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Organic produce and production system conformity assessments

eggs and isotope analysis

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Summary

On request of the Department of Agriculture, Nature and Food Quality (LNV) of the Netherlands the analytical options for verification of productions systems of eggs labeled organic were evaluated. Scientific literature was searched and reviewed with particular attention to eggs and isotope analysis. Furthermore scientific experts were consulted for their vision on the state-of-the-art regarding isotope analysis for organic produce authentication. Few studies on egg authentication have been reported, and none on egg isotope analysis with the aim to authenticate organic produce. Nevertheless, stable isotope analysis has made a substantial contribution to other forms of authenticity testing. This type of analysis is based on the premise that each species has its own unique patterns of naturally occurring stable isotopes. Stable isotope analysis of the bioelements H, C, N, O, S has been applied for more than 20 years in food authentication control, e.g. for wine, flavors, aromas, honeys and fruit juices. Literature showed and fellow scientists agreed that a scientific base exists for discrimination of organic produce from conventional produce by isotope ratio analysis within certain boundaries. Success will depend on the product under investigation. The isotope ratios in livestock are primarily determined by the animals' diet. External effects on carbon isotopes mainly result from the ingested proportion of C_3- to C_4-plants, as C_4-plants, such as maize or sugar cane, have a higher relative $\delta^{13}C$ content. This approach may be considered if the diet of animals in both production systems differ considerably. Crops grown with artificial fertilizers differ generally in $\delta^{15}N$ values from those grown under organic regimes in which manure is used. Livestock feeding on these crops will reflect (within boundaries) the crops' isotopic signature. However, many confounding factors exist, such as the definition of organic produce, use of leguminous plants (clover) for enhancement of the nitrogen fertility of soils, temporal and spatial variations within fields where crops for feed are grown due to nitrogen turnover factors, precedent land use, variability in both synthetic and non-synthetic fertilizers, enrichment of heavier isotopes during metabolism, etc. For this reason, stable isotope analysis is promising but the complicating factors should be kept in mind.

For future work on organic egg authentication, a survey on products from farms (both organic and conventional) produced under known production conditions is recommended in order to explore the practical use of a selection of analytes for discrimination of products originating from different production systems. This sort of experimental data is not readily available. Isotopes and fatty acid composition are, according to literature and scientists, promising analytical variables. Conditions would need to cover the variation encountered in regular market samples. Good documentation of conditions (geographics, livestock, feeding, fertilization, field history, sampling, etc.) is essential for a correct evaluation and reliable prediction of the method's successful use later in the real world. For extraction of information from the data a chemometric approach (advanced statistics) is recommended.

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1 Introduction

Given people's growing awareness of health and the environment, public interest is increasingly focusing on the quality of foods and food production. Considerable attention is being paid to organic farming (Woese et al., 1997). Organic farming is likely to receive a major boost in the EU and probably also worldwide. Consumers have lost some trust in food derived from conventional production due to recent crises (e.g. mad cow disease, foot-and-mouth epidemic) and also due to concerns regarding use of pesticides in farming and antibiotics in livestock feed. The large increase in organic farming stems from a variety of rationales: (a) to preserve the earning capability of farmers in a world that needs less producers to feed the well-fed part of the world's population; (b) to preserve the rural countryside as such; (c) to use cultivation methods that will conserve the soil and contribute to sustainability (Mayfield et al., 2001). Organic farming seems to be a relevant tool, which contains the potential to participate in solving simultaneously a range of problems related to food production, environment, animal welfare, and rural development (Siderer et al., 2005).

The organic farming movement started almost a century ago (Balfour, 1943). During the past two decades, organic farming and organic food markets became large enough to call for legislation in order to organize farming procedures and marketing routes. The standards of the organic agriculture were developed in the past mainly by private organizations of farmers (Kahal, 2000). In the past decade more standards were included in governmental regulations. However, there is no worldwide standard for organic agriculture. Harmonisation of standards or an international process is necessary to make the comparison between various standards and for the development of worldwide trade in organic products (Siderer et al., 2005). The International Federation of Organic Agriculture Movements (IFOAM) developed a basic standard for organic production in 1998 that was revised in 2002 (IFOAM, 2002) as a framework for further developing organic standards around the world. Another international organisation that develops standards for organic products is the Codex Alimentarius Commission (intergovernmental body under the auspices of the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) of the United Nations). This organization established in 1999 (revised in 2001) a proposal for a world wide standard of production and marketing for organic foodstuffs (Codex Alimentarius Commission, 2001). Its aim is to develop free international trade by creating international standards. The EU has regulated the organic products market in a set of laws such as EC Regulation No. 2092/91, EC Regulation No. 1804/99 and EC Regulation 834/2007. According to this legislation, a product can only be marketed as an organic product, when it is produced according to the productions rules given in the law. It includes the definition of the principles of organic production by the farmer, the materials that are allowed to be used for fertilization and plant protection, the minimal requirements for control and guidelines applying to processed products. Organic products of plant origin are grown without the aid of chemical-synthetic pesticides and largely without the use of readily soluble mineral fertilizers within a diverse range of crop rotation and extensive soil tillage. Livestock farming is undertaken in line with the needs of the animals (Woese et al., 1997). European law is based in many parts on the basic standards of IFOAM and there are few essential differences (Siderer et al., 2005). Farmers and stakeholders have been facing new challenges in the EU agricultural policy area: the EU Action Plan for Organic Food and Farming and the Luxemburg Agreements (CAP reform). With the European

Action plan, the EU recognizes the dual societal role of organic farming: (a) organic land management generating public benefits; and (b) organic food as a direct response to consumer concerns relating to quality, safety, health and animal welfare. The 2003 CAP reform emphasized the long-term economic and social viability of the EU agricultural sector, providing safe, high quality products by methods offering a high degree of consideration for the environment. The CAP reform provides therefore a positive framework for the development of organic farming in Europe (Siderer et al., 2005).
Organic produce tends to retail at a higher price than its conventionally grown equivalent mainly because of higher production costs. Following a farmer's decision it takes at least 2-3 years to convert from conventional to organic farming. In order to take such a decision, the farmer should be convinced that the conversion will pay off and that there will be buyers for the organic crops. The consumers, on the other hand, are entitled to know what they buy. Fraud in organic farming may become an increasing concern as the sector experiences rapid year-on-year growth. For both producer (fair competition) and consumer (reassurance) there is, therefore, an urgent need for scientific, independent technologies for organic produce authentication.

3.1 Aim of the study

A number of scientific studies have compared the nutritional components, sensory quality and food safety aspects of organically and conventionally produced foods (Bourn & Prescott, 2002; Woese et al., 1997). However, existing analytical approaches cannot provide a general, consistent reliable designation of the production system of foods (Ulberth, 2004). A robust scientific technique or combination of techniques, for the authentication of foods labeled organic would be of immense value for consumers, government agencies and the agri-food industry.
In the present study, on request of LNV, scientific peer-reviewed literature on (a) methods for the authentication of eggs labeled organic, and (b) isotope analysis and the reasoning for its value for organic produce authentication was reviewed and discussed. Furthermore, the present scientific state-of-the-art was discussed with Prof. Förstel and Dr. Boner of Agroisolab which provides isotope analysis for this type of authentication as an analytical service in Germany. Furthermore, other EU scientific experts from Germany, Ireland and the UK were consulted on the topic. Finally, conclusions are drawn from both the literature review and the network information, and recommendations are provided regarding the required scientific approach for evaluating methods for organic egg authentication in the Netherlands.

