.1	3.1	15.8	4.1	12.1	1.8	8.8	1.4	6.5	0.7	3.5	0.4	1.6	0.0	0.0	71.5	58.5	24.7

APPENDIX D

SUPPORTING INFORMATION FOR CHAPTER 5

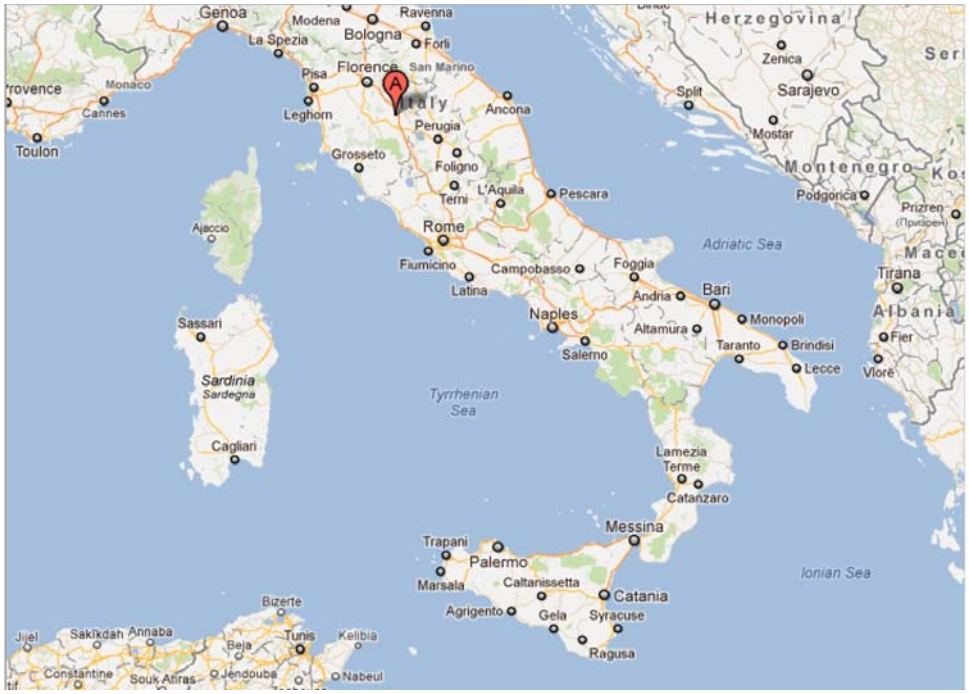

Figure D1. The location of the olive grove in Ciggiano, Italy. The image adopted from Google maps at http://www.maps.google.co.uk (date accessed: 15 May 2012).

Figure D2. Mechanical (A) and manual (B) olive harvesting in Ciggiano. Photographs were taken by Dr. Nikolai Pedentchouk (University of East Anglia).

Table D1. The mean temperatures during August-December in Arezzo region, Italy. Data obtained from the weather history database at http://www.wunderground.com (date accessed: 1 May 2012).

Oil production year	Mean temperature, °C					
	August	September	October	November	December	**Mean**
2004	25.0	22.0	18.0	12.0	8.0	**17.0**
2005	23.0	20.0	16.0	10.0	5.0	**14.8**
2006	23.0	22.0	18.0	12.0	9.0	**16.8**
2007	24.0	20.0	16.0	10.0	7.0	**15.4**
2008	25.0	20.0	17.0	11.0	7.0	**16.0**
2009	27.0	22.0	15.0	12.0	7.0	**16.6**
2010	23.0	19.0	14.0	11.0	5.0	**14.4**

Table D2. The *n*-alkane average chain length (ACL) and $\delta^{13}C_{C29}$ values of olives and leaves from Frantoio, Leccino and Moraiolo varieties.

