Food quality control and studies on human nutrition by mass spectrometric and nuclear magnetic resonance isotope ratio determination

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Lebensmittel-Qualitätskontrolle und Untersuchungen zur menschlichen Ernährung durch Isotopenverhältnis-Analyse mit Hilfe der Massen- und NMR-Spektrometrie

Zusammenfassung. Die natürlichen Häufigkeiten der stabilen Isotope von Bioelementen in biologischem Material unterliegen kleinen Variationen, die durch Isotopeneffekte bei physikalischen Prozessen und chemischem Reaktionen in den natürlichen Kreisläufen der entsprechenden Elemente bedingt sind. Daraus resultieren typische relative Isotopenhäufigkeiten (δ-Werte) von Nahrungsmitteln und deren Inhaltsstoffen, die Zuordnungen zu Ursprung und Verarbeitung sowie den Nachweis von Verfälschungen gestatten.

Nach einer Beschreibung der Aufbereitung entsprechender Proben für die massenspektrometrische Isotopenverhältnis-Analyse werden als Beispiele der Anwendung erläutert: Der Nachweis der Fälschung von Honig und Ahornsirup mit high fructose corn syrup, die Möglichkeiten zur Bestimmung von Zucker- und Wasserzusätzen bei Wein und Fruchtsäften, der Nachweis einer natürlichen Herkunft von Ethanol und Essigsäure und der Echtheit von Aromastoffen und Gewürzen.

Weiterhin wird die Bedeutung von „natürlich markierten" Nahrungsmitteln zur Erkennung von Nahrungsgrundlagen und als Tracer in der Ernährungswissenschaft dargelegt. Schließlich werden künftige Möglichkeiten einer Bestimmung der intermolekularen und intramolekularen Isotopenverteilung in Nährstoffen durch NMR-Analyse diskutiert.

Summary. The natural abundances of the stable isotopes of the main bioelements in biogenic material are submitted to small variations caused by isotope effects of physical processes and chemical reactions in the natural cycles of these elements. Resulting typical relative abundances (δ-values) of food and food ingredients permit assignments to their origin and treatment and allow the proof of the addition of adulterants.

After the explanation of the sample preparation for mass spectrometric isotope ratio measurements, examples are given for the proof of adulteration of honey and maple sirup by high fructose corn sirup, for the determination of sugar and water addition to wine and juices, for the recognition of the natural origin of ethanol and acetic acid, and for the investigation of the authenticity of flavours and spices.

The use of "naturally labelled" foodstuffs as indicators of human diet and tracers in nutrition research is demonstrated. Finally, future possibilities of the determination of the intermolecular and intramolecular isotope distribution by means of NMR are discussed.

Introduction

The aim of any food adulteration is the substitution of high quality or expensive products by less expensive substances in order to attain larger profits. Through the addition of beet sugar to grape juice one can obtain a wine of seemingly higher quality, and through the addition of sugar and water one can even produce more wine. Sometimes the added substances may be chemically identic to the genuine ones and possess the same nutrient and physiological value, however, our food laws and tax regulations stress on the genuineness of natural products, and often the commercial value of food is only determined by this genuineness and origin from a natural source.

The task of a corresponding food control is therefore, to assign chemically identic products, like sucrose from cane or beet, to their natural origin. The most valuable method for the solution of this task is the determination of the natural abundance of stable isotopes in the foodstuffs or their ingredients.

Natural abundances of stable isotopes and causes for their variations

The main bioelements are consisting of mixtures of stable isotopes, in which the light ones are by far dominant, while the heavy isotopes are occuring only in small abundances (Table 1). In general, the heavy isotopes are – in accordance with their mean natural abundance – statistically distributed over and within the molecules of an organic substance (restrictions see last chapter). Hence, any organic substance is a mixture of isotopomers. Due to the different number and arrangement of the neutrons within the isotope nuclei of a given element, these differ in mass, charge distribution and nuclear spin. This gives rise to differences in the forces which they exert to neighbour nuclei, and has for consequence differences in the binding forces and interactions within and between isotopomer molecules. Therefore these isotopomer molecules may have physical properties slightly distinct from each other, and may, because of different binding and activation energies, show different velocities in chemical reactions. The quotients of the cor-

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responding equilibrium or velocity constants are called thermodynamic or kinetic isotope effects.