2 Comparison of organic and conventional produce

2.1 Studies in general

Most of the studies reported in literature evaluate physicochemical properties of the products in terms of (desirable and undesirable) ingredients, pesticide residues, contaminants and sensory analyses.

There are three ways of undertaking studies to compare conventionally and organically produced foods: (a) market-orientated supply studies, (b) surveys at farms, and (c) cultivation tests. Market-orientated supply studies tend to monitor the situation of the consumer. A large number of samples are taken from shops. The disadvantage of this method is that the origin of the samples cannot be identified. Surveys are conducted on products from selected farms with different forms of cultivation for which the production conditions are recorded. However, it is difficult to select the farms and fields in such a manner that they truly represent the cultivation forms which are to be compared. Cultivation tests are often viewed by scientists as the most accurate form of comparative studies. They can ascertain whether foods from different forms of production show differences in quality and also which kind of cultivation this is attributable. The remaining problem is, that the results of these tests only apply to the specific location and farming situations. Only a relatively small number of samples can be examined. It is, therefore, difficult to make general statements (Vetter et al., 1987).

In an extensive literature review (Woese et al., 1995) more than 150 comparative studies, which were published between 1926 and 1994, on the quality of foods from conventional and organic production or foods produced with the use of different fertilizer systems were summarized and evaluated. The study compiled findings spanning several decades. During this period organic farming methods hardly changed in respect of the use of fertilizers and pesticides, but 'conventional' farming changed considerably since it became increasingly oriented towards the sparing and optimum use of chemical agents. Hence, there was a great variety in 'conventional' farming produce. Despite difficulties in the compilation and generalization of results, the overview revealed some difference in quality between conventionally and organically produced foods. In some studies conventionally cultivated or minerally fertilized vegetables had a higher nitrate content than organically produced or fertilized vegetables. In respect of the pesticides, lower residue levels can be expected in both vegetables and fruit from organic production. Contamination with persistent, chlorinated hydrocarbons, which have been banned for some time now, do not constitute today for any of the product groups examined a suitable differentiation criterion. For vegetables (in particular leaf vegetables) a higher dry matter concentration was observed in the organically grown or fertilized products. With regard to all other desirable nutritional values, either no major differences were observed in physico-chemical analyses or contradictory findings did not permit any clear conclusions. The same applied to sensory tests. For cereals, there may be differences in terms of processing properties.

2.2 Studies on eggs

Few research studies were reported on components of eggs for discrimination of organic vs. conventionally produced eggs. Lambing (1992) reported a comparative study on genetically identical but differently kept hens. The eggs showed differences in the contents of protein, lecithin and total carotenoid contents. Schlatterer and Breithaupt (2006) evaluated commercial eggs which were divided in groups according to the in the EU used classification to rearing method: ecological, free range, barn, cage. The eggs were evaluated for their xantophylls compositions. Lutein and zeaxanthin were the predominant xanthophylls in egg yolks produced in accordance with ecological husbandry. Concentrations of the compounds in ecological eggs were significantly different from those determined in the eggs of the other classes. A third study reported in literature dealt with quality characteristics of eggs from different housing systems and included organic eggs (Hidalgo et al., 2008). The authors concluded that in their study, which included 41 physical and chemical parameters

on 28 eggs, organic eggs had the highest whipping capacity and foam consistency but the lowest albumen quality. Statistical multivariate data analysis did not allow correct classification of the eggs from the four housing systems examined.

Cherian et al. (2002) compared five types of specialty eggs, among which were organic eggs, for their fatty acid compositions and yolk/white/shell compositions. Present authors decided to look more in detail on the data of Cherian and co-workers by applying a chemometric (multivariate statistical) approach to the data. Principal Component Analysis allows easy visual interpretation of the samples and measured analytes (fatty acid, yolk fraction, e.g.). Principal Component Analysis was carried out on the white, yolk and shell weights of five types of eggs (data Cherian et al., 2002). A plot is shown for the yolk/white/shell composition in Fig. 1. When the sample codes are close to each other in the plot, this means that these samples have a similar composition. For example, the organic samples are in the plot positioned near SP4 (vegetarian diet cage-free brown eggs), so they are similar in yolk/white/shell composition. When an analyte is plotted close to a sample, this indicates that this analyte is present in a relatively high concentration in this particular sample compared to the other samples. Fig. 1 illustrates that the forementioned two types of egg are closely positioned near 'white', this implies that they consist of a relatively high proportion of egg white. As yolk is in the opposite direction along the horizontal axis, this suggests that the two types of eggs have relatively low proportions of yolk. In Fig. 2 analysis data on various fatty acids measured in the different types of eggs were added. To facilitate easier interpretation the plot of the samples (Fig. 2 upper plot) was separated from the plot of the analytes (Fig. 2 lower plot). Nevertheless, interpretation is done similarly to the previous example. In the upper plot, again organic eggs and SP4 eggs (vegetarian diet cage-free brown eggs) are in the same area, which indicates that they show a similar composition (also in terms of fatty acids). In the same location in the lower plot, i.e. left of the vertical axis, near the horizontal axis, the components palmitic acid, palmitoleic acid, total SFA (saturated fatty acids) and white are positioned (circle). These components are present in relatively high concentrations in the organic and the vegetarian diet cage-free brown eggs. Opposite these components along the horizontal axis are other components located, e.g. the % yolk, docasaheaenoic acid, etc. This implies that the two types of eggs mentioned present low concentrations of these components.
Multivariate statistical analysis can provide an interesting additional approach as it may show correlations between samples and between samples and analytes which may not be picked up when analytes are evaluated one-by-one. Subsequently, the approach can be used to select the most discriminating analytes for authentication on a sound statistical base. This is particularly important when a large range of analytes is evaluated and correlations become more important, and a one-by-one approach becomes too tedious. Principal Component Analysis has been presented here to visualise underlying relationships between samples and analytes. Many other relevant techniques, such as statistical classification techniques, are important in authentication studies.

