IRMS METHODS FOR ASSESSING THE QUALITY AND ORIGIN OF HONEY USING $\delta^{13}C$ AND $\delta^{15}N$ ISOTOPIC FINGERPRINTS

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ABSTRACT

Multi-isotope ratio analysis proved in the last decade its potential in authentication of raw material in different foodstuff (eg. wine, spirits, vinegar, juice fruit, and honey). Currently, consumer demand for high quality food is something usual, the botanical origin of the raw material being the one of the main traced parameters. Due to its natural origin and health properties, honey has a special status among consumers. For this reason to certify the floral origin or to detect the substitution or extension of the main components of honey i.e. water and sugar became a necessity. The aim of this study was to characterise and classify honeys of different floral varieties according to their botanical and geographical origin based on the signature of stable isotopes (carbon 13 and nitrogen 15). Since honey contains only small quantities of nitrogen, a special emphasis was given to the determination of nitrogen isotope ratio $^{15}N/^{14}N$ (expressed in $\delta^{15}N$) in honey protein. To obtain measurable amounts of this element, the honey protein was precipitated. The results showed that the stable isotope ratio of the bio-elements carbon and nitrogen in honey and honey protein, respectively, can be applied to verify the origin of honey.

Keywords: *authentication, bio-elements, honey, stable isotopes.*

1. INTRODUCTION

The Isotope ratio mass spectrometry (IRMS) proved since the beginning to be a valuable tool capable of measuring isotope ratios in various matrixes, over a wide range of values, with a good precision and accuracy, and with many applications to the study of natural variations of stable isotopes in geological and biological systems. [Platzner I. T., 1997]. Considerable improvements and innovations achieved in the last decade have left their mark on the today's isotope ratio mass spectrometers and their associated sample preparation systems. For measuring $^{13}C/^{12}C$ and $^{15}N/^{14}N$ in organic and inorganic samples, the new IRMS instruments were designed with on-line sample preparation built- in the Elemental Analyzer (EA). Prior to the analysis of carbon and nitrogen, the samples undergo combustion in an O_2 atmosphere. The transition in the ion source of the reaction

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gases obtained from the sample combustion in the EA is performed by using a carrier gas (Helium with purity better than 99.9992%). For calibration, repeated analyses of working standard materials are done regularly, for both liquid and solid samples.

The first applications of measuring carbon and nitrogen stable isotope ratios were in biochemistry and pharmacology, emphasizing the metabolic pathways in human's body: $^{13}CO_2$ breath test for detection of metabolic diseases and ^{15}N isotopic fingerprint from amino acids and proteins [Matwiyoff N. A. and Walker T. E., 1977]. In 1990, Sugino et al. continue to use the ^{15}N-labelled urea as marker in studying chronically renal failure or dialysis. Later, $\delta^{15}N$ and $\delta^{13}C$ values were used to detect the use of organic manures versus synthetic fertilizers with the aim to discriminate between organic and conventional plants [Camin F. et al., 2011; Laursen K. H. et al., 2014].

An increased interest was felt worldwide in the last decade on the certification of food/beverages authenticity using as markers the multi-elemental composition and stable isotopes fingerprint and thus employing high performance technology and instrumentation like Isotope Ratio Mass Spectrometry (IRMS). To apply the isotopic investigation in food quality control several mandatory requirements are to be fulfilled: to understand the stable isotope fractionation in nature and to establish a relevant stable isotope database from authentic natural substances for a statistical evaluation [Rossman A., 2001].

One of the most appreciated natural products on the market is honey especially for its health-promoting benefits. That's why, when discussing about the authentication of honey two aspects are pursued, the raw material (eg. to detect the fraudulent adulteration with cheap High Fructose Corn Syrup (HFCS) and the declaration on the label concerning the botanical (distinguishing honey floral varieties) and geographical origin [Dinca O. et al., 2014]. In 1987, Croft L.R. discussed about the possibility to detect the adulteration of genuine honey with HFCS using carbon 13 as origin marker, knowing that the $\delta^{13}C$ values for HFCS are around -10‰ and for honey between -28‰ and -23 ‰.

The growth and ripening period for plants depend of the geographical origin: in regions with warmer climates plants have a much longer period of vegetation than in temperate climates, which influence the metabolism kinetics, respectively the δ13C and δ15N values, and indirectly the isotopic fingerprint of honey [Camin F. et al., 2008]. These parameters can be used as discriminating factors for the geographic and botanical origin of honey [Farquhar, Ehlenringer&Hibick, 1989].