Isotope effects can have for consequence shifts of the relative isotope abundances during transfers of isotopomers from one pool to another. This is used for example in multistep destillation or diffusion processes with NO or CO for the artificial enrichment of stable isotopes. The enriched simple compounds serve as starting material for the synthesis of labelled molecules needed in biochemistry or medicine for tracer investigations.

Table 1. Mean natural abundances and standards of the main heavy stable isotopes of the bioelements (after [17])

Element and main heavy isotope	Mean natural abundance [Atom-%]	Name[a]	Standard abundance [Atom-%]	Isotope ratio[b] R
Hydrogen (^{2}H)	0.0145	SMOW	0.0158	0.00015576
Carbon (^{13}C)	1.108	PDB	1.111	0.0112372
Nitrogen (^{15}N)	0.3663	atmosph.N_2	0.3663	0.0036765
Oxygen (^{18}O)	0.2039	SMOW	0.2000	0.0020052
Sulfur (^{34}S)	4.215	CDT	4.275	0.0450045

[a] *SMOW* Standard mean ocean water, *PDB* PeeDeeBee-belemnite (carbonate), *CDT* Canyon diablo Troilite (FeS)
[b] [heavy isotope]/[main isotope]

$$\delta\text{-value: } \frac{R_{sample} - R_{standard}}{R_{standard}} \cdot 1000\,[‰]$$

In general, the mean relative natural abundance of the stable isotopes of the bioelements is regarded as being constant, however, isotope effects implied in natural processes cause small but perceptible differences between the pools of biological material [15]. As the abundance variations are in the range of ppm they are expressed relative to international standards (δ-value scale, Table 1). The main reasons for the natural abundance variations are briefly discussed in the following (Fig. 1).

Water is the sole primary source of hydrogen and the main primary source of oxygen for the biosynthesis of organic molecules. Isotope effects of evaporation and condensation in the global water cycle give rise to a depletion of the heavy isotopes in vapour and clouds and a relative enrichment in precipitates. The isotope abundance of meteoric water is dependent on the distance from the sea and other circumstances of its occurrence [14]. Correspondingly, the evaporation of ground water in the leaves of plants is accompanied by an enrichment of the heavy isotopes, and finally the photosynthetic biomass production with leaf water as the starting material proceeds with isotope effects [13] (Fig. 2).

The primary carbon pools in nature are HCO_3^- in the hydrosphere and CO_2 in the atmosphere; a thermodynamic isotope effect provides a difference of the δ^{13}C-value between these two pools of 7‰. Two distinct primary photosynthetic carbon fixation reactions, the ribulose-bisphosphate-carboxylase reaction, leading to the three-carbon compound 3-phosphoroglycerate, and the phosphoenolpyruvate-carboxylase reaction, producing the four-carbon product oxalacetate, proceed with different kinetic isotope effects. These reactions have for consequence that the organic material of "C_3-plants" is more depleted in the heavy carbon isotope (δ^{13}C-values -32 to -25‰) while that of "C_4-plants" is "heavier" (δ^{13}C-values -15 to -9‰) [39]. A third group, the so-called CAM-plants (crassulacean acid metabolism) produces material with δ^{13}C-values be-

