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STABLE ISOTOPES DETERMINATIONS IN SOME FRUIT JUICES TO DETECT ADDED SUGAR

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Abstract

A method was developed to detect the adulteration of fruit juices. By means of stable isotope determinations, it is possible to detect exogenous sugar addition to natural juices. Since sugars photosynthesized by the Calvin cycle (orange, apple, beet) have $\delta^{13}C$ values much lower than their Hatch-Slack counterparts (cane sugar). Thus, it is possible to detect cane sugar addition. The δD in nitrated sugars enables the determination of beet sugar addition to natural juices. Commercially available Slovenian fruit juices were investigated. The results showed that none of the juices investigated was illegally adulterated.

INTRODUCTION

Stable isotopes have been extensively used for more than 15 years for the authentication and quality control of foodstuffs [1]. The most commonly employed stable isotopes in food analyses are ^{13}C, ^{2}H, ^{18}O, ^{14}N and ^{34}S, but the most important, regarding

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the analytical information, are ^{13}C, ^{2}H and ^{18}O, whereas ^{14}N and ^{34}S are used only occasionally. The abundance of a stable isotope in a compound is easily measured by means of Isotope Ratio Mass Spectrometry (IRMS).

Natural products exhibit small variations in their isotopic composition that are particularly amenable for provenance studies. The fluctuations are a result of isotopic fractionation that occurs during the synthesis of a given constitutional compound of a foodstuff. The causes for discrepancies in the isotopic composition are mostly different photosynthetic cycles used by plants. Moreover, milieu conditions, geographical position, rainfall and other factors also contribute to isotopic fractionation but to a lesser extent [2]. Synthetic products and compounds show different isotopic abundances, thus, enabling the differentiation among compounds of natural and those of artificial origin.

Natural variations in the stable isotope abundances in plant components provide a useful means to detect the adulteration of food. Carbon ^{13}C is especially useful in this regard. To incorporate CO_2 during photosynthesis, plants follow three biological cycles, resulting in carbohydrates with different isotopic compositions. The most plants use the C3 or Calvin cycle, whereas the fewer plant species, C4 or Hatch-Slack and CAM mode [3]. Most fruits and vegetables follow Calvin cycle, with the most well-known exceptions being corn and sugar cane which obey Hatch-Slack cycle. Due to the kinetic isotope effect, C4 plants and their metabolites are slightly enriched in ^{13}C compared to their C3 counterparts. By measuring the $^{13}C/^{12}C$ ratio, it is possible to distinguish between natural products produced by C3 plants and those synthesized by C4 plants.

Juices - both fruit and vegetable - are very popular commercial products, and there have been many attempts to adulterate them with low-cost beet, cane sugar or corn syrups. Adulterations of juices can be achieved in various ways. Exogenous sugar can be added to natural juice or to juice made of concentrate, and then diluted with water. It is also possible to dilute concentrate and sell such juice as genuine. It was expected that in Slovenia the first case of adulteration might be present.

Fortunately, with the advances in measuring stable isotope abundances, adulteration of juices has become increasingly risky, since the addition of exogenous sugar is easily traced [4 - 8]. It is possible to distinguish, e.g. between orange juice sugar (Calvin pathway) and cane sugar (Hatch-Slack pathway) by measuring the ^{13}C content of the sugars. Though, the compounds may have the same chemical composition, they differ in isotopic content due to different photosynthetic pathways.

The problems arise when the exogenous sugar added was photosynthesized using the same cycle as the fruit plant (e.g. beet sugar added to orange or apple juice). Such a case of adulteration is expected in Slovenia since beet is the main sugar source. Though, there are slight variations in ^{13}C content among C3 plants, these differences are not sufficient for the verification of adulteration from the point of view of analytical reliability. That is why other stable isotopes and approaches are utilized to establish a reliable method for all possible cases of juice adulteration. The deuterium content is a useful tracer which can differentiate between plants and their metabolites belonging to the same photosynthetic pathway.