The plants use different photosynthetic pathways who caused consistently the depletion of isotope ^{13}C in favour of ^{12}C and this is a reason why scientists divide the plants in three categories: C3 plants (the Calvin cycle, were the range value for $\delta^{13}C$ is from -29‰ to -23 ‰), C4 plants (the Hatch-Slack cycle, with $\delta^{13}C$ value around -10 ‰) and CAM plants (Crassulacean acid metabolism, were the range values for $\delta^{13}C$ is between -17‰ and -13 ‰) [Platzner I. T., 1997].

In crops, the $\delta^{15}N$ value is mainly determined by the size and isotopic composition of soil nitrogen as nitrate and ammonia [Wagner B. et al., 1995; Evans R. D., Ehleringer J. R., 1994; Ledgard S. A. F. et al., 1984] value that depends on

the geographic and climatic conditions [Heaton T. H. E., 1987] and agricultural practices such as fertilization. Distinction between different nitrogen sources is also reflected by the isotopic composition of nitrogen in plants. The metabolism of plants who fixing the nitrogen is based on both atmospheric nitrogen and from the soil (the N15 content in soil is enriched compared to the atmosphere). Therefore, the nitrogen-fixing plants have $\delta^{15}N$ values lower than nitrogen nonfixing plants grown on the same conditions [Delwiche C. C., Steyn P. L., 1970; Shearer G. and Kohl D. H., 1986]. This is a reason why between C3 and C4 plants there is no difference at $\delta^{15}N$ values. The difference is given by the climatic conditions and the plants potential for fixing nitrogen. For example, high concentrations of N15 are found in cotton compared with barley and soybeans. The soybeans has an lower content of N15 because it is a nitrogen fixing plant and therefore shows values close to those present in atmospheric nitrogen and the cotton plant shows a higher content due to the region where it was produced, with a warm climate. In the honey case, the nitrogen will be present exclusively as protein and as amino acids from which the protein is synthesized. Studies showed that the essential amino acids can vary in time [Young V. R., El-Khoury E., 1995].

The main aim of this paper is to present the IRMS methods who can assure the classification of honeys from different floral varieties according to their botanical and geographical origin using $\delta^{13}C$ and $\delta^{15}N$ isotopic fingerprint. The work is the sequel of a study started in 2013, in which we reported data on $\delta^{13}C$, $\delta^{18}O$, and $\delta^{2}H$ of honey measured by IRMS together with the stable isotopes (deuterium and carbon 13) in ethanol from fermented honeys. It was demonstrated that the use of δ13C value as single parameter in distinguishing honeys floral variety is not practicable, but a great characterization and separation can be provided by using SNIF-NMR as complementary technique, the (D/H)I values being specific to a given botanical and geographical origin. (Dinca O. et al., 2014).

2. MATERIALS AND METHODS

2.1. Sampling

Honey samples used for this study were collected from different geographical regions of Romania during the 2013 harvesting season, from both trusted beekeepers. Additionally, 1 commercial sample was purchased from the market, origin Turkey. Were employed honey samples of acacia (*Robinia pseudoacacia*), sunflower (*Helianthus annus*) and multifloral. The information about origin sources (floral variety and growing region) and harvest date were obtained by the declared data on the label. The samples were stored in the dark, in screw-cap jars at 20÷22 °C prior to analysis [Dinca O. et al., 2014].

2.2. Sample preparation

Preparing honey for IRMS analysis: if the samples honey show appreciable amounts of solid impurities, there were filtered on nylon stocking material because any insoluble material heavier than water will contaminate the protein precipitation [AOAC Official Method 998.12, 1999].

Protein isolation and purification from honey: for this step we used the repetitive washing procedure from AOAC: in a 50 ml flask was added 4 ml water to 10-12 g honey and mix well. In a small flask were mixed 2 ml of 10% aqueous solution $Na_2WO_4x2H_2O$ and 2 mL 0.335M H_2SO_4 and the mixture was immediately added to honey solution. In a water bath, at 80 °C, the flask was shacked until a precipitate was formed under a transparent supernatant. If there isn't precipitate or the supernatant is cloudy, 2 ml of 0.335M solution of H_2SO_4 is added successively, with repeated heating, until the precipitate is formed. The flask was brought up to 50 ml with water and then the sample was centrifuged for 5 min at 1500 rotations, thoroughly dispersing pellet each time. The last step was repeated by washing, stirring and centrifuging for 5 times, adding each time up to 50 ml of water in the flask containing the protein precipitate.