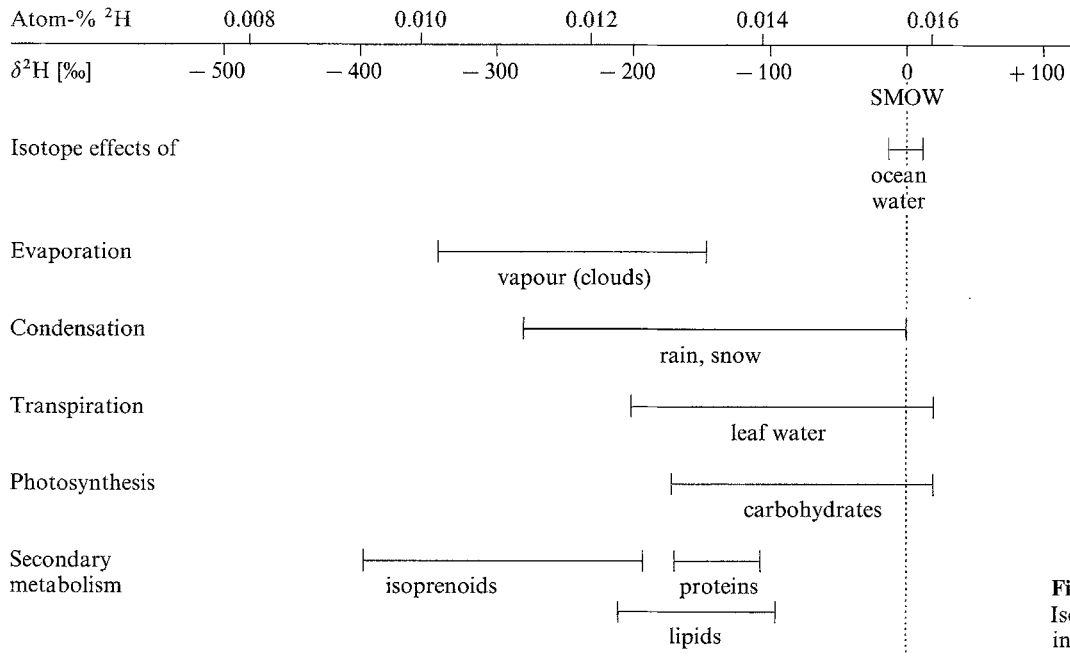

Fig. 1
Isotope effects and abundances in the hydrogen cycle

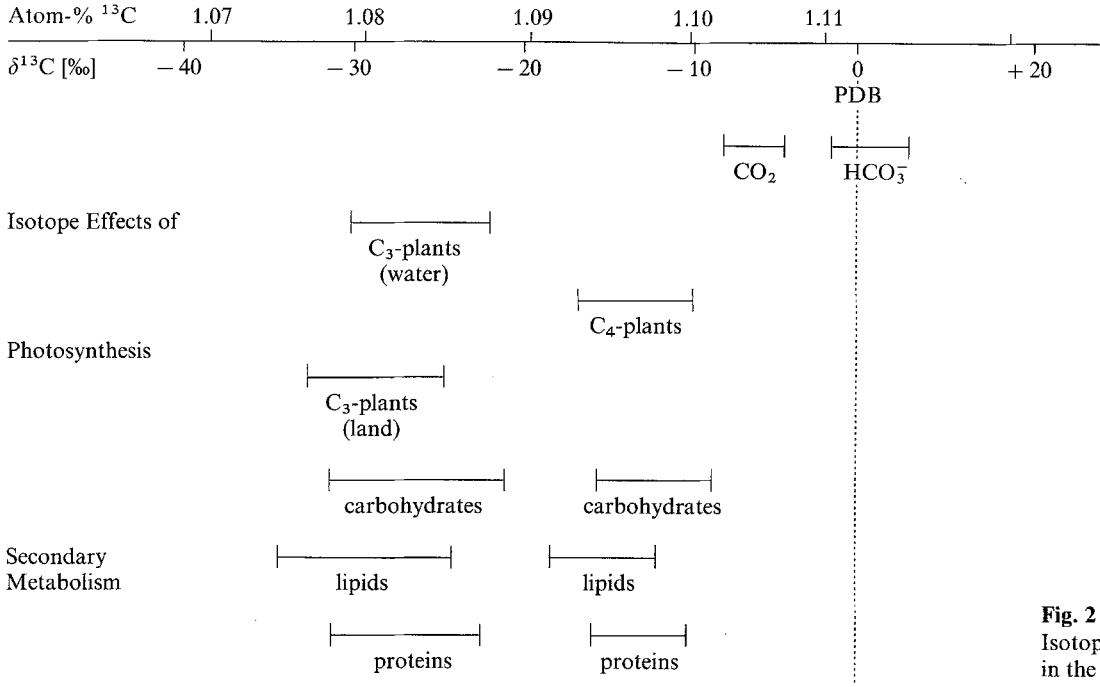

Fig. 2
Isotope effects and abundances in the carbon cycle

tween −30 to −10‰, because it is capable of using both CO_2-fixation mechanisms [24]. Economically important C_4-plants are corn, sugar cane, sorghum and millet, while most other cereals, potatoes, sugar beets and vegetables belong to the C_3-plant group. The CAM-plants do not have a comparable economic importance. In any of these plant groups isotope effects in the secondary metabolism cause further isotope ratio shifts, among which the isotopic depletion of lipids relative to that of carbohydrates by about −6‰ is the most important. Fossil organic material (petrol) shows $\delta^{13}C$-values between −45 to −20‰.

Atmosphere N_2 is the main nitrogen pool and simultaneously the standard of the $\delta^{15}N$-scale. The assimilation (nitrogenase reaction) occurs without isotope effect. Therefore the $\delta^{15}N$-value of legumes is close to that of N_2. However, isotope effects are implied to most of the other metabolic reactions of nitrogen, but as the intercorrelations between the different nitrogen pools and oxidation states are very complicated, and as nitrogen is transferred between any of these pools, an isotope scrambling is inevitable. Therefore, unequivocal assignments of isotope abundances to pools are not possible, however, a general observation is that the heavy nitrogen isotope becomes enriched in metabolic chains e. g. from plants to animals [43].

Sample preparation for the mass spectrometric isotope ratio determination

The natural abundance variations are in the ppm-range, and therefore their quantitative analysis requires very sophisticated and sensitive methods. The quantitative isotope ratio mass spectrometry (IRMS) is performed on simple gas molecules. Usually, mg-amounts of the organic compounds are combusted in vacuum lines or in sealed tubes in order to produce these gases [38]. The informations obtained by this