Olive variety	ACL		$\delta^{13}C_{C29}$, ‰	
	olives	leaves	olives	leaves
Frantoio	27.7	31.6	-32.6	-32.0
Frantoio	26.8	31.7	-31.9	-32.4
Frantoio	27.1	31.8	-32.7	-32.1
Leccino	27.0	31.9	-34.9	-31.7
Leccino	27.0	32.1	-33.3	-32.2
Leccino	27.3	32.0	-35.1	-31.5
Moraiolo	27.1	31.9	-33.6	-31.8
Moraiolo	27.2	32.1	-34.0	-31.6
Moraiolo	27.3	32.2	-32.9	-31.4

APPENDIX E

SUPPORTING INFORMATION FOR CHAPTER 6

Figure E1. Olive trees marked with waterproof colour in Ciggiano (Tuscany, Italy).

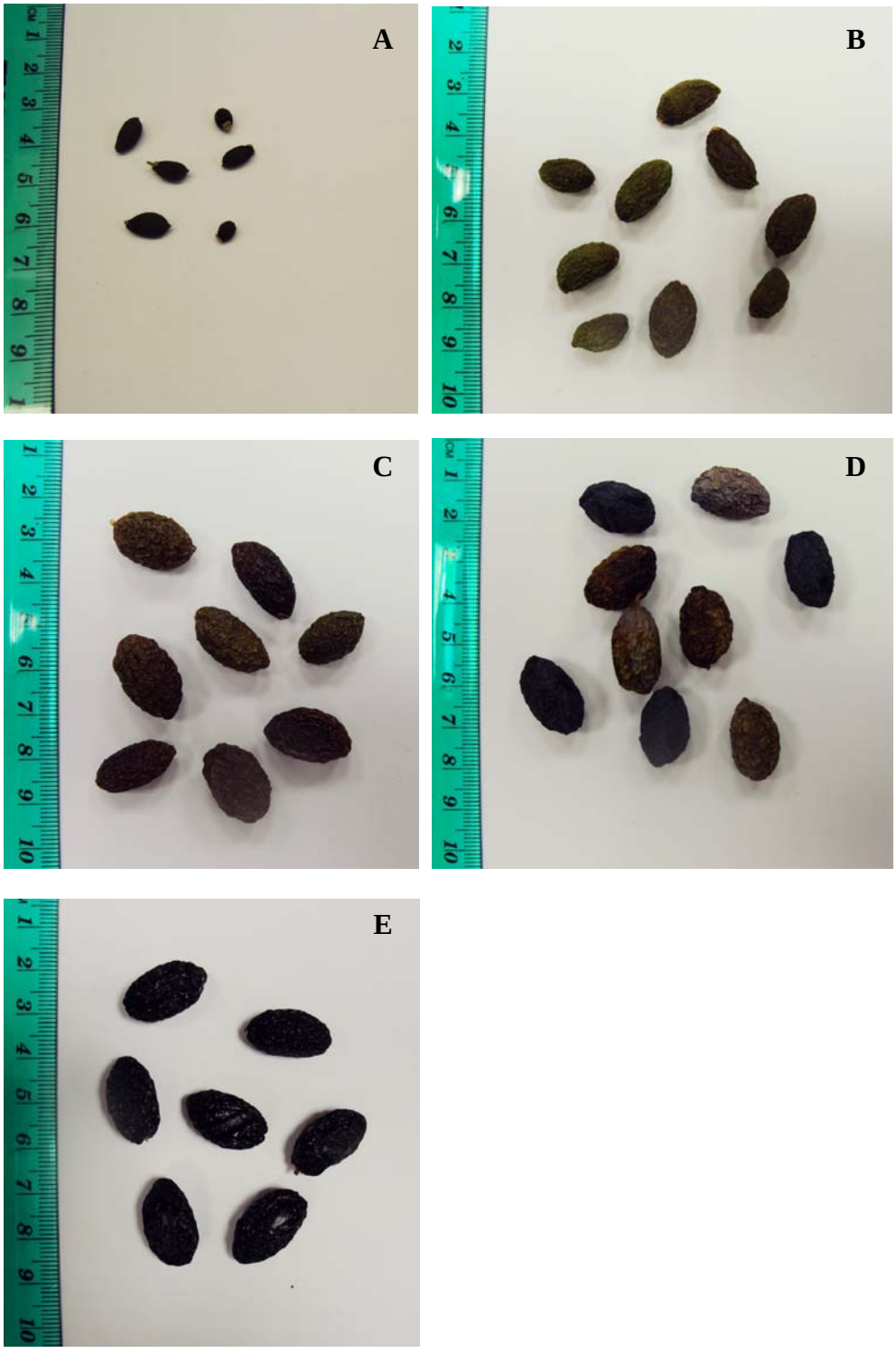

Figure E2. Different development stages of olives from Frantoio, Leccino and Moraiolo varieties. A – July, B – August, C – September, D – October, E – November.