The protein was subjected to combustion by the same method used to determine $\delta^{13}C$ (‰) from honey [AOAC Official Method 998.12, 1999]. The difference between the isotopic ratios $|\delta^{13}C_{protein} - \delta^{13}C_{honey}|$ should not be more than 1 ‰.

$$A\ (adulteration)\ (\%) = 100 \times (\delta^{13}C_{protein} - \delta^{13}C_{honey})/[\ \delta^{13}C_{protein} - (-9.7)]$$

If the A values are higher or at least equal to 7 %, the honey is considered to be adulterated (official method 998.12 AOAC).

2.3. IRMS instrument and methods

The instrument used for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratio analysis was an elemental analyzer Flash EA 1112 HT coupled at a CF-IRMS Delta V Plus spectrometer (Thermo Scientific, Germany). The Dumas combustion into CO_2 and NO_2 was at 960 ^{0}C, using as carrier gas the Helium 6.0 (purity 99.9999 %). The reference gases (CO_2 and respectively NO_2) and the reaction gases generated in the combustion furnace were diluted subsequently with ultra-pure Helium in a ConFlo III interface located prior to IRMS. With an autosampler for liquids, the sample was injected directly into the furnace at high temperature, using a 10 µl micro syringe.

For quality control of the analysis, at the beginning of each sequence a reference material was analysed (for $^{13}C/^{12}C$ determination we used as reference a BCR 656 provided by Institute for Reference Material and Measurements Belgium, with a certified value of $\delta^{13}C_{VPDB} = -26.91 \pm 0.07$ ‰; for $^{15}N/^{14}N$ ratio analysis we used as reference a IA-R045 ammonium sulphate purchased from Iso_Analytical Laboratory Standard Cheshire UK, with a certified value of $\delta^{15}N_{air} = -4,71 \pm 0.07$ ‰) and also two working standards, at the beginning and the end of each sequence (Table 1). The difference between the measured values of the same working standard, at the beginning and at the end of the sequence, was less than the reported uncertainty (k = 2). To ensure the quality control of our measurements, systematically participations at international proficiency tests are done. The calibration of the instrument was performed with CO_2 4.5 (99.995 %) from a cylinder, which was previously calibrated with BCR 656. The analytical precision for honey and protein was 0.5 ‰.

The results are reported as deviations versus international standards, in δ (‰) notation

$$\delta^{*}X/X(‰) = \left(\frac{(^{*}X / X)_{sample}}{(^{*}X / X)_{s\tan dard}} - 1 \right) x1000$$

were X is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. The $^{13}C/^{12}C$ ratio was measured and reported as $\delta^{13}C$ relative to the Pee Dee Belemnite (PDB) standard, from South Carolina-USA, and $\delta^{15}N$ relative to the air [Nier A. O., 1950].

Table 1. Experimental conditions for nitrogen 15 and carbon 13 isotopic analyses using the elemental analyser Flash EA 1112HT coupled at CF-IRMS

	$\delta^{13}C_{VPDB}$ (‰) from honey and protein extract from honey	**$\delta^{15}N_{air}$ (‰) from protein extract from honey**
Sample quantity	1÷3 µl	4÷5 µl
Reactor temperature	950 ^{0}C	950 ^{0}C
GC column (Pora Plot Q) temperature	45 ^{0}C	45 ^{0}C
Carrier flow	60 ml/min	60 ml/min
Reference (He) flow	90 ml/min	90 ml/min
Oxygen flow	100 ml/min	50 ml/min
O_2 injection time	1 s	1 s
Reference materials	BCR660: $\delta^{13}C/^{12}C_{vsPDB}$ (‰) = -26.72±0.09	IA-R045 (Ammonium Sulphate): $\delta^{15}N/^{14}N_{vsAIR}$ (‰) = -4.71 ± 0.30

Working reference for $\delta^{13}C_{VPDB}$ (‰) measurements		**Working reference for $\delta^{15}N_{air}$ (‰)measurements**	
Ethanol from wine	-28.01 ± 0.28	Urea	-2.01 ± 0.40
Ethanol absolute	-24.50 ± 0.25	NH_4Cl	-6.21 ± 0.40
BCR 660	-26.72 ± 0.09	IA-R001 wheat flour	+2.55 ± 0.22

3. RESULTS AND DISCUSSION

Since the conventional analysis of honey based on the chemical composition, physical characteristics and the pollen analysis was not satisfactory to establish criteria for classification and origin (geographic and floral) discrimination purposes, we used as descriptors in the analyzed honey samples the stable isotope ratios of the bio-elements carbon and nitrogen.