The hydroxyl hydrogen atoms of sugars are easily exchanged with water and so the overall deuterium content of sugars no longer corresponds to that in the original fruit. Thus, it is necessary to first remove the readily exchangeable hydroxyl hydrogen atoms before performing deuterium isotopic analysis. The best solution is the nitration of isolated and lyophilized sugars. One can measure the deuterium content in juice water (plus the content of ^{18}O) to detect the dilution of a juice concentrate [9 - 11]. By measuring the deuterium content of sugars isolated from a juice, it is possible to detect the addition of exogenous sugar [12 - 14]. All these approaches use Isotope Ratio Mass Spectrometry (IRMS) to determine the stable isotope content. The deuterium content can also be established using Site-specific Natural Isotope Fractionation NMR (SNIF/NMR) [2, 15, 16]. In this case, sugars must be converted to ethanol upon which the deuterium content at a specific site is measured.

Besides fruit juices, stable isotopes can be used to detect the adulteration of honeys [17, 18] brandies and addition of artificial aromas to different foodstuffs [19, 20].

In the present study, a method was introduced to detect the suspected adulteration of commercially available juices in Slovenia. The research focused on ^{13}C and the deuterium content of sugars. The juices under investigation were products from domestic companies but for comparison imported products were also tested. Although only orange and apple juices were investigated, the method can be easily modified to detect adulteration of other fruit and vegetable juices and also wine [12, 21].

EXPERIMENTAL

Isolation of sugars

The method for isolating sugars from juices was taken from Koziet *et al.* [7]. The samples comprised of freshly squeezed oranges and lemons, 7 commercially available orange juices, an apple juice and a juice made of mixed fruits.

The insoluble constituents of cca. 50 ml of a juice were eliminated by centrifugation (30 min at 4000 rpmin). The supernatant was decanted. To precipitate soluble organic and amino acids, 2 g of calcium oxide p.a. (Merck, Germany) were added to the solution while stirring well and heating on a water bath at 90 ^{o}C for three minutes. In the case of natural lemon juice 4 g of CaO were added since lemon juice contains more organic acids than orange and apple juice. The sample was then centrifuged (10 min at 4000 rpmin) and the supernatant, which contained mainly sugars, was acidified to cca. pH = 5 using sulfuric acid (1 M). The solution was refrigerated at 4 ^{o}C overnight to allow the calcium sulfate to precipitate. The final solution contained mainly sugars (fructose, glucose and sucrose), with dissolved calcium sulfate and some colorants as minor constituents. Such a solution is directly amenable to the combustion unit preceding the instrumentation unit for ^{13}C isotopic analysis, whereas, the preparation of samples for deuterium isotopic analysis acquires additional preparation.

Solutions of sugars were lyophilized (66 hours) and a mixture of sugars was obtained.

Nitration of sugars

Two nitration methods were attempted, both leading to successful results [12]. The first method uses a mixture of fuming nitric acid, acetic anhydride and acetic acid as a nitration reagent, while the second reagent is a mixture of fuming nitric acid and concentrated sulfuric acid. Since the second method closely resembles the former, only one procedure is given.

25 ml of fuming nitric acid (Fluka) was cooled to 0 °C (on ice) and a mixture containing 15 ml of acetic anhydride and 15 ml of acetic acid (both Fluka) was slowly added. The temperature was kept below 5 °C, since at higher temperatures an extremely vigorous reaction can occur. To the nitration mixture approximately 3 g of sugars were added and allowed to react overnight. The mixture was poured into icy water where the nitrated sugars precipitated. The precipitate was thoroughly washed with water to remove all traces of acid. The nitrated sugars were dried under vacuum for 3 days to completely remove the water. The nitrated sugars formed a sticky yellowish residue and could be directly used for the deuterium isotopic determination.

Isotopic measurements of ^{13}C

The isotopic ratio of a sample is expressed as a relative value against the PDB (Pee Dee Belemnite from South Carolina, USA) standard. However, since the PDB no longer exists, a secondary standard NBS 22 (mineral oil, IAEA, Vienna) was used to calibrate the working standard (urea, 232 mg/ml).

All the measurements are expressed in relative terms against PDB. The assigned value of $^{13}C/^{12}C$ ratio for PDB is:

$$R = {}^{13}C/{}^{12}C = 0{,}0112372$$

For the other samples delta notation ($\delta^{13}C$) is used in isotopic measurements with the expression:

$$\delta^{13}C = (R_{sample} - R_{standard})/R_{standard} \cdot 1000 \qquad [‰]$$

The instrumentation comprises of a combustion unit coupled to a mass spectrometer modified to achieve a high resolution [22, 23, 24]. In our case, isotope-ratio mass spectrometer ANCA-NT (Europa Scientific, Great Britain) was used. ANCA is a specific line for use with raw samples in environmental and nutritional research enabling to measure $^{13}C/^{12}C$ ratio of solid, liquid and gaseous samples.