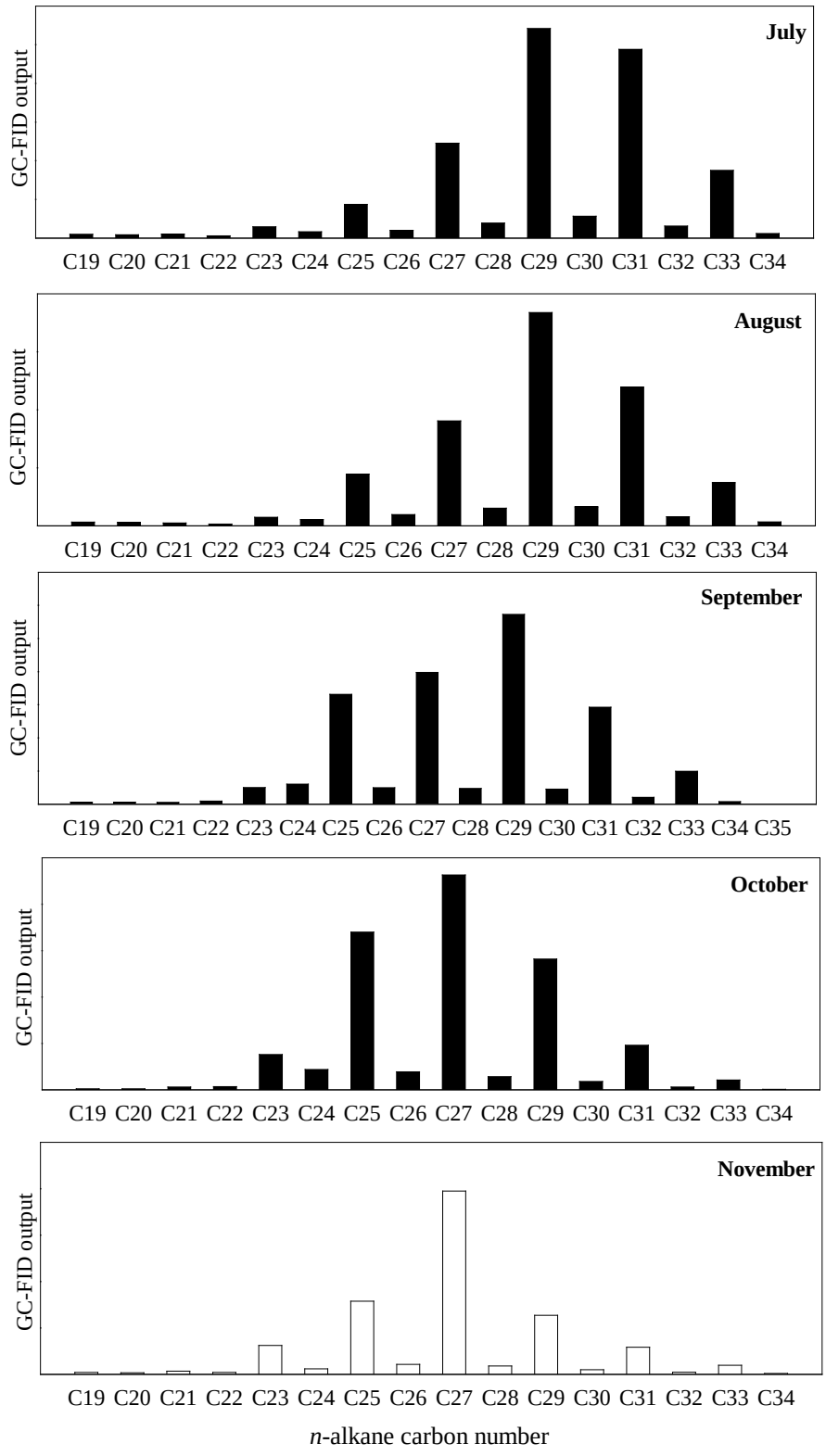

Figure E3. The distribution of *n*-alkanes in the olives of Leccino variety in 2011.