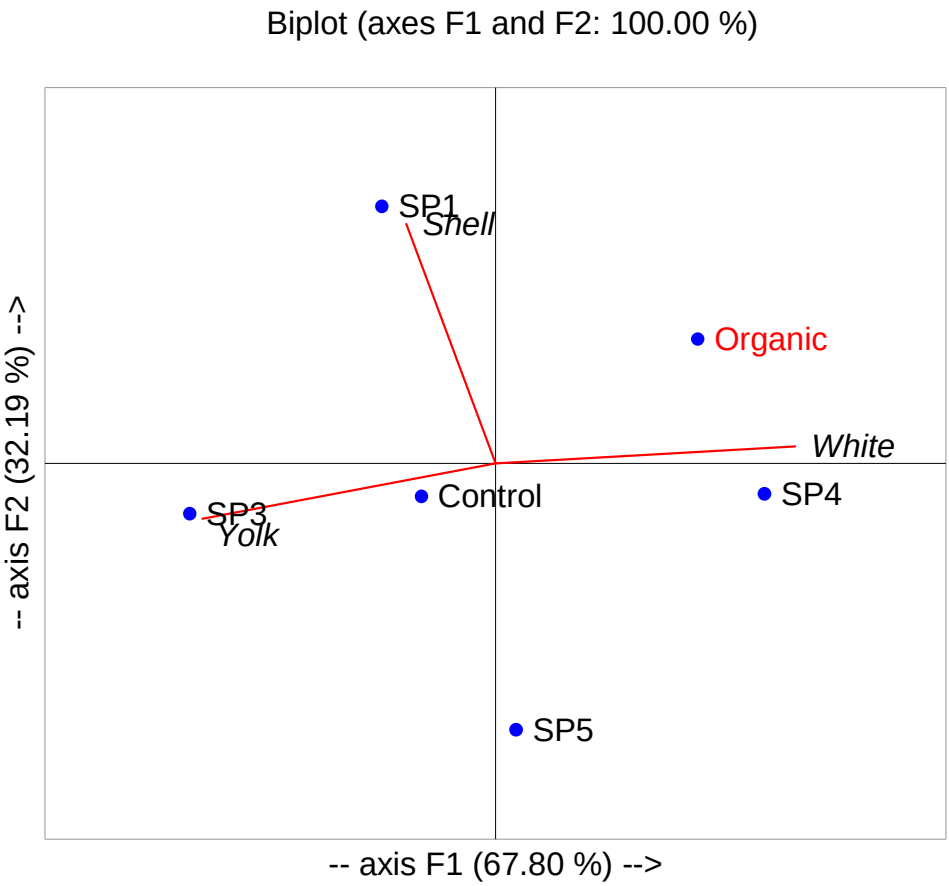

Fig. 1 Plot of the first two dimensions of Principal Component Analysis on the white/shell/yolk data of organic and other specialty eggs (SP1= animal fat free-high n-3; SP3= uncaged-non-medicated brown eggs; SP4= vegetarian diet cage-free brown eggs; SP5= cage-free naturally nested no steroid no stimulants brown eggs. Data from Cherian et al. (2002) used for Principal Component Analysis after normalization to control group.

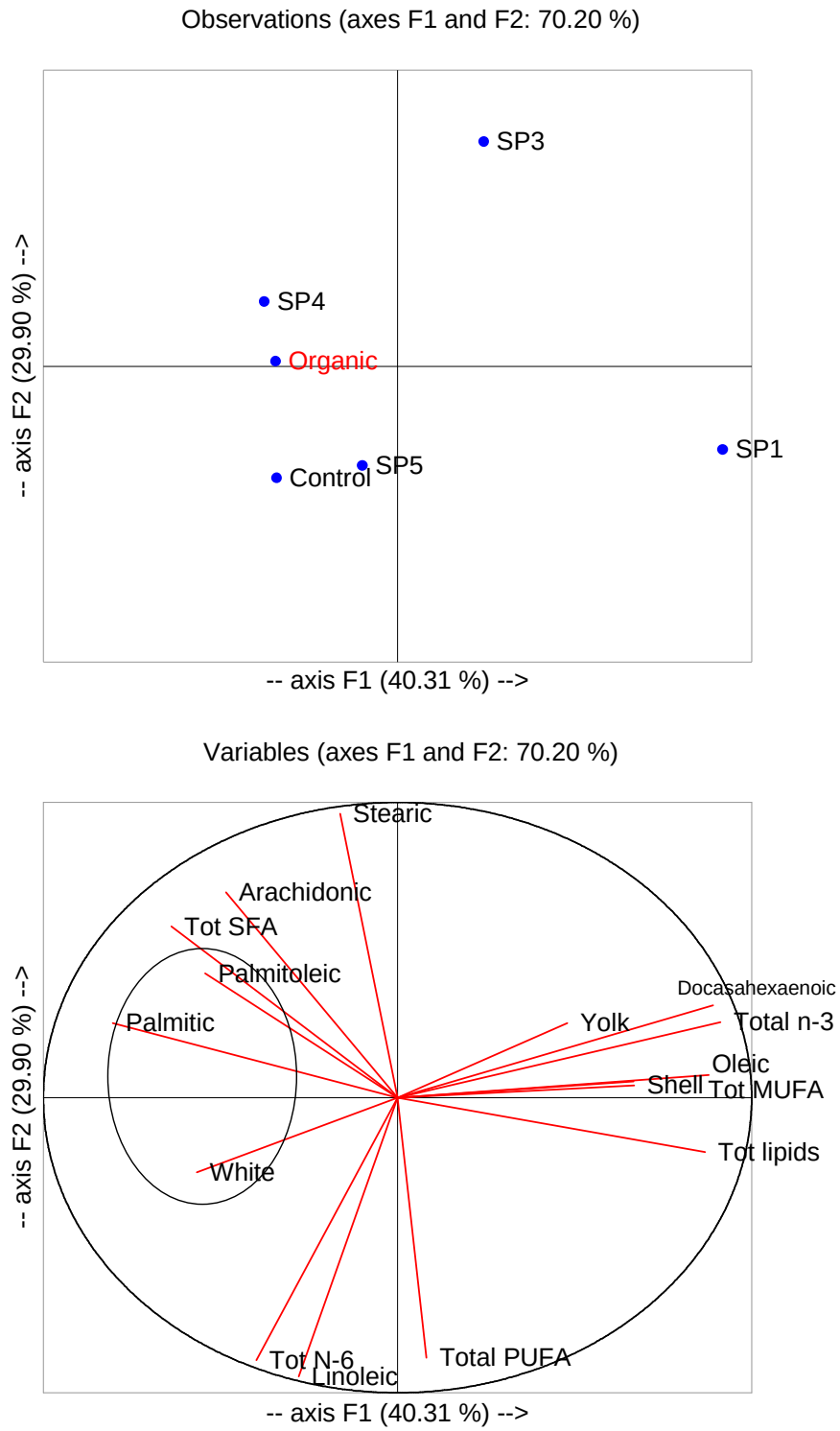

Fig. 2 Plot of the first two dimensions of Principal Component Analysis on the white/shell/yolk data and fatty acid composition of organic and other specialty eggs (SP1= animal fat free-high n-3; SP3= uncaged-non-medicated brown eggs; SP4= vegetarian diet cage-free brown eggs; SP5= cage-free naturally nested no steroid no stimulants brown eggs. Data from Cherian et al. (2002) used for Principal Component Analysis after normalization to control group.

3 Isotope analysis in authentication testing

A brief introduction to isotopes and their analysis is given below, followed by its use in authentication which is described in greater detail in the subsequent sections.

3.1 Introduction to isotopes

A feed or food molecule is composed of different atoms (e.g. nitrogen (N), carbon (C), hydrogen (H)). In turn an atom comprises a kernel (nucleus) and an outer shell of electrons. The nucleus consists of protons and neutrons. The structure of an atom is presented in Fig. 3. Isotopes of an element have 'kernels' (nuclei) with the same number of protons (same atomic number) but different numbers of neutrons. Therefore, isotopes have different mass numbers, which give the total number of nucleons – the number of protons plus neutrons. Different isotopes of the same element have similar chemical properties though.
In IUPAC nomenclature, isotopes are specified by the name of the particular element, implicitly giving the atomic number, followed by a hyphen and the mass number (e.g. carbon-12, uranium-238). In symbolic form, the number of nucleons is denoted as a superscripted prefix to the chemical symbol (e.g. ^{12}C, ^{238}U). Elements are composed of one or more naturally occurring isotopes, which are normally stable. Some elements have unstable (radioactive) isotopes, for example because their decay is so slow that a fraction still remains since they were created (e.g. uranium, potassium), or for other reasons (http://en.wikipedia.org/wiki/Isotope). Isotopic signatures of elements find their way from biosphere (unpolluted environment), geosphere (rocks), and anthroposphere (polluted environment) into plant, animal and human tissue. They, therefore potentially constitute markers or tracers for determination of the provenance and, implicitly, origin and authenticity of any kind of food- or feedstuff (Hölzl et al., 2004).