The combustion unit is maintained at 1000 ^{o}C and the reduction column at 600 ^{o}C. Carbon dioxide is formed in the combustion column and analyzed in the mass spectrometer which discriminates between $^{12}CO_2^+$ and $^{13}CO_2^+$. By dividing the areas under peaks for these two ions, the ratio $^{13}C/^{12}C$ is obtained which is used to calculate the $\delta^{13}C$ value of a sample.

Isotopic measurements of ^{2}H

The determination of the deuterium content is a rather laborious and time-consuming procedure since the samples have to be combusted in a separate unit (off-line). The water formed has to be converted to hydrogen before the δD value can be measured.

However, the reduction of water to hydrogen is a rather sensitive procedure in terms of analytical reproducibility. It can be achieved in three ways: using zinc metal [25], chromium metal [26] or equilibrating water with hydrogen gas of known isotopic composition [27, 28]. In our study, the method based on zinc was utilized.

A home-made combustion unit was used (Figure 1), assembled according to Dunbar *et al.* [12] with some modifications. Approximately 30 mg of vacuum-dried nitrated sugars is combusted in a flow of air, previously passing through silicagel and active charcoal. During combustion at cca. 600 ^{o}C water and other gases (nitrous oxides, carbon dioxides) are formed which flow through a reduction column packed with copper oxide CuO and maintained at 900 ^{o}C. Water is trapped in an ethanol-liquid nitrogen trap, while the other gases are pumped away. Afterwards, the water is transferred into a special vacuum-tight reaction vessel, containing zinc, and lyophilized.

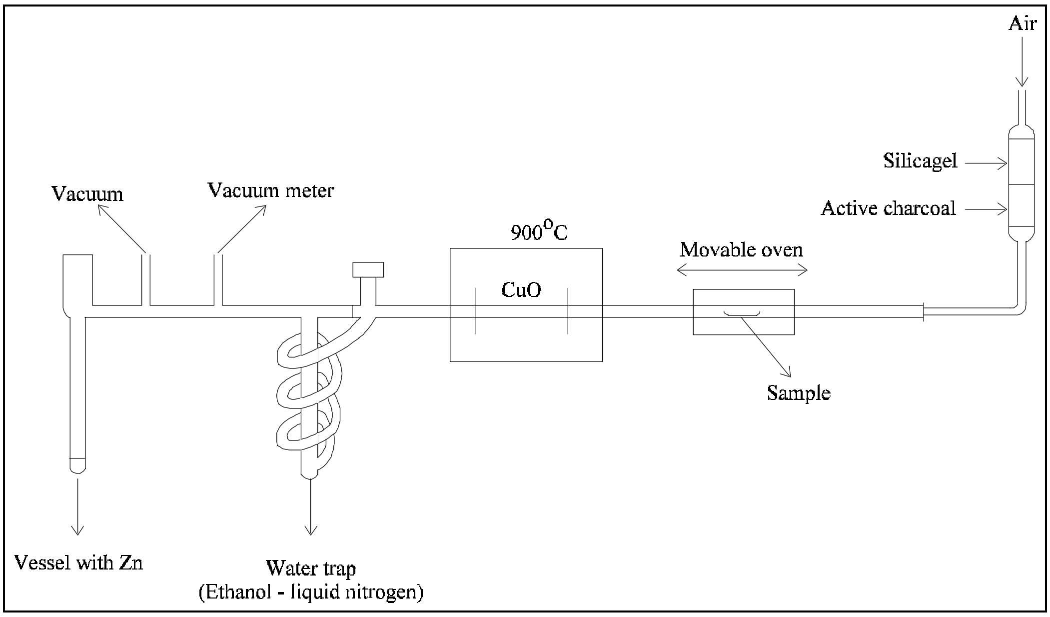

Figure 1: Schematic representation of the combustion unit

The reaction vessels, which are vacuum-tight closed, are then placed in an aluminum-block furnace at 490 °C and left to react for 4 hours. Thus, hydrogen is formed which is then measured with the mass spectrometer (MAT 250, Varian) against a suitable standard. As a primary standard in deuterium isotope determinations SMOW (Standard Mean Ocean Water) is used, nevertheless, in our study secondary standards were used: Water1 with $\delta D = -90‰$, Water2 with $\delta D = -55‰$ and polyethylene with $\delta D = -110‰$.