method are therefore average values of the material, and as any organic compound contains the stable isotopes in their natural abundance, in some cases complicated purification procedures (which must be checked to proceed without isotope effects) must preceed the combustion process. A very promising recent development is the direct connection of a gas chromatograph to a combustion unit before a mass spectrometer inlet [2] (Fig. 3).

The ^{13}C-analysis is performed on CO_2 which is directly obtained by the combustion. The preparation of N_2, the gas for the ^{15}N-analysis of organic material, is possible according to the Dumas technique, to which some complications are connected. A recently developed improvement uses the isolation of N_2 by condensation on a molecular-sieve [16]. Very promising seems to be the direct combination of a nitrogen analyzer to an isotope ratio mass spectrometer [34]. More common in use is the digestion of biological material by the Kjeldahl-method in combination with a NaOBr-oxidation of the NH_3 formed [30].

Rather laborious methods are necessary for the preparation of CO_2 for the oxygen isotope analysis in organic material. The methods used are pyrolyses in an oxygen free medium followed by the conversion of the CO formed to CO_2. A very reliable version is the pyrolysis in welding-sealed Ni-tubes [6]; however, obviously this method does not work with nitrogen containing material. For the oxygen isotope analysis of water (about 1 ml is needed) an equilibration with CO_2 by (catalyzed) exchange is performed [13].

The deuterium analysis of organic material is made through combustion and subsequent reduction of the water to hydrogen by reaction with Zn or U [11, 38].

Mass spectrometers used for IRMS have double gas inlet systems, because the isotope ratio of the sample has always to be compared to that of a standard. In the analyzer the ion beams of adjacent molecular ions (e. g. $^{12}CO_2$ and $^{13}CO_2$) are collected on fixed Faraday cups, and the ion current ratio is used for the computing of the δ-values.