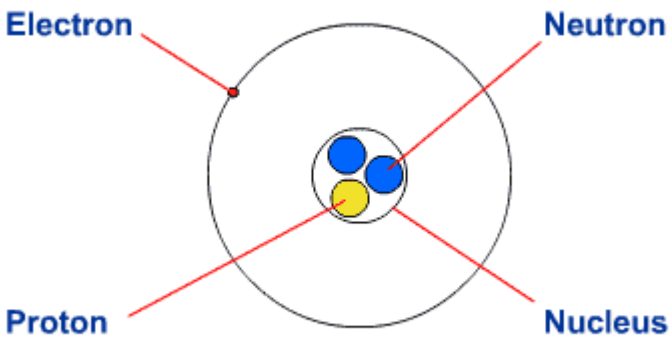

Fig. 3 Schematic overview of an atom presenting the protons and neutrons in the nucleus and the outer shell of electrons.

In isotope analysis the amounts of the various isotopes of a single element are measured. Minute variations in very small amounts of the heavier isotope (e.g. ^{15}N) are detected in the presence of large amounts of the lighter isotope (e.g. ^{14}N). Due to the small variations of the heavier isotope habitually

measured, the δ-notation in units of per mill (‰) has been adopted to report changes in isotopic abundance in comparison with an isotopic standard.

$\delta s = \{(Rsample - Rstandard)/Rstandard\} * 1000$ [‰]

where Rsample is the measured isotope ratio for the sample and Rstandard the measured isotope ratio for the standard.

3.2 Isotopes in general authentication testing

Amongst the analytical methodologies available, stable isotope analyses have made a substantial contribution to authenticity testing. These analyses are based on the premise that each species has its own unique pattern of naturally occurring stable isotopes. The variations in isotopic composition between different species or different sources of the same species are due to isotopic fractionation caused by a variety of factors, from biochemical processes and reactions to physical phenomena such as evaporation and precipitation (Lees, 1994). So, isotope analysis is the determination of isotopic signature, the relative abundances of isotopes of a given element in a particular sample. For biogenic substances in particular, significant variations of isotopes of carbon, nitrogen and oxygen (O) can occur (http://en.wikipedia.org/wiki/Isotope). Despite the large variety of organic objects and tissues, the fundamental principles of isotope variations in nature, and the analytical techniques, methods, and methodological approaches, are similar. In terms of material and method therefore, it makes no difference whether one analyzes bacteria or complex human tissues (Hölzl et al., 2004).

Stable isotope analysis of the light elements (or bioelements) hydrogen, carbon, nitrogen, oxygen and sulfur (S) has been applied for more than 20 years in food authentication control (Kelly et al., 2005; Rossmann, 2001). These methods are based on stable isotope ratio measurements of a product or of a specific component such as an ingredient or a target molecule of the product. The determination provides information on the botanical and geographical origins. Over the last 15 years, the EU and the Organisation International de la Vigne et du Vin (OIV) have adopted analyses of $^{2}H/^{1}H$, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in their regulations as official methods to assure the authenticity of wine (Calderone et al., 2003; EC regulation No. 2676/90, No. 822/97, No. 440/2003). In the same way the industrial production has implemented the use of routinely isotope ratio analyses also to assess the origin of flavors and aromas, honeys (Association of Official Analytical Communities methods), fruit juices (Comité de Européen de Normalisation methods) and sometimes of the residual sugar (Calderone et al., 2007). Presently, no official methods exist in the field of food control for muli-element stable isotope analysis of animal products (Camin et al., 2007).

3.3 Isotopes as indicator of agricultural production system

3.3.1 Introduction

In order to differentiate products by their agricultural production systems, scientists have been searching for specific markers. The relationship between livestock product-animal-feed/crop-soil-

fertilizer is an interesting but complicated chain to be considered in authentication (Fig. 4). The concentrations of these isotopes of animal tissues are useful isotopic markers of diet (Bahar et al., 2005). In organic production systems legislation exclude the use of synthetic fertilizers. It has been suggested that inputs of chemically synthesized nitrogen fertilizers, used in conventional agricultural regimes, may produce crops that can be differentiated from crops grown under organic regimes on the basis of their nitrogen isotope composition. The possible use of nitrogen isotopes to differentiate between crops grown with or without inputs of synthetic nitrogen is based on the hypothesis that the application of synthetic nitrogen fertilizers with N isotope ratios near 0‰ will result in plants grown in conventional regimes with lower N isotope ratios than those in organic regimes.

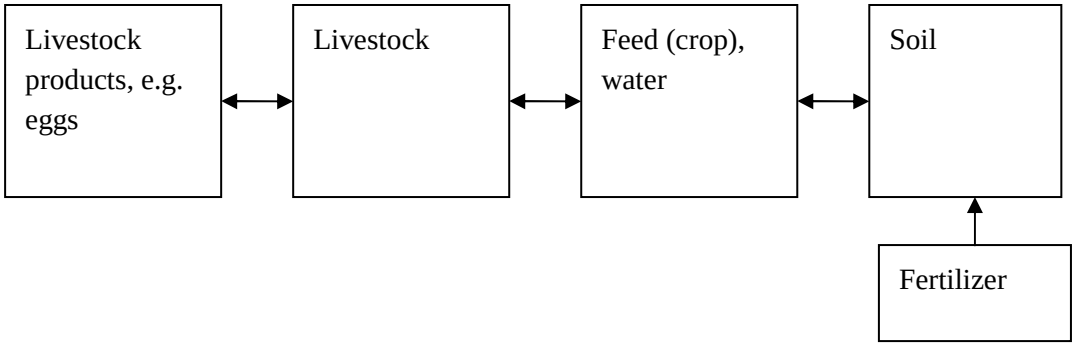

Fig. 4 Schematic overview of chain influencing the isotopic signatures of livestock products.

3.3.2 Crops

3.3.2.1 C isotopes

Plants can be classified into three groups depending on the photosynthetic path followed in sugar synthesis: C_3 or Calvin cycle (Calvin & Bassham, 1962), C_4 or Hatch-Slack cycle (Hatch & Slack, 1970) and Crassulacean Acid Metabolism (CAM; Whelan, Sackett & Benedict, 1973). C_3 species such as grape and beet contain sugars with a lower ^{13}C content than the sugar from C_4 plants like cane sugar and maize. This difference is maintained in the ^{13}C content of the fermentation products of sugars, ethanol and CO_2. CAM species are in-between C_3 and C_4 and so is the ^{13}C content of their metabolites (Calderone et al., 2007). The average $\delta^{13}C$ values obtained from C_3 plant tissues are typically 10-15‰ lower than the values of C_4 plants. If the type of feed used in organic or conventional productions differ in terms of C metabolism, livestock (products) may reflect the isotopic signature of the feed. Although the vast majority of plants (>300,000) belongs to the C_3 group with $\delta^{13}C$ values of <-24‰, differences in enzyme kinetics of biochemical pathways (mainly caused by environmental factors, such as climate and geographical location) result in subtle variations in the ^{13}C signature at natural abundance level of bioorganic compounds, such as fatty acids (Meier-Augustein, 2002).
For instance single seed vegetable oils of C_3 plant origin, such as groundnut, palm, rapeseed and sunflower oils could be distinguished by their $\delta^{13}C$ values (Kelly et al., 1997). The authors found that the $\delta^{13}C$ values for the authentic vegetable oil fatty acids fell within a narrow range of -27.6 to -32.1‰. Employing canonical discriminant analysis, ^{13}C data from sunflower oil could be separated from other oils, exploiting small yet significant differences in $\delta^{13}C$ values within the oil varieties. Thus, these subtle differences in ^{13}C data may allow in specific cases discrimination of produce.