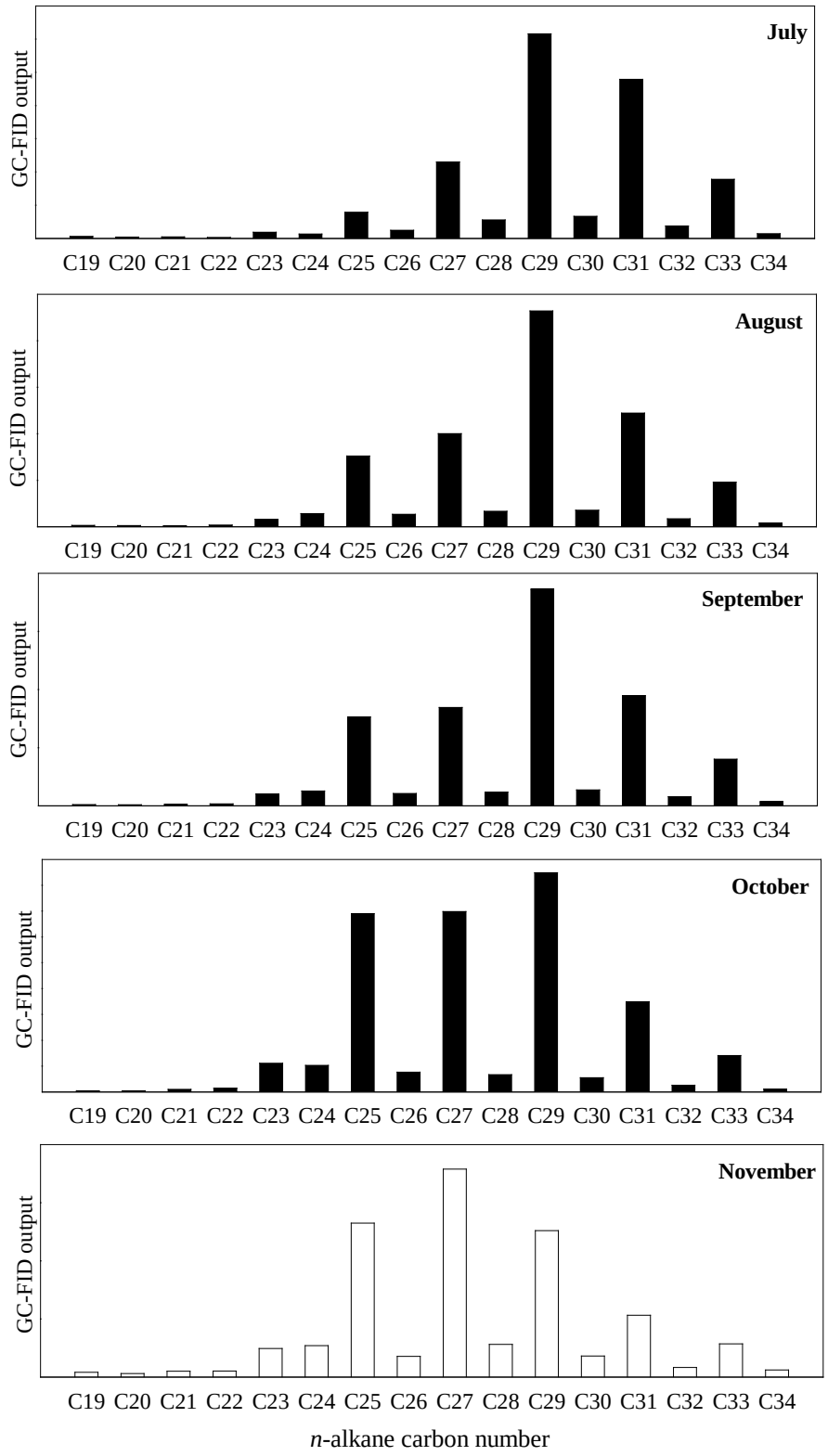

Figure E4. The distribution of *n*-alkanes in the olives of Moraiolo variety in 2011.