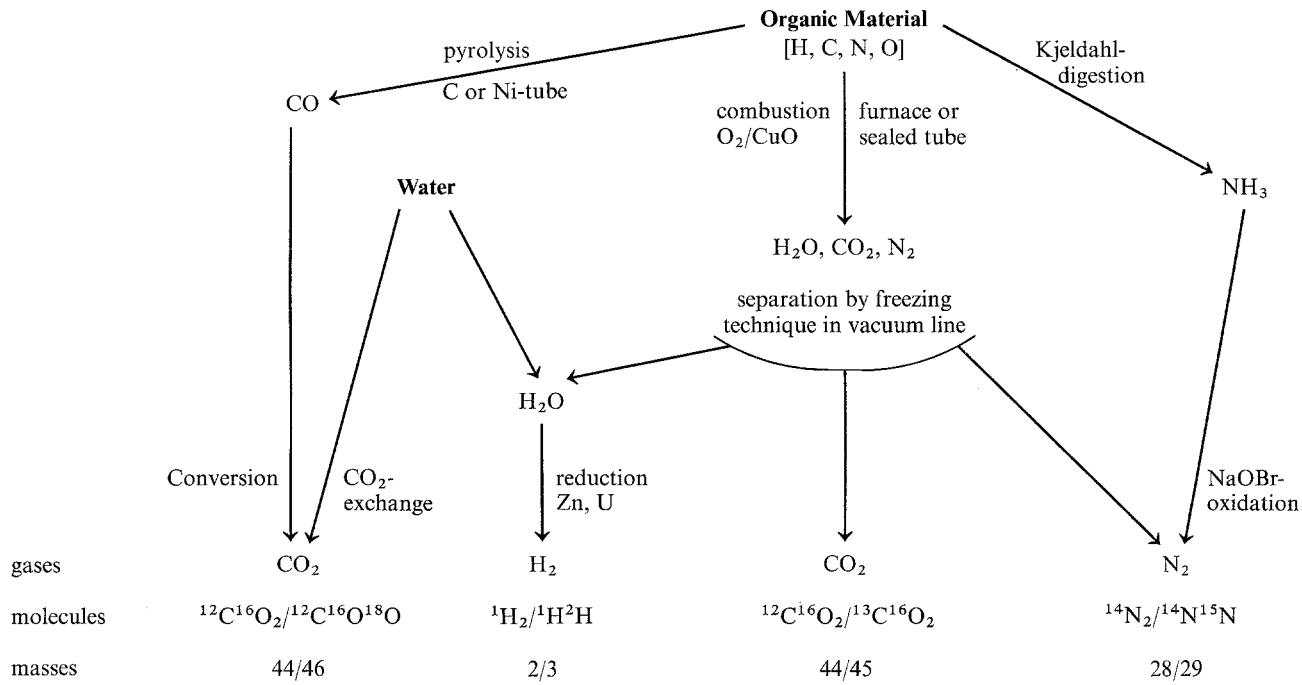

Fig. 3. Sample preparation for stable isotope analysis

Isotope abundance as indicator for the origin of food ingredients

It is not the intention of this paper, to repeat data collected and presented in recent reviews [18, 36, 44, 45]. The aim is more to complete them by some new results and to condense them under practice-oriented aspects. Any biological material is directly or indirectly originating from plants. An assignment is possible on the base and within the limits given by the typical isotope abundance variations explained before. From here derived realistic chances and possibilities for assignments can be summarized as follows: On the base of their $\delta^{13}C$-values, products originating directly or indirectly from the groups of C_3-, C_4- and CAM-plants can be distinguished from each other and sometimes from substances originating from fossil carbon. δ^2H-values and $\delta^{18}O$-values of organic material and water can be indicative within any of these plant groups, because they are not only caused by plant physiological but also climatological isotope discriminations. On the base of these prerequisites the following paragraphs discuss the identification of food adulterants.