3.3.2.2 N isotopes

Why nitrogen may work

Synthetic nitrogen fertilizers are not permitted in organic farming. Instead, soil fertility is maintained through the use of crop rotations that include green manures and also by the application of selected fertilizers which may be permitted where the need is recognized by an inspecting authority. It has been suggested that inputs of chemically synthesized nitrogen fertilizers, used in conventional agricultural regimes, may produce crops that can be differentiated on the basis of their nitrogen isotope composition from crops grown under organic regimes (Choi et al., 2003). Synthetic nitrogen fertilizers tend to have $\delta^{15}N$ values within a few per mil of zero (Shearer et al., 1974; Freyer & Aly, 1974) since their nitrogen is derived from atmospheric nitrogen ($\delta^{15}Natm = 0‰$) and there tends to be little fractionation during the production process. Animal manures with $\delta^{15}N$ values around +5‰ have been reported to produce nitrate with $\delta^{15}N$ values in the range of +10‰ to +22‰ (Kreitler, 1979). This enrichment is mainly due to the preferential volatilization of ^{14}N depleted ammonia from the manure. Nitrogen isotope values for other fertilizers which may be permitted under organic regimes are not all well documented but are likely to exhibit a much wider range of compositions than synthetic fertilizers due to their more diverse origins.

Previous studies have found that grain crops grown in soils to which synthetic nitrogen fertilizers were added had lower $\delta^{15}N$ values than plants grown in the same soil to which manure was added. Kohl et al. (1973) reported that the $\delta^{15}N$ value of a maize crop decreased with increasing applications of a synthetic nitrogen fertilizer. Bateman et al. (2005) showed that the fertilizer type is an important factor influencing the nitrogen isotope compositions of crops (carrots, tomatoes, lettuce). Under controlled conditions, applications of synthetic nitrogen fertilizer resulted in crops with lower $\delta^{15}N$ values when other factors that are also potentially important in determining crop $\delta^{15}N$ are not variable. Recently Rogers (2008) reported a study on discrimination of a limited number of organic and nonorganic crops purchased in a supermarket by N isotope analysis. Faster growing vegetables (time to harvest < 80 days) showed significantly higher $\delta^{15}N$ values. For slower growing vegetables organic and conventional counterparts showed smaller differences in $\delta^{15}N$ values.

Why nitrogen may not work

In order for $\delta^{15}N$ to be used more generally as an indicator of whether synthetic nitrogen fertilizers have been applied to crops, the tendency for applications of synthetic nitrogen fertilizer to result in crops with lower $\delta^{15}N$ values must predominate over many other factors which may also influence plant $\delta^{15}N$. Such factors include variability in the (i) field, temporal and spatial variations, (ii) the use of leguminous plants (clover) for enhancing the nitrogen fertility of soils, (iii) $\delta^{15}N$ of synthetic nitrogen fertilizers (used recently or in the past), (iv) the form of nitrogen in the applied synthetic fertilizer (NO_3^-/NH_4^+/urea), (v) variability in the $\delta^{15}N$ of nonsynthetic nitrogen fertilizers, (vi) timing of the fertilizer application, and (vii) the pedoclimatic conditions of the location.

<u>Leguminous plants</u>

Use of leguminous plants for fertilization affects the $\delta^{15}N$ of the soil considerably since minimal isotopic fractionation occurs during N_2 fixation by leguminous plants (Bedard-Haughn et al., 2003). Therefore, the $\delta^{15}N$ signature of leguminous plant material is usually close to that of atmospheric nitrogen (i.e. close to 0‰), like when synthetic fertilizers are used. Legume root nitrogen is likely to

represent the largest source of nitrogen to soils in rotational farming systems although legumes with a large harvest nitrogen index will make only a marginal contribution to the nitrogen status of a soil. However, even when only a marginal contribution is made to soil total nitrogen, the influence on the $\delta^{15}N$ of the available soil nitrogen pool may still be significant. Symbiotically fixed nitrogen is contributed to soil when plant remains of N_2-fixing legumes decay. This is probably because fertilizer nitrogen is easily leached from the soil while legume residues reside for longer. Determining the effect of the use of leguminous crops on the $\delta^{15}N$ of subsequent crops is important since some studies suggest that the fixation of atmospheric nitrogen in this way may cause a decrease in the $\delta^{15}N$ of subsequent crops similar to the effect seen when a synthetic fertilizer is applied.

Turnover

Apart from the use of leguminous plants, many of the factors presented in the first part of this section may influence the turnover of nitrogen in the soil through the processes of nitrification, denitrification, mineralization, volatilization, leaching, etc. Isotopic fractionations are associated with these nitrogen turnover processes and could potentially override any variations in plant $\delta^{15}N$ due to fertilizer influence. Overall, crops will usually have $\delta^{15}N$ values that lie between the $\delta^{15}N$ values of the applied fertilizer and the $\delta^{15}N$ value of the soil nitrogen. However, total soil $\delta^{15}N$ values have been reported showing that variability in soil may occur spatially (Broadbent et al., 1980) and vertically through a soil profile (Shearer et al, 1978). Changes in plant $\delta^{15}N$ over time during the growth period have been observed: during the early stages of growth, the $\delta^{15}N$ values of crops receiving synthetic nitrogen fertilizer were significantly lower than those not receiving the synthetic fertilizer. However, increases in the $\delta^{15}N$ of the crops was observed during the later stages of growth attributable to a decrease in the availability of the synthetic fertilizer over time due to uptake, losses, and immobilization, and an increasing contribution of natural soil nitrogen to plant total nitrogen. Therefore, both spatial and temporal changes affect the $\delta^{15}N$ of the soil, and subsequently the $\delta^{15}N$ of the crops.

Definition of organic produce

An additional complicating factor is the definition of organic produce. Even if plant $\delta^{15}N$ were a perfectly suitable marker of synthetic nitrogen fertilizer application, it is still the question whether this is a watertight criterion determining the organic production system. Legislation may allow (partly) other types of fertilization in organic productions, which may lead to false negatives. In the contrary, it is evenly important to emphasize that even if plant $\delta^{15}N$ can be used to distinguish satisfactorily between crops that have been grown with and without the application of synthetic nitrogen fertilizer, it does not follow automatically that the crop can be described as ‘organic’ since the crop may not have been grown in conditions which comply with all the requirements of organic cultivation, i.e. result in false positives (Bateman et al., 2005).

3.3.3 Livestock products

Plants and non-migratory animals feeding on them, have potentially region-specific isotopic compositions determined by climatic and environmental conditions (Boner & Förstel, 2004). However, the isotopic authentication of milk (Kornexl, Werner, Rossmann, & Schmidt, 1997), meat and other livestock products is more complex because livestock can consume foodstuffs of various origins and can also be raised on several different farms during their lifetime. Further, extensive research on wildlife species (Kelly, 2000) suggests that most biological and physiological factors influencing the isotopic compositions of animal tissues are still poorly understood.