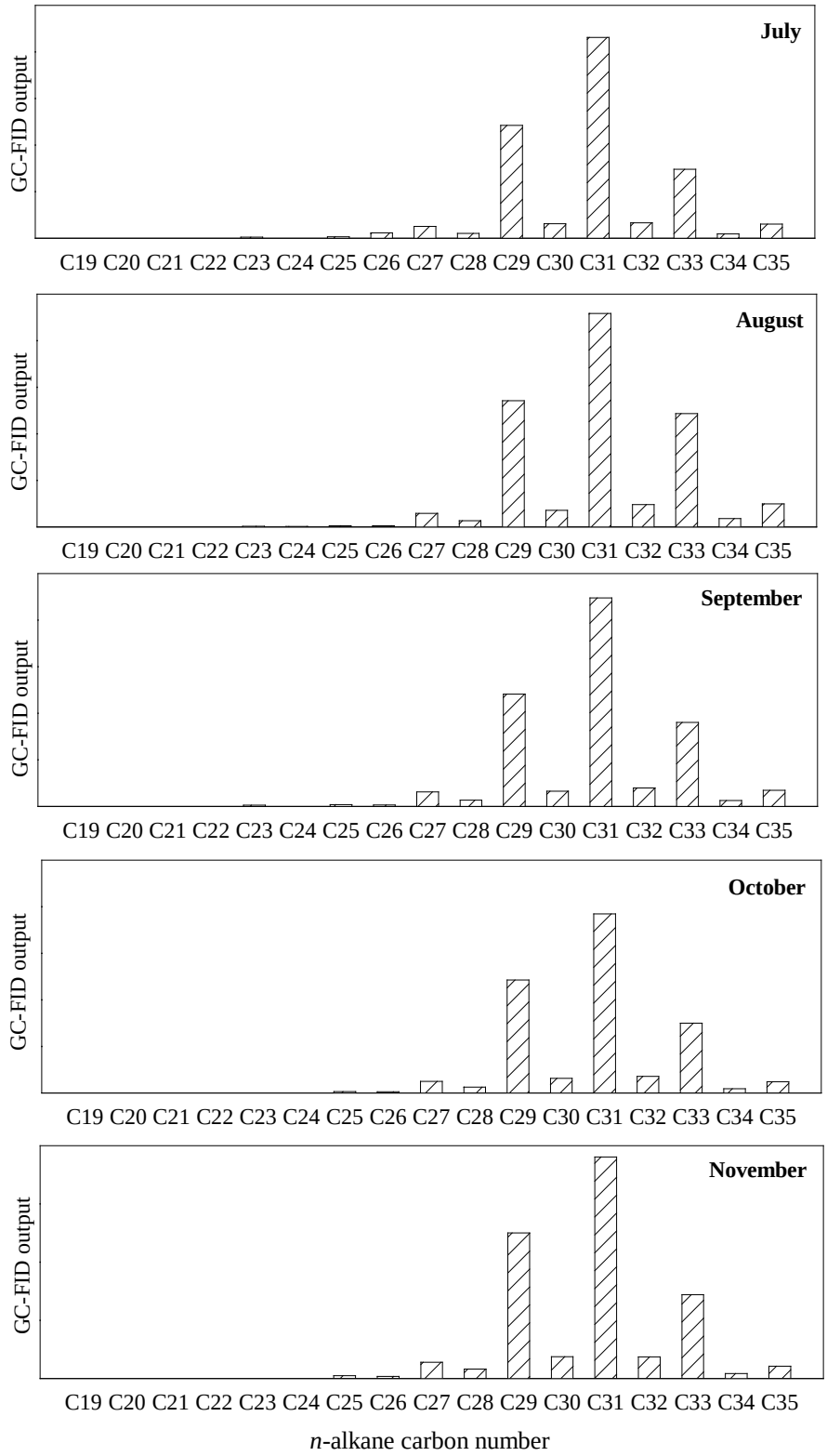

Figure E5. The distribution of *n*-alkanes in the leaves of Leccino variety in 2011.