Proof of C_4-plant sugars as adulterants

High Fructose Corn Syrup (HFCS) is prepared by hydrolysis of corn flour and enzymatic isomerization of the glucose obtained [18]; it contains about 50% glucose and 43% fructose. Because of its low prize it is used in the United States to sweeten limonades and other beverages. However, its addition to natural fruit juices, maple sirup or honey is strictly prohibited. Maple sirup and honey are relatively rare and expensive high quality products, and their adulteration with HFCS would be a very profitable business. Fortunately, the sugar maple and nearly any nectar producing plants (with the exception of some South African grasses and aloe) are C_3-plants. Series of analysis from different laboratories have provided standard values for the unchanged natural products and thus permitted to establish limits for the proof of their adulteration (Table 2).

In completion to former results [45] Krueger and Reesman [18] determined the δ-values of many pure honey samples from various countries. Upon their recommendation the AOAC (Association of Official Analytical Chemists) has accepted the $\delta^{13}C$-value determination as an official control method and has settled as limit for adulteration proof -21.5‰. In the case of maple sirup the standard deviation for natural products is even smaller than for honey, and the recommended limit for adulteration proof is -23.5‰. Morselli and Baggett [32] claim, that a 99% confidence limit for adulteration is attained when the $\delta^{13}C$-value is more positive than -23.6‰. Krueger and Reesman [18] also determined average $\delta^{13}C$-values of apple juice (-25.4 ± 1.2‰), orange juice (-25.7 ± 1.0‰) and grape juice (between -24 and -27‰ with an average of -25.5‰). In the case of apple juice the official AOAC method claims -20.2 as a limit for adulteration, and on this base many apple juices with the declaration "No sugar added" had been found to contain up to 50% cane sugar.

The alcoholic fermentation has for consequence only small $\delta^{13}C$-value shifts. The remaining sugar and the ethanol formed do not show more than 1‰ deviation relative to the original sugar [10, 35, 40]. This served as a base for the proof of cane sugar addition to New Zealand wines down to a limit of about 15% by isotope analysis of the alcohol [10]. A very careful study on the correlation between the $\delta^{13}C$-values of sugar and ethanol in sake (rice wine) [27] even permitted to distinguish the addition of cane or corn sugar before or after the fermentation.

Recognition of beet sugar addition to juices and wine

In Europe sucrose is exclusively originating from sugar beets. Sugar beets are C_3-plants, and hence adulterations of

Table 2
δ^{13}C-values [‰$_{PDB}$] of C_3-sugar products and C_4-adulterants. Results from different laboratories

Honey	Maple sirup	HFCS	Cane sugar	References
-25.5 ± 2.5	-24.0 ± 1.0	-10.0 ± 0.5	-11.5	[18]
-25.0 ± 2.5	-24.21 ± 0.27	-11.29	-11.85	[32, 44, 45]
-24.0	-24.0 ± 0.3	-10.8 ± 0.9	-11.4 ± 0.7	[23, 45]
> -21.5[a]	> -23.5[a]			[18, 32]

[a] Recommended limit for adulteration proof

juices or wine with beet sucrose (mean δ^{13}C-value -25.5 ± 1.0‰) will not alter their average ^{13}C-content. Ziegler et al. [46] showed, that the δ^2H-value of plant material can, independently on the δ^{13}C-value, vary within large limits. Accordingly in 1978 Bricout [7] found differences of the δ^2H-values for the sugars from the C_3-plants beet, cane and grape. A systematic study of Dunbar et al. [11], basing on the non-exchangeable (C-bound) hydrogen only, revealed that – at least in Germany – the δ^2H-value of sucrose from beets (-118.7 ± 4.9‰) is distinctly different from that of the sugars in grape juice (-69.8 ± 2.9‰) or wine (-67.0 ± 5.8‰). These findings permit the proof of an adulteration of wine, preferably when the sugar addition had been made after fermentation. The detection of an addition of beet sugar to juices will be generally possible on this base with an accuracy range of about 20%.