For this work, a series of 28 Romanian honey samples were used. The Carbon 13 isotope composition of honey of different floral varieties is presented in Table 2, where we presented the $\delta^{13}C$ values of honey [according AOAC Official Method 998.12, 1999) and $\delta^{13}C$ of honey sample subjected to a fermentation process.

Table 2. Carbon 13 isotope composition of Romanian honey samples of different floral varieties

Honey	$\delta^{13}C_{VPDB}$ (‰) Honey					$\delta^{13}C_{VPDB}$ (‰) Ethanol extracted from fermented honey
	Mean	SD	Min	Max	Median	Mean
Acacia (n=13)	-23.14	0.50	-24.23	-22.34	-23.10	-24.41
Sunflower (n=6)	-25.38	0.89	-26.65	-24.03	-25.32	-27.27
Multifloral (n=9)	-24.90	0.98	-26.33	-23.29	-25.00	-26.22

Was observed a significant difference between $\delta^{13}C_{honey}$ and $\delta^{13}C_{ethanol}$ values (fig. 1) which can be explained by the isotopic fractionation associated with the production of CO_2 during the fermentation process. From this data, a good discrimination of acacia honey from sunflower honey can be achieved.

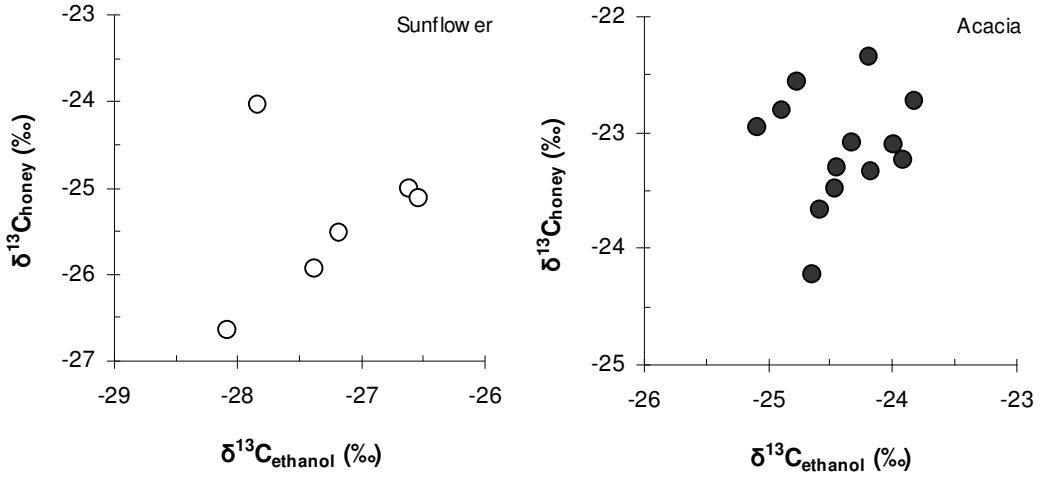

Figure 1. The differences between the various types of honey using the correlation $\delta^{13}C_{honey}$ with $\delta^{13}C_{ethanol}$

The influence that botanical origin has on the isotopic ratios of honeys can also be observed in Table 2, highlighting the large differences on $\delta^{13}C$ values between the acacia, sunflower and multifloral honey. The range of $\delta^{13}C$ values for the Romanian honeys are from -22.34 to -26.65 ‰, specific to the nectar-bearing plants (C3 plant group). If we evaluate the variation of $\delta^{13}C$ content in the honey samples of different varieties, Carbon 13 it is more depleted in the sunflower honey (average of -25.38 ‰) and more enriched in acacia honey (average of -23.14 ‰), behaviour related with the different vegetation period.

In Table 3 we highlighted the isotopic analysis results (carbon 13 and nitrogen 15) from honey, 2013 year product, from different geographical area.