RESULTS AND DISCUSSION

At the beginning of the study, the isotopic measurements were performed on pure sugars and natural juices. Natural juices of orange and lemon were obtained by squeezing fresh fruits, thus, there were no doubts about the authenticity of the juices. The samples of sugars included beet sugar from Slovenia (Tovarna sladkorja Ormož) and two samples of cane sugar: one from Brazil and the other one from Australia.

Genuine juices were prepared according to the procedure described above, whereas, sugars were directly analyzed for $\delta^{13}C$ and nitrated for δD determination.

Table 1 shows the results for $\delta^{13}C$ determinations.

Table 1: $\delta^{13}C$ values for natural juices and some commercially available sugars

Sample	$\delta^{13}C$ (average)	No. of replicates	Standard deviation
Beet sugar (SLO)	-25.4	3	0.1
Cane sugar (BRA)	-11.3	3	0.2
Cane sugar (AUS)	-10.8	3	0.2
Orange juice	-22.93	5	0.07
Lemon juice	-24.1	5	0.2

The data in Table 1 clearly demonstrate that sugar cane is a C4 plant whereas oranges, lemons and beet are plants using C3 photosynthetic pathway. The standard deviation of the $\delta^{13}C$ determination is better than 0.2, or in relative terms, better than 1%. The difference between $\delta^{13}C$ for beet sugar and the same value for orange juice is more than one unit, however, this is not sufficient to detect beet sugar adulteration since climate and geographic position greatly affect the $\delta^{13}C$. The interval of the $\delta^{13}C$ values for orange juices is between -22.1 and -25.6‰ [29], thus, covering the interval of the $\delta^{13}C$ values for beet sugar. That is why the deuterium content of a sample must be taken into account to detect an illegal addition of beet sugar to a pure juice. The data of the deuterium content determinations are collected in Table 2.

Table 2: δD values of sugars and a natural orange juice

Sample	δD*
Nitrated beet sugar	-118
Nitrated cane sugar (BRA)	-78
Nitrated cane sugar (AUS)	-38
Natural orange juice	-48

*an average of two replicate measurements

Due to the laborious and time-consuming procedure of the deuterium content determination, only two replicate measurements were performed. The deuterium content determinations suffer from memory effects during combustion and due to incomplete combustion. Both hindrances can be partly eliminated by prolonged heating of the sample container before and during combustion. The data in Table 2 clearly demonstrate that δD values for beet sugar differ from orange juice, thus, giving a reliable basis for detecting the addition of exogenous beet sugar. The range of the δD values for nitrated beet sugar is -130.4 to -108.2 permil in Germany [12], from -178 to -108 permil in USA [9] and for North America from -160 to -109 permil according to [8]. The δD values for Slovenian beet sugar resemble to the German values which is logical considering a similar geographical position and the climate milieu. On the other hand, the δD values for oranges are less negative: from -50 to 0 permil [8] and from -43 to -13 permil [9]. An adulterated orange juice (to which beet sugar was added) should have a δD value between -100 and -60 permil. Moreover, the δD value itself can serve as a tracer for the estimation of the percentage of sugar added.

The next step involved an attempt to adulterate natural orange juice with beet and cane sugar. To aliquots of 10 ml of orange juice (representing approximately 1 g of sugars) 0, 0.05, 0.1, 0.5 and 1 g of beet or cane sugar was added, thus, obtaining adulterated juices with 0, 5, 9, 33 and 50% adulteration, respectively. Afterwards, the ^{13}C content was established. The results are plotted on Figure 2.