A very extensive systematic study of Misselhorn et al. [31] on the deuterium, carbon-13 and oxygen-18 abundances in alcohol from various sources demonstrates that only the δ^2H-value can be suitable to distinguish ethanol from grape and beet sugar. However, these results are still basing on the total average of the deuterium content. In the course of the alcoholic fermentation, only hydrogen atoms in position 2 of ethanol are not submitted to an exchange with water and only these would therefore be indicative for the carbon bound deuterium of the original sugar. On the base of these considerations Martin et al. [28, 29] developed a very sophisticated NMR-method for the identification of the origin of natural alcohols. In a proton decoupled deuterium NMR-spectrum of about 5 ml of ethanol the signals for the deuterium in position 2 relative to that of an internal standard can be used for the abundance determination of deuterium in this position. The method can be used for the proof of beet sugar addition to grape juice. Unpublished results of our laboratory indicate, that also the mass spectrometric D-determination on C-atom 2 of ethanol is suitable for the proof of beet sugar addition before fermentation.

Proof of water addition to beverages

The deuterium and oxygen-18 content of ground and tap water are typical for the occurrence and do not show large variations. On the other hand, plants enrich isotopes by transpiration, and the enriched water is preserved in their fruit. δ-values of fruit juices are therefore often typical for their origin, and concentration and re-dilution implies changes. The determination of δ^{18}O- and δ^2H-values of water in juices can thus prove their natural genuinesses [8]. Oxygen-18 determinations have also been used to prove the water addition to New Zealand wines [10]; however, relative large error limits are implied in the method. An extensive systematic work of Förstel and Hützen [14] on factors influencing the δ^{18}O-values of German wines showed that the proof of the addition of tap water to wine is possible, provided the climatological, hydrological and ecological conditions during the growth of the grapes are well known and reference samples from the same area are available. According to our own experience the local and temporal variation of the δ^{18}O-value of wine will not permit the unequivocal proof of a water addition of less than 20%.

Table 3. Carbon-13 content (δ^{13}C [‰$_{PDB}$] in the different C-atoms of acetic acid. Methods for degradation see references

Synthetic		Biogen		References
H_3C	COOH	H_3C	COOH	
-15.1	-44.4	-34.9	-16.5	[8]
-22.8	-39.0	-25.8	-21.9	[37]
-36.1	-35.3	-25.7	-22.5	
-25.0	-30.1			
-27.4	-25.2	-26.4	-20.0	[19, 20]
-21.4	$-5,9$	-28.4	-21.9	
-18.0	-31.9	-36.3	-24.6	[this work]

Determination of the natural origin of ethanol and acetic acid

The main problem with these substances is the proof of their natural origin in demarcation to possible petrol-basing products. ^{14}C-dating is very useful [21], but needs special equipment, is very laborious and not absolutely secure from fraudulence. A determination of the δ^{13}C-values would only be indicative to prove the addition of C_4-products, because the δ^{13}C-values of C_3-products and fossil carbon do overlap. As already pointed out the δ^2H-values are more significative [35], and in addition the carbon isotope distribution within the molecules would be of value. Natural acetic acid from vinegars is in general relatively enriched in carbon-13 in position 1, while this is not so with synthetic acetic acid (Table 3). Corresponding informations for ethanol are not available at present.

Investigation on the natural origin of flavours and spices

Natural flavours and spices have always been of high commercial value. Their main components are often relatively simple organic compounds, which can easily be synthezised or prepared from inexpensive other natural material.

The vanilla orchidee is a CAM-plant, natural vanillin shows δ^{13}C-values between -19.5 and -21.5‰. A partial synthesis of vanillin can be performed by the oxidative degradation of lignin, yielding a product of a δ-value close to -28‰ (Table 4) [8, 19, 22, 26]. Adulterations do not only

Table 4
Isotope content of flavours and spices [8, this work]

	δ^2H-value [‰$_{SMOW}$] of			δ^{13}C-value [‰$_{PDB}$] of	
	natural	synthetic		natural	synthetic
Linalool[a]	−257	−170	Vanillin[a]	−20.5	−27.0
	−269			−20.0	−27.8
	−244				−29.5
Citral[a]	−258	−174	Benzaldehyde	−26.8	−26.8
	−276			−28.3	−27.3
	−251			−28.9	−30.0
Menthol[a]	−394	−196		−29.5	
	−358	−242		[−25.6][b]	