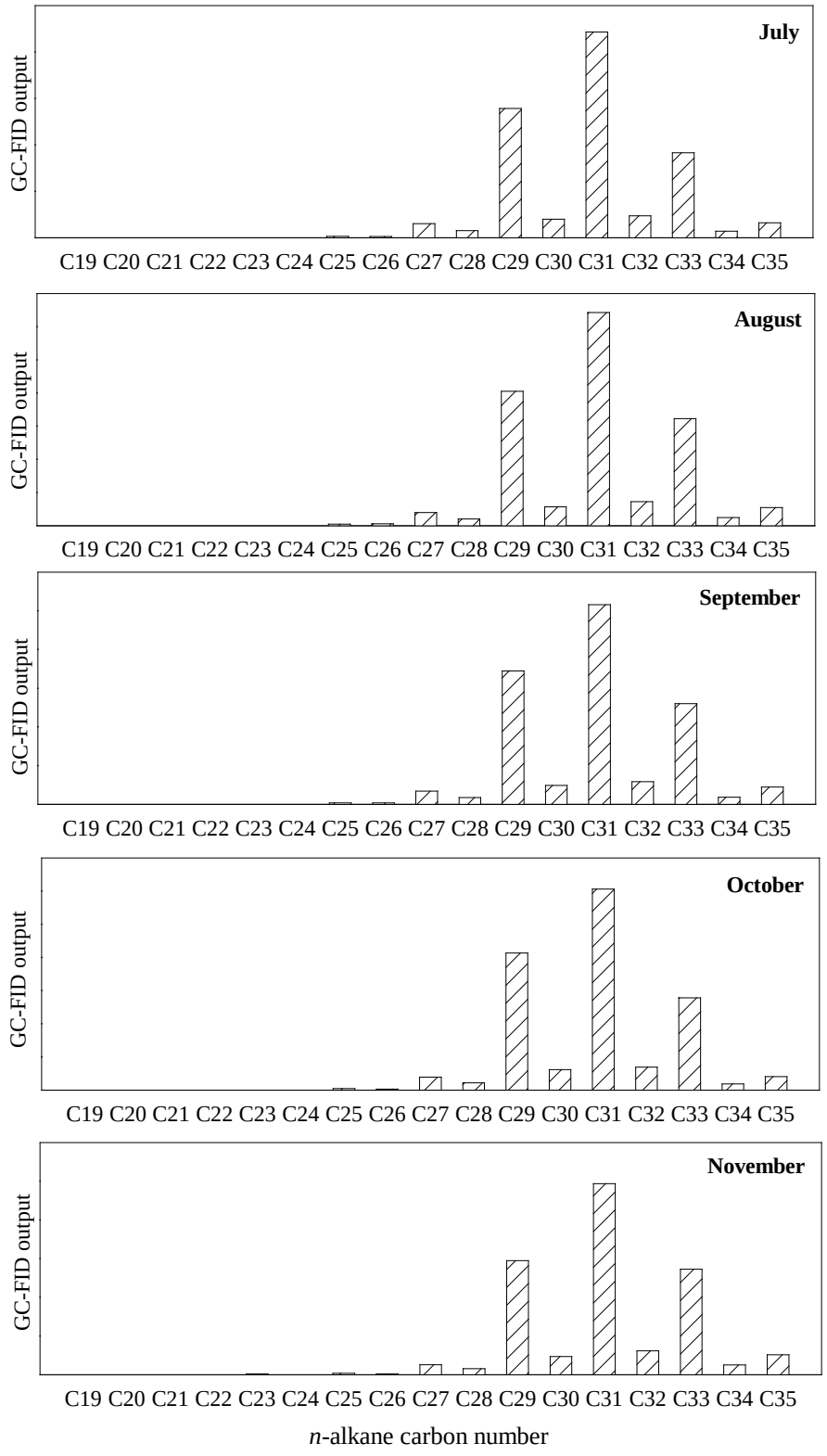

Figure E6. The distribution of *n*-alkanes in the leaves of Moraiolo variety in 2011.