3.3.3.1 C isotopes

When particular crops are used as feed separately in conventional and organic productions, the ^{13}C isotope may be an interesting marker. Examples of studies evaluating this hypothesis are presented below.

Meat

Boner and Förstel (2004) concluded from their study on authentication of conventional and organic beef that the $^{13}C/^{12}C$ ratio would be a reasonable tool to distinguish between conventional en organic farming although advanced statistics were not applied. The difference in $^{13}C/^{12}C$ ratio was explained by the fact that conventional farming used maize intensively in order to obtain a rapid growth and consistent meat production. Organic farming usually did not rely on feeding of maize, or only occasionally as a minor component.

In the study reported by Schmidt et al. (2005), the combined isotopic composition of C, N and S distinguished between conventional and organic Irish beef. The two groups differed significantly (MANOVA $F_{3,28} = 10.3$, $P<0.001$). Conventional Irish beef had a less negative and sometimes a somewhat more variable $\delta^{13}C$ value (-24.5‰±0.7‰) than organic beef (-26.0‰±0.2‰). These data suggest that more concentrated feedstuffs were fed in conventional than in organic production. The latter relies more on grass which has more negative $\delta^{13}C$ values than concentrates (Schmidt et al., 2002). Although the discrimination worked for the Irish beef, it should be kept in mind that its success when applied broader depends fully on the consistent feeding of particular feedingstuffs (concentrated vs. grass). This may not always be the case.

Milk

A study on the differentiation of organically and conventionally produced milk by stable isotopes and fatty acid analysis was reported by Molkentin and Giesemann (2007). Thirty-five samples from both production systems in Germany were collected and reflected seasonal variation. Stable carbon isotope analysis ($\delta^{13}C$) enabled complete distinction of both types of milk. For conventional milk fat $\delta^{13}C$ values were -26.6‰ or higher whereas for organic milk fat values were always lower, with a maximum of -28.0‰. The time resolved mean difference was 4.5±1.0‰. The $\delta^{13}C$ value of milk reflected the proportion of C_3-plants to C_4-plants in the feed. The substantial difference of ca. 5.2‰ between the values for the farm samples can be explained by the different percentage of maize in the feed. Whereas the basic feed on the conventional farm was made up of 60% maize silage during the whole year, the organic farm used only small amounts of maize silage during the pasture period. Smaller, but still significant, was the difference for the retail samples, with a maximum of -28‰ for organic milk and a minimum of -26.6‰ for conventional samples. Consequently, the origin of all the samples could be correctly assigned on the basis of their $\delta^{13}C$ values. It should however be kept in mind that a difference between $\delta^{13}C$ levels in organic and conventional milk can only be expected in regions where maize is grown and used for milk production. Milk from alpine highland would be identified as organic due to the special conditions irrespective of the type of farm management. A similar situation exists in Ireland, where all-year pasture feeding is widely used.

3.3.3.2 N isotopes studies

If production systems are defined by the use of type of fertilizer, livestock that is fed on crops grown with these fertilizers are expected to reflect the feed's isotopic signature. Remarks about the isotopic signature of a crop reflecting the fertilizers' signature are discussed in section 3.2.3.2.. A few studies

were reported on the use of $^{15}N/^{14}N$ ratios of livestock products for discrimination of production systems.

Meat/fish

Studies of Schmidt et al. (2005) showed higher $\delta^{15}N$ values (7.8‰±0.4‰) in conventional beef than in organic beef (6.6‰±0.4‰). It can be hypothesized that, in these predominantly pastoral production systems, this result reflects accumulative, plant-soil-system ^{15}N enrichment under conventional, more intensive management which is associated with external N inputs and hence higher N (preferentially ^{14}N) losses from such 'open' systems.

N isotope ratio analysis was reported to allow discrimination of organically farmed salmon from wild salmon, but not from conventionally farmed salmon based on $\delta^{15}N$ values (Molkentin et al., 2007). Stable isotope data and fatty acid data were combined and subjected to neural network modeling. This approach allowed complete correct assignment of the organically farmed, conventionally farmed, and wild salmon.

Milk

Regarding $\delta^{15}N$ measurements on organic and conventional milk, all year variation of $\delta^{15}N$ in retail and farm samples of both organic and conventional milk overlapped substantially (Molkentin and Giesemann, 2007). Seasonal resolution of the data did not much improve the distinction between the production systems. The higher level of $\delta^{15}N$ expected in organic milk samples, because of the effect of fertilizers, was not observed. The tendency of organic milk to have lower $\delta^{15}N$ values may indicate the cows consumed a greater amount of leguminous material, for example clover. Whereas legumes fix atmospheric N_2 with $\delta^{15}N=0$, non-legumes depend on soil nitrogen and thus, among other things, on the kind of fertilizer. Although artificial fertilizers used in conventional farming also depend on atmospheric $\delta^{15}N$, liquid manure from animal husbandry is also widely used in conventional farming. Thus $\delta^{15}N$ values clearly above 0 in conventional milk can be understood. Overall, $\delta^{15}N$ values alone did not enable the differentiation of organic from conventional milk. In addition certain fatty acids (e.g. C18:3ω3) were promising for separation of conventional and organic milk. Combination of properties by means of statistical modeling was considered to have further potential.

4 Opinions on the application of isotope analysis for organic produce authentication

4.1 Agroisolab

On July 1st (2008) Dr. Saskia van Ruth met with Prof. Dr. Hilmar Förstel and Dr. Markus Boner of Agroisolab (Jülich, Germany). This laboratory offers analytical services regarding the authentication of organic products. With Prof. Förstel and Dr. Boner the scientific progress in the area of isotope analysis for organic produce authentication was discussed, as well as the pros and cons of the application and their normal procedures in this type of testing. It should be mentioned that Prof. Förstel has been one of the pioneers in the field of isotope analysis for whine authentication, a procedure which is now generally applied in the EU.
Agroisolab consists of 10 scientists/technicians, and runs ca. 100-200 isotopic analyses per day on their six machines. The approach of Agroisolab is to develop databases on isotopic patterns on request for trade and authorities. When databases are developed, isotope ratios in offered products are compared with those in the database in a univariate way, i.e. isotope by isotope (target approach). Scientists indicate that they can only discriminate organic from conventional produce to some extend by the effect of fertilizer use. For them it is also obvious that there is a risk of false positives and that negatives (samples which do not conform) always need to be confirmed by information on the fertilization and other conditions in order to prevent false negatives. An example of a false positive are for instance products from a conventional farmers using manure, the samples of which will look like organic produce. False negatives may concern organic produce samples that look like conventional ones in terms of isotope ratios, but that the reason for this unusual pattern is due to e.g. the use of clover or particular local conditions. The Agroisolab scientists indicated that additional knowledge about all sorts of conditions (e.g. the field, fertilization, etc.) is important for interpretation of the data. This information needs a constant updating as productions are dynamic, new factors may appear over time or exceptional circumstances may apply which were not included in the original database. Regarding quality control, Agroisolab participates in isotope proficiency testing, sample material concerns wine, juice, honey, vanilla and alcohol. The scientists of Agroisolab stressed that although the use of the equipment has been simplified over the last decades, internal standards, calibration and sample preparation need careful attention.
The scientists of Agroisolab indicated that they have not published any studies on organic produce authentication as they are a private business and generally customers do not wish databases which were developed for them to be presented to the public. The scientists mentioned that there is a considerable reluctance in sharing isotopic data among groups within countries and between countries in the EU, but that the sharing would be of great value. Although the method is employed on a commercial base, it is a drawback that its principles have not been published in scientific literature and thus not been refereed by scientific peers.