Table 3. The isotopic fingerprint ($\delta^{13}C$ and $\delta^{15}N$) from honey, 2013 year product, from different geographical area

Honey samples	Provenance area	$\delta^{15}N_{air}$ (‰) protein[(1)]	$\delta^{13}C_{VPDB}$ (‰) protein[(1)]	$\delta^{13}C_{VPDB}$ (‰) honey[(1)]	Adulteration (%)
Sunflower	Dobrogea, Romania	+1.89 ± 0.40	-25.55 ± 0.77	-25.54 ± 0.77	0.06
Sunflower	Olt, Romania	+1.13 ± 0.40	-26.27 ± 0.80	-25.93 ± 0.78	1.57
Acacia	Transilvania, Romania	+4.14 ± 0.40	-24.39 ± 0.73	-24.08 ± 0.76	2.11
Acacia	Dobrogea, Romania	+5.11 ± 0.40	-25.19 ± 0.76	-24.34 ± 0.76	5.49
Acacia	Campulung Arges, Romania	+2.18 ± 0.40	-25.40 ± 0.76	-24.62 ± 0.74	4.97
Multifloral	Romania	+2.46 ± 0.40	-25.56 ± 0.73	-24.90 ± 0.72	4.16
Multifloral	Turkey	+4.56 ± 0.40	-25.01 ± 0.73	-24.15 ± 0.72	5.62

It was observed that increased values of $\delta^{15}N$ and $\delta^{13}C$ from the protein extracted from honey are registered in geographical region with warm climate (eg. Dobrogea – region from Romania South-east or dry climate (e.g. in country like Turkey), compared to region area with lower mean temperatures or higher air humidity in flourished period (eg. Campulung-Arges) (fig. 2).

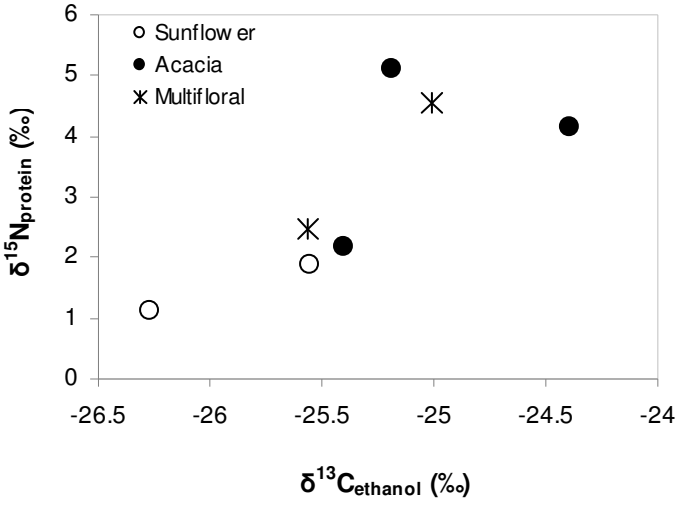

Figure 2. The differences between the various types of honey using the correlation $\delta^{15}N_{protein}$ with $\delta^{13}C_{protein}$

Considering the variation of $\delta^{15}N$ content in protein extract from different varieties of honey, the Nitrogen 15 it's depleted in the sunflower honey (average +2 ‰) and enriched in the acacia honey (average +4 ‰) (eg. in the same region, the difference between $\delta^{15}N$ values in sunflower and acacia honey is +3.22 ‰). The nitrogen isotopic composition of honey protein fraction reflects the soil conditions of the area where the honey has been collected by the bees, but it is also an

important nutrition parameter for the plants, thus $\delta^{15}N$ values are expect to reflect the botanical origin too.

Another explanation for the enrichment of nitrogen 15 may be due to the fact that in areas such as Dobrogea and Transylvania there are crops to which nitrogen fertilizer has been used or in the neighbourhood are crops with nitrogen-fixing plants.

To get a more detailed interpretation of $\delta^{15}N$, data related with nitrogen values of soil samples taken from the same locations as honeys should be achieved.

4. CONCLUSIONS

The isotope ratio mass spectrometers continue to proving the efficiency and accuracy in determine stable isotopes, with applications in determination of the geographical and botanical origin of the raw materials from different foodstuff. The stable isotope fingerprints of carbon 13 and nitrogen 15 could be used as potential origin markers together with other analytical methods, to assure the classification of honeys of different floral varieties according to their botanical and geographical origin.

The goal of the current study was mainly to present the methods of measuring stable isotopes honey and honey protein fraction with the aim to establish the potential origin markers and not to build a model for prediction of origin.

This research will continue with development of a database with potential markers for honey discrimination by combining various analytical techniques like SNIF-NMR, IRMS, GC-MS, HPLC, etc. to a better authentication of the product according to the statement of the label.

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