[a] Values from Bricout (1982) [8]
[b] Amygdaline

Table 5
Average δ^{13}C-values [‰$_{PDB}$] of common food stuffs. Values in ( ) US-products. After [33, 42, 44]

δ^{13}C [‰]		δ^{13}C [‰]	
Corn flour	−12.3 (−10.7)	Sunflour oil	−29.0
American whisky	−12.7	Butter	−28.6
Cane sucrose	−12.2 (−10.3)	Corn germ oil	−14.3
Jamaica Rum	−13.0 (−10.1)	Porc lard	−27.9
Barley malt	−25.6	Porc meat[a]	−24.8 (−13.6)
Malt whisky	−24.2	Cod liver oil	−26.5
Beer	−25.5	Fish	−23.1 (−16.8)
Rice	−26.0	Cheese	−27.3 (−20.0)
Sake	−25.7	Eggs	−18.1 (−14.8)

[a] Protein carbon only

base on this semi-synthetic product but also use the addition of artifically ^{13}C-enriched vanillin. As these labelled products contain ^{13}C only in defined molecule sites, recent publications on the proof of vanillin adulteration [19, 22, 26] give highly sophisticated degradation methods for δ-value determinations in distinct molecule positions.

After Schmid et al. [36] natural benzaldehyde, the main component of bitter almond oil, should have δ^{13}C-values in the range of −22‰. This is rather unlikely, because almond trees belong to the C_3-plant group. Systematic research in our own laboratory showed, that there is no difference between natural and synthetic benzaldehyde, however, that a distinction between them is probably possible on the base of the δ^2H-values. Further results of deuterium determinations in flavours are indicated in Table 4. According to our experience a systematic research on δ^2H-values will provide many more possibilities in food assignment.

Importance of isotope abundances in food and food chains

The isotope abundance of the biomass of herbivores and carnivores is primarily determined by that of their diet. According to Table 5 most of our foodstuffs are from C_3-plants, but some of them are C_4-plant originating. The average of our diet counts for that of the body proteins, however, isotope effects of the metabolism are responsible for slight enrichments of heavy isotopes. "Lighter" molecules are faster metabolized while "heavier" serve for the biomass synthesis. Thus the δ^{13}C-value of the hair of man is by 3‰ more positive than the average value of the diet [33]. These correlations served for the identification of prehistoric diet on the base of collagen δ^{13}C- and δ^{15}N-values [5]. Similar results were obtained on the base of δ^2H-values [12].

A further practical application of "naturally labelled" food (C_4-products) is its use as tracers in so-called "breath tests": The kinetics of the metabolism of a ^{13}C-labelled compound are indicated by the ^{13}C-excess in the breath CO_2. Naturally labelled carbohydrates have been used in glucose loading tests with diabetics and obeses and in studies on the consumption of exogenous energy sources during physical exercise [41].

Future aspects: intermolecular and intramolecular isotope distributions

The biosynthesis of distinct constituents of natural compounds can occur through independent pathways, from where different parts of these molecules may differ in their isotopic compositions. In context with our studies on benzaldehyde we found a δ^{13}C-value of −25.65‰ for amygdalin, while the sugar part had −23.85‰, and the benzaldehyde −30.07‰. Similar results have been shown for the components of lipids [9]. Early in 1961 Abelson and Hoering [1] had already found that the δ^{13}C-value of the different amino acids of a protein hydrolysate differ, and that even discriminations between the distinct carbon atoms can occur. This has recently been confirmed by Macko and Estep [25], and a systematic study of Bengsch and Schulten [4] seems to reveal regularities about the non-statistical isotope distribution within molecules. A highly sophisticated development of NMR-technique will give the possibility for

a direct measurement of intramolecular isotope distributions [3], as already shown for deuterium in ethanol [28, 29].

This will not only give insights into the intercorrelation between biosynthetic pathways, but also become a very powerful means for the control of the biological origin of natural products. The same possibilities will be true on the base of the mass spectrometric isotope analysis of different compounds from the same foodstuff, a field for the already mentioned combination of GC with IRMS, and the simultaneous determination of different isotope ratios in the same compound.

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