Table E1. *n*-Alkane average chain length (ACL) values of olive leaves from Frantoio, Leccino and Moraiolo varieties in 2011.

Sampling time	Variety	ACL	Average	SD
June	Frantoio	30.5		
	Frantoio	30.7	30.6	0.1
	Frantoio	30.6		
	Leccino	30.4		
	Leccino	30.4	30.4	0.0
	Leccino	30.3		
	Moraiolo	30.9		
	Moraiolo	30.8	30.9	0.1
	Moraiolo	30.9		
July	Frantoio	30.1		
	Frantoio	30.8	30.5	0.4
	Frantoio	30.5		
	Leccino	30.8		
	Leccino	30.8	30.7	0.1
	Leccino	30.6		
	Moraiolo	31.2		
	Moraiolo	30.6	30.8	0.3
	Moraiolo	30.6		
August	Frantoio	30.3		
	Frantoio	30.8	30.6	0.3
	Frantoio	30.6		
	Leccino	30.6		
	Leccino	31.0	30.9	0.2
	Leccino	31.1		
	Moraiolo	30.9		
	Moraiolo	30.8	30.9	0.1
	Moraiolo	31.0		
September	Frantoio	30.2		
	Frantoio	30.9	30.6	0.4
	Frantoio	30.8		
	Leccino	30.6		
	Leccino	31.0	30.7	0.3
	Leccino	30.5		
	Moraiolo	30.7		
	Moraiolo	30.4	30.7	0.3
	Moraiolo	31.0		
October	Frantoio	30.1		
	Frantoio	30.6	30.5	0.3
	Frantoio	30.8		
	Leccino	30.4		
	Leccino	30.9	30.7	0.3
	Leccino	30.8		
	Moraiolo	30.8		
	Moraiolo	30.8	30.8	0.0
	Moraiolo	30.8		
November	Frantoio	30.8		
	Frantoio	30.7	30.8	0.1
	Frantoio	30.8		
	Leccino	30.9		
	Leccino	30.6	30.6	0.3
	Leccino	30.3		
	Moraiolo	31.2		
	Moraiolo	31.0	30.9	0.4
	Moraiolo	30.4		

SD – standard deviation based on three replicate samples of each variety

Table E2. The $\delta^{13}C$ values of *n*-C_{29}, *n*-C_{31}, and *n*-C_{33} alkanes from leaves of Frantoio, Leccino and Moraiolo varieties in 2011.