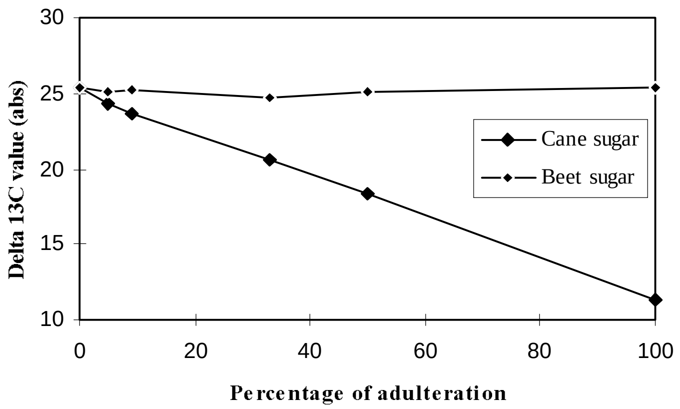

Figure 2: δ^{13}C values of orange juices adulterated with cane or beet sugar

The graphs show a decreasing $\delta^{13}C$ value (in absolute terms) with an increased percentage of adulteration with cane sugar, however, there is no trend observed in the case of beet sugar addition since the beet sugar and orange sugar are produced by C3 plants and their $\delta^{13}C$ values show similar patterns. The 'cane sugar plot' has an inherent analytical value because it enables an estimate of the percentage of cane sugar added to juices of the fruits photosynthesized by the Calvin pathway.

A list of the tested commercially available juices and the measured $\delta^{13}C$ and δD values are given in Tables 3 and 4, respectively.

Table 3: The list of juices analyzed

Number	Type of juice	Producer and mark	Specification
1	natural orange juice	lab-squeezed	100%, no sugar added
2	orange juice	FRUCTAL (SLO)	100%, no sugar added
3	orange nectar	FRUCTAL (SLO)	50%, sugar added
4	orange juice	DANA, Dan (SLO)	100%*****
5	orange juice	VITAL Mestinje (SLO)	100%, no sugar added
6	orange juice	FRUCTAL, Sunny day	100%, no sugar added
7	orange juice	Happy day (Austria)	100%, no sugar added
8	orange juice	Santal (Spain)	100%, no sugar added
9	apple juice	FRUCTAL (SLO)	100%, no sugar added
10	mixed fruits	Kasfruit (Spain)	100%, no sugar added

*****no declaration that no sugar is added, though, they declare it is an 100% orange juice

Table 4: Results of isotopic measurements of various juices

Number	Producer and mark	$\delta^{13}C$ [permil]*	δD[permil]**
1	lab-squeezed	-22.9	-48
2	FRUCTAL (orange, 100%)	-24.8	-53
3	FRUCTAL (orange nectar)	-17.8	-28
4	DANA, Dan	-24.9	-23
5	VITAL Mestinje, Frupi	-25.1	-25
6	FRUCTAL, Sunny day	-24.9	-37
7	Happy day (Austria)	-24.6	-27
8	Santal (Spain)	-24.7	-15
9	FRUCTAL (apple, 100%)	-25.9	-57
10	Kasfruit (Spain)	-23.3	-32

*an average of three replicate measurements
**an average of two replicate measurements

The results of isotopic measurements in the Table 4 lead to interesting conclusions. On the basis of ^{13}C determinations one can conclude that one of the juices contains cane sugar. Nevertheless, the producer declares that sugar was added to the juice. It is interesting to note, however, that cane sugar was added, though, the predominant sugar source in Slovenia is beet sugar. The deuterium content determinations agree with the values for natural juices. Thus, no exogenous beet sugar seems to have been added to the juices analyzed.

The isotopic ratio determinations of sugars in juices prove to be a reliable method for detection of adulteration through exogenous sugar addition. The main disadvantage of the method are the time consuming steps in deuterium determination: isolation, nitration of sugars and their combustion in a special unit. Thus, a more promising approach seems to be a novel method by which deuterium content is measured by means of SNIF/NMR [15, 16].

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Povzetek

Uvedli smo metodo, s katero je možno zaslediti potvarjanje sadnih sokov. S pomočjo merjenja izotopskih razmerij je mogoče določiti dodatek sladkorja naravnim sokovom. Sladkorji, ki nastanejo preko Calvinovega cikla (pomaranča, jabolka, sladkorna pesa), imajo $\delta^{13}C$ vrednosti mnogo nižje kot ustrezni sladkorji, ki nastanejo preko Hatch-Slackovega cikla (sladkorni trs). Na ta način je mogoče zaslediti dodatek sladkorja iz sladkornega trsa. Na drugi strani pa δD vrednosti nitriranih sladkorjev omogočajo določitev dodatka sladkorja iz sladkorne pese. Metodo smo uporabili na nekaterih komercialno dostopnih slovenskih sadnih sokovih. Ugotovili smo, da noben izmed preiskanih sokov ni vseboval ilegalno dodanega sladkorja.