Agroisolab approach
Agroisolab uses a two-step approach. Initially the measurement of the isotopes and comparison with the database is a form of screening. When samples do not match (see for criteria below) the original set for organic produce, additional information is requested in order to determine whether the unusual data obtained can be explained. The Agroisolab will usually require six samples from a single field

when crops are assessed for their production system. The resulting data will then be subjected to outlier tests in order to determine whether a normal (t) distribution exists. If not, 12 samples of the field are required. If there are still outliers, the customer is informed. We have to keep in mind that two situations may exist: (a) a non-normal distribution exists and/or (b) there may be outliers. Values may not be identically distributed because of the presence of outliers. Outliers are anomalous values in the data. They may be due to recording errors, which may be correctable, or they may be due to the sample not being entirely from the same population. Apparent outliers may also be due to the values being from the same but non-normal population. Furthermore, if the sample size is small (and 6 is very small from a statistical point of view) it may be difficult to detect assumption violations. Moreover, with small sample sizes, no-normality is difficult to detect even when it is present. It should be kept in mind that normality tests assume normality and are looking for sufficient evidence to reject the null hypothesis of a population (dataset) having a normal distribution. So, for very small sample sizes, there needs to be striking evidence of deviations from normality in order to reject the null hypothesis. In the meeting with Agroisolab no statistically hard procedures for cut-off points or confirmation were obvious, the approach may differ from case to case depending on the client's wish. The order of evaluating isotopes for either origin/production system conformity assessments is usually (1) H and O (most discriminating for region), S and N (geological information), and C (climate factor). Cut-off points in the screening are not based on hard statistical criteria, but have an empirical base. Regarding crops, Agroisolab considered N values below 0 extremely unlikely to originate from organic products based on general literature and own experience. In practice they use two cut-off points. N values between 1 and 3, and N values below 1. In both cases additional information is requested from the producer, with the latter group being more suspicious than the first. In the confirmation step Agroisolab decides on the base of additional information whether the N values are likely to originate from normal organic procedures (e.g. fertilization etc.) or whether it concerns an adulterated sample.

4.2 Other European scientists

In order to shed a light on the state-of-the-art of isotope ratio analysis application for verification of organic vs. conventional produce, four fellow top scientists in the field were contacted. In an email the scientists were asked the following queries.

'The Department of Agriculture and Food Quality of the Dutch government asked us to evaluate the possibility to determine the authenticity of organic livestock produce vs. conventional productions. The idea exists that the measurement of N isotopes may be used for this purpose. I am very interested in your opinion, do you think science has progressed sufficiently to use these measurements to determine the agricultural regime in soil and plants, and do you think that if these plants are consumed by animals the isotopic composition in tissues and products (e.g.) eggs will reflect this pattern consistently as well. The next step is, is evidence in science so strong that it could be used for legal actions?'

The following scientists were asked about their opinions:

- Dr Simon Kelly, Institute of Food Research, Norwich, UK.
- Dr Olaf Schmidt, University College Dublin, Dublin, Ireland.
- Dr. Andreas Rossmann, Isolab GmbH, Schweitenkirchen, Germany.

- Dr. Joachim Molkentin, Max Rubner - Institute (MRI), Federal Research Institute of Nutrition and Food, Kiel, Germany

With permission of the scientists, their response to the queries is detailed below.

(a) Dr Simon Kelly. Crop differentiation on the basis of N-isotope data is not definitive because of confounding factors such as clover rotation, root crops that have a low N requirement such as carrots, soil nitrogen cycling, time of harvest, precedent land use and so on… What is clear for crops, is that certain conventionally cultivated horticultural products (or perhaps more correctly, hydroponically cultivated products) possess a clear difference in N-isotopes because the soil dynamics are removed from the 'equation'. Investigating the question "organic or conventional?" with respect to animal products adds another layer of complexity compared to crops. Essentially one would try and reconstruct diet influences in the hope that the organic feeding practices give rise to a deviation from conventional feeding. With regard to beef the two attached papers (see section 3.3) suggest that purely pasture fed organic herds tend to possess lower $\delta^{13}C$ values at certain times of the year due to winter feeding practices. This is probably due to the feeding of supplements to conventional herds that contain non-photosynthetic plants parts that generally possess slightly enriched $\delta^{13}C$ values and/or the feeding of a proportion of C_4 maize or maize gluten in the diet. The milk study suggested that $\delta^{15}N$ and $\delta^{34}S$ were not useful for production assignment. If cattle are feeding on pasture then it is unlikely to have been amended with synthetic N with a low $\delta^{15}N$ value. Even if it has, organic herds might be grazing on pasture that contains clover which would give rise to low 'synthetic N like' $\delta^{15}N$ values. Furthermore, in times where organic feed is not available there is a caveat in the legislation allowing a proportion of conventional feed to be incorporated into an organic herd's diet.
Chickens in the UK are predominantly fed a wheat based diet as far as I know, but I do not know what the basis would be for differentiating organic chickens or eggs. From what I have seen of Schmidt's paper ^{15}N was not definitive for differentiating organic and conventional wheat due to large variations and overlap of $\delta^{15}N$ values. Furthermore, there will be a trophic shift due to the chicken's metabolism that will further complicate issues. Having said all of that a pilot-screen of some organic and conventional eggs may be worth undertaking, due to unanticipated effects.
In short, animal tissues will reflect feed isotopic composition given time for the tissues to reach equilibrium with the diet. Half-lives for cattle C_3 to C_4 diet switch are of the order of 3-months. This time will be significantly shorter for smaller animals/birds. However, one is reliant upon consistent differences in feeding practice due to organic legislation. There will always be the risk of false negatives when a conventional farmer chooses to use organic feeding regimes.
In conclusion, answers will not be definitive but may provide useful intelligence in a court case.
There is a significant amount of literature regarding organic authenticity and it would be timely to bring this together in a European network of excellence or project of some kind.

(b) Dr. Olaf Schmidt. The use of C + N isotope ratio measurements for distinguishing organic from conventional animal products still requires more R&D. They will probably provide one line of evidence that could be used together with others, but on their own they will only be useful in some circumstances. For example, extensive maize (a C_4 photosynthetic plant) feeding produces a certain $^{13}C/^{12}C$ ratio that is not possible if the cattle were supposed to be grazed only (all Western European pasture plants are C_3) or if organic farmers are not supposed to grow maize because of its negative effects on soil fertility. Nitrogen is more complex and the isotope ratio is not usually very specific, but comparative studies can be useful. N especially would not stand up on court at present, the N cycle in

soil-plant-animal systems is too complex. I haven't heard of studies on eggs either. Several papers have been published on vegetables recently, this can work for hydroponic systems.

(c) Dr. Andreas Rossmann. Success depends mainly on the product under investigation (it has been tried to express this with the publications, too). If you consider e.g. cereals or milk (milk products), or field grown products in general, it is very difficult to differentiate conventional and organic products using only stable isotope (nitrogen isotope) analysis. If you consider horticultural products, as tomato, paprika, potato, herbs and vegetables grown under horticultural culture practices in general, this works pretty well. Anyway it is very useful to have additional information regarding geographical provenance, cultivation systems and fertilization practices, and to check if this information and the isotopic data are well in agreement.