Sampling time	Variety	$\delta^{13}C$, ‰		
		n-C_{29}	*n*-C_{31}	*n*-C_{33}
June	Frantoio	-30.6	-30.4	-31.5
	Frantoio	-30.6	-30.3	-31.0
	Frantoio	-30.5	-30.5	-30.6
	Leccino	-32.5	-31.7	-33.1
	Leccino	-32.3	-31.6	-33.1
	Leccino	-31.7	-31.7	-32.3
	Moraiolo	-31.6	-30.5	-31.8
	Moraiolo	-31.5	-31.1	-31.6
	Moraiolo	-30.8	-30.2	-31.2
July	Frantoio	-30.6	-30.7	-31.7
	Frantoio	-30.4	-29.9	-30.6
	Frantoio	-30.5	-30.3	-31.1
	Leccino	-32.9	-32.1	-33.3
	Leccino	-32.1	-31.1	-32.4
	Leccino	-31.2	-30.8	-31.3
	Moraiolo	-31.1	-30.4	-31.2
	Moraiolo	-31.2	-31.0	-31.6
	Moraiolo	-31.7	-31.5	-32.1
August	Frantoio	-30.7	-30.6	-32.0
	Frantoio	-30.6	-30.4	-31.4
	Frantoio	-30.8	-31.3	-31.4
	Leccino	-31.5	-30.9	-32.4
	Leccino	-31.1	-30.2	-31.6
	Leccino	-31.5	-31.2	-31.7
	Moraiolo	-30.8	-30.2	-31.5
	Moraiolo	-31.2	-31.0	-31.6
	Moraiolo	-30.4	-30.1	-30.8
September	Frantoio	-31.0	-31.3	-32.5
	Frantoio	-30.3	-30.4	-31.2
	Frantoio	-30.2	-30.6	-30.8
	Leccino	-32.2	-31.6	-33.3
	Leccino	-31.8	-31.2	-33.0
	Leccino	-30.5	-30.6	-31.3
	Moraiolo	-31.1	-30.4	-31.3
	Moraiolo	-31.3	-31.3	-31.7
	Moraiolo	-30.9	-31.0	-31.6
October	Frantoio	-30.9	-31.1	-32.3
	Frantoio	-30.8	-30.9	-31.4
	Frantoio	-30.5	-30.6	-30.6
	Leccino	-31.2	-30.9	-32.7
	Leccino	-30.6	-29.8	-31.6
	Leccino	-31.4	-31.4	-31.8
	Moraiolo	-32.1	-31.2	-32.0
	Moraiolo	-32.6	-32.9	-33.0
	Moraiolo	-31.4	-31.3	-31.7
November	Frantoio	-30.5	-30.7	-31.9
	Frantoio	-31.7	-31.7	-32.2
	Frantoio	-31.3	-31.4	-31.6
	Leccino	-31.7	-31.1	-32.8
	Leccino	-31.6	-31.8	-32.3
	Leccino	-32.0	-31.9	-32.6
	Moraiolo	-31.7	-30.7	-31.5
	Moraiolo	-32.0	-31.9	-32.1
	Moraiolo	-32.1	-31.9	-32.3

Table E3. The $\delta^{13}C$ values of *n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31} alkanes from olives of Frantoio, Leccino and Moraiolo varieties in 2011.

Sampling time	Variety	$\delta^{13}C$, ‰			
		n-C_{25}	*n*-C_{27}	*n*-C_{29}	*n*-C_{31}
July	Frantoio	-30.6	-31.3	-31.4	-31.6
	Leccino	-32.0	-32.4	-32.3	-32.4
	Moraiolo	-31.4	-31.9	-31.9	-32.0
August	Frantoio	-29.3	-29.3	-30.0	-30.5
	Leccino	-32.6	-32.9	-32.6	-32.6
	Moraiolo	-31.2	-31.1	-31.2	-31.7
September	Frantoio	-28.7	-29.4	-29.7	-30.6
	Leccino	-31.9	-32.3	-31.9	-32.2
	Moraiolo	-32.0	-31.9	-31.9	-31.7
October	Frantoio	-29.8	-29.8	-29.8	-30.1
	Leccino	-32.0	-32.1	-31.9	-31.7
	Moraiolo	-33.0	-33.0	-32.6	-32.5
November	Frantoio	-31.3	-31.2	-31.1	-31.4
	Leccino	-33.7	-33.2	-33.5	-33.1
	Moraiolo	-33.8	-33.3	-32.8	-32.1

APPENDIX F
MANUSCRIPTS PUBLISHED AND IN PREPARATION

- **Mihailova A.** and Kelly S.D. 2012. Organic food authenticity – recent advances in isotope ratio mass spectrometry. Review. *Food Science and Technology* 26 (1): 26-28.

- Laursen K.H., **Mihailova A.**, Kelly S.D., Epov V.N., Bérail S., Schjoerring J.K., Donard O.F.X., Larsen E.H., Pedentchouk N., Marca-Bell A.D., Halekoh U., Olesen J.E. and Husted S. Is it really organic? - multi-isotopic analysis as a tool to discriminate between organic and conventional plants. Submitted to *Food Chemistry* in September 2012.

- **Mihailova A.**, Pedentchouk N. Geographical classification of extra virgin olive oils from the Mediterranean region on the basis of molecular and carbon isotope composition of *n*-alkanes. In preparation for submission to *Food Chemistry*.

- **Mihailova A.** and Pedentchouk N. Composition of *n*-alkanes in olives and leaves from three Italian varieties – implications for virgin olive oil authentication. In preparation for submission to *Food Chemistry*.