(d) Dr. Joachim Molkentin. Differentiation of conventional from organic milk was tried using N isotopes as well. The consideration was that differences in the manure or fertilizers used in the respective feed production system might result in a different N isotopic ratio in feed and milk. Provided that you have a typical isotopic signature in the feed, this will most probably also be reflected in the animal product (milk, meat, eggs ...). However, it was found that the identification of organic milk by N isotopes was not possible. There are some papers describing different $\delta^{15}N$ values in conventionally and organically grown plants. However, the products analyzed normally originate from rather controlled sites. If you investigate an unknown sample to prove its organic origin, the situation is far more complex. On the one hand you have organic manure in organic production and synthetic fertilizers in conventional production, each probably having distinguishable $\delta^{15}N$ values. On the other hand, liquid manure from animal husbandry is also widely used in conventional feed production. Further, $\delta^{15}N$ values in plants and soils are influenced by leguminosae, such as clover, which fixate atmospheric N (organic and conventional production). Thus, I question the reliable identification of organic food by only considering N isotopes.
A quite useful aspect is the accumulation of ^{15}N along the food chain. Thus, animal protein has a higher $\delta^{15}N$ than plant protein. Moreover, animals standing high within the food chain have a higher $\delta^{15}N$ than those at the beginning. There was a study about eggs, showing higher $\delta^{15}N$ values for free living hens than for battery hens, because the free living hens took up some animal protein from worms, snails or bugs in addition.
In conclusion, stable isotope analysis is a very promising technique for food authentication. However, if you consider the whole variation in food composition, it will not identify 100% of samples correctly. You can improve the robustness of such methods by using multi element IRMS or by combining IRMS data with other analytical methods. With respect to legal actions to be implemented it can be assumed, that in many cases no fixed limits can be applied. The decision has to be made individually and in case of doubt completed by further evidence.

4.3 Conclusions on scientists' opinions

All scientists agreed that there is a sound scientific base for using isotope analysis for discriminating between organic and conventional production systems. They also all agree that there are some serious confounding factors. These factors have to be taken into account if one wishes to set up a database for any product. Extremes should be included in the database, and information on the production factors

(geographical origin, feed, etc.) are essential for interpretation of the data. Agroisolab is applying isotopic analysis for organic produce conformity assessments as analytical service. They circumvent the complexity to some extend by avoiding a hard statistical approach and applying a more empirical approach for deciding on conformity or non-conformity of samples instead. Both from a legal and scientific perspective, sound statistical boundaries are to be preferred. Again, this can only be achieved if conditions are extensively included in the initial database for both organic and conventional produce and the database is updated regularly.

5 Conclusions and Recommendations

5.1 Conclusions

Literature and discussions with scientists show a sound scientific basis for the use of isotopic patterns, possibly in combination with fatty acid profiles for differentiation between organic and non-organic produce. However, several factors add complexity and need to be considered when evaluating for a particular application, e.g. discrimination of organic and conventional eggs. In the literature no scientific evidence was found for the use of isotope analysis on eggs for discrimination of production systems. Consequently, conclusions are based on the general knowledge on isotopic analysis for organic produce authentication.

In general

The isotope ratios in livestock are primarily determined by the animals' diet. External effects on carbon isotopes mainly result from the ingested proportion of C_3- to C_4-plants, as C_4-plants, such as maize or sugar cane, have a higher relative $\delta^{13}C$ content. This approach may be considered if the diet of animals in both production systems differ considerably. Crops grown with artificial fertilizers differ generally in $\delta^{15}N$ values from those grown under organic regimes in which manure is used. Livestock feeding on these crops will reflect (within boundaries) the crops' isotopic signature.

Remarks

Differentiation of organic vs. conventionally produced plants on the basis of N-isotope data only is not definitive because of confounding factors such as

- Clover rotation
- Root crops that have a low N requirement such as carrots
- Soil nitrogen cycling, temporal and spatial effects
- The N cycle in soil-plant-animal systems is fairly complex and not fully understood yet
- Time of harvest
- Precedent land use
- EU regulations (EU Council regulation 2092/91) demand the use of a variety of products for use in fertilization. $\delta^{15}N$ values may vary with the type of products used
- The risk of false negatives when conventional farmers choose to use manure as fertilizers.

Animal products (e.g. eggs) add another layer of complexity compared to crops due to

- Further enrichment of the heavier isotopes occurring during metabolism
- The fact that animal tissues will only reflect the feed isotopic composition if time is allowed for equilibration with the diet.
- EU regulations allow partly use of non-organic feeds (presently 10% of feed for chickens e.g.).
- The risk of false positives: organic produce has to fulfill more requirements than the application of particular fertilizers for feed.
- The risk of false negatives when conventional farmers choose to use organic feeding which will increase $\delta^{15}N$ values (e.g. animal protein: worms, snails, bugs) consumed also by conventional free living hens).

5.2 Recommendations

From an analytical chemistry perspective possibilities exist for verification of produce from organic and conventional production systems. This conclusion is based on scientific expertise in this area on particular crops and livestock produce. However, no scientific proof of the use of isotope analysis or other methods for egg authentication have been reported in the scientific literature yet. For each type of produce the suitability of analytical markers needs to be examined with application of the correct statistics. As there is no data on eggs readily available and methods on egg authentication using isotope analysis have not been scientifically peer-reviewed, for future work on the analytical authentication of eggs originating from conventional and organic production systems, a survey is recommended on products from selected farms with different forms of cultivation for which the production conditions are known. Conditions should be selected to cover as much variation originating from normal production conditions as possible. C isotopes, N isotopes and possibly fatty acid composition would need to be considered. Three parts of the eggs could be studies: shell (mainly C information), white (proteins: C and N information), yolk (lipids: mainly C and fatty acids). C may allow discrimination due to the difference in feed between organic and conventional productions, N may allow discrimination due to the fertilizers applied while growing crops for feed. Fatty acids has been shown useful in discrimination of organic and conventional livestock products such as milk and salmon, and may therefore be considered. Good documentation of information on conditions (geographics, livestock, feeding, fertilization used for crops/feed, sampling, etc.) is an essential part of the work. To allow for better interpretation in the future, additional analysis on chicken feed and water is recommended when compiling the database. For extraction of information from the data a chemometric approach (advanced statistics) is recommended.

Summarizing the approach:

- Formation of a group of experts (government representatives; branch representatives; scientists on authentication, on isotope analysis, on statistics, possibly other analytical chemistry experts)
- Experts' evaluation and selection of most promising analytical tools (for eggs, e.g. isotope analysis, fatty acid analysis)
- Experts' evaluation and selection of critical production conditions and other conditions in the chain which may influence the analytical results
- Experts: development of experimental design for database generation (how many samples, which conditions, when sampling, type of analysis, what sort of information is required, etc.)

- Collection of samples, documentation of conditions, and analysis of samples
- Collection of data and statistical evaluation
- Scientific peer-review and publication of scientific manuscript(s)
- Deliverable 1: Knowledge on the best analytical method for the authentication of specific organic produce, its limitations, its discriminatory power based on statistical criteria, and finally its usefulness in practice for control purposes
- Deliverable 2: Availability of a transparent database for future use

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