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RAPID COMMUNICATION

Detection of honey adulteration with beet sugar using stable isotope methodology

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A usual aspect of our work involves the analysis of honey samples for later sale, following current Spanish legislation. Such analyses essentially consist of studying pollen sediments, and sensory and physicochemical analyses. With this background, it seemed appropriate to investigate possible adulterations due to the addition of sugar (beet and cane). To do this, we selected 49 samples of honey obtained from 14 floral types and used them for pollinic and sensory analyses and to detect possible adulterations due to the addition of beet sugar products (treating the oligosaccharide fraction contained in the honey with the galactose oxidase reaction) or due to corn syrup addition (with normal $\delta^{13}C$ stable carbon isotope ratios). After classifying the samples according to the results of the pollen and sensory analyses, further assays were conducted. From the results it was concluded that 15% of the samples had been adulterated with beet sugar and 4% with cane sugar. The implementation of many analyses for each sample means that the results can be intercorrelated very well.

INTRODUCTION

There are some 200 000 hives in the province of Salamanca, representing 1/8th of all those exploited in Spain. They are located mainly in the mountainous regions of the area, with a special density in Valero de la Sierra where all the inhabitants are devoted to different aspects of the bee-keeping industry, most of them on a professional basis. Almost 2.5 million kg of honey and more than 900 000 kg of pollen are collected from these hives, the latter figure representing more than half of the pollen harvested in the whole of Spain.

To determine the geographic and botanical origins of honeys, qualitative, quantitative and sensory analyses are performed on all pollen samples, because such tests provide reliable information about the origins of the products.

Quantitative analyses are used to check the amounts of pollen present in honey samples (which vary with the extraction method employed) and are also able determine whether a filtering technique has been used to remove pollen, selectively or wholly, a practice not permitted by current Spanish legislation (BOE, 1983). Additionally, the characteristic elements of the honeydew are identified (remains of fungal hyphae, spores, algae). Qualitative analyses aim at identifying the pollen grains isolated by a method to be described below. Study of the corresponding pollen spectrum allows a botanical origin to be assigned to each sample and hence shows whether the labelling information is correct and whether the samples should be considered as 'monoflor' (coming from a single plant species) or 'multiflor' (from many different plants). For a honey to be classified as monoflor, the percentage of pollen from a given plant species is taken into account.

In the identification of honey-dew honeys, apart from study of their characteristic elements and above all the amounts of these, other parameters such as the amount of ashes and, especially, electrical conductivity are determined. Thus, by studying the elements characteristic of honey-dew honey and using conductivity measurements (above 9×10^{-4} S m cm^{-1}) it is possible to clearly differentiate nectar honeys from honey-dew honeys. In the present work, only the honeys from *Erica* spp had such a high conductivity, although in these cases the pollen sediment left no doubt as to their classification.

Concerning 'forest' honeys, Spanish legislation (BOE, 1983) considers these (and they can be labelled thus) as honeys that are natural or artificial mixtures (at the hive or in the bottling process) of nectar honey with honeydew honey. To differentiate 'forest' from other honeys,

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a criterion that states that honeys with an electrical conductivity below 9×10^{-4} Sm cm^{-1} cannot be considered as honey-dew honey is followed. According to this, honeys whose electrical conductivity is below this figure and that have abundant honey-dew elements should be classified as 'forest' honeys.

Sensory analyses are routinely carried out at our laboratory as a complement to pollen analysis to classify honey samples. Such tests are customarily used to check product quality and are especially valid in the case of monoflor honeys, which for the most part are well defined from the sensory point of view.

With this set of analytical possibilities (pollen and sensory analyses and electrical conductivity measurements), the samples in question are classified as 'monoflor', 'multiflor', 'honey-dew', or 'forest' honeys for later assessment with the stable isotopes technique or to detect possible adulteration by beet sugar. Using the stable isotopes technique, it is possible to detect certain adulterations made to honey such as the addition of cane sugar to the honey or to the bee's winter food or the addition of corn syrup. This is not the case, however, for beet sugar since beet is a C_3 plant while sugar cane is C_4. Cane sugar has a $\delta^{13}C$ value of about -11.5% while the $\delta^{13}C$ value of beet sugar is around -25.5%. Corn syrup and cane sugar have high ^{13}C contents and hence it is easy to recognize when they have been mixed with honey on account of their isotope contents. Doner and White (1977) in the USA and Zeigler *et al.* (1979) in Europe have identified honeys by their ^{13}C contents. In both regions, bees produce honey mainly from C_3 plants (with $\delta^{13}C$ contents around -25%). Only a few honeys from tropical regions have different $\delta^{13}C$ values.

In the present work, to detect the addition of beet sugar, a method based on the treatment of the oligosaccharide content of the honey (the same fraction as that used in the thin layer chromatography (TLC) test) with galactose oxidase was used White *et al.* (1986). We were interested first in characterizing the flora of the surrounding countryside with respect to the pollen contents of the honey samples through pollen and sensory analyses. A second aim was to check whether the honey samples had been adulterated by sugared syrups for later commercialization by measuring the $\delta^{13}C$ of the different samples and using the galactose oxidase assay.

MATERIAL

Honey samples

Forty-nine samples of honey were studied. Most of the samples were collected directly from the bee-keepers. Commercial samples (C) were purchased from food stores and those referenced 46–49 (I) were taken from the research hives of the University of Salamanca during 1992–1994.

Regarding the geographic origin of the samples, most of them (except the commercial samples) were from the province of Salamanca. Many of them were collected from the mountainous regions of the province and a few were taken from provinces limiting with Salamanca (Cáceres, Avila and Zamora).

METHODS

Pollen analysis

Qualitative

Acetolytic method. For qualitative analyses, the acetolytic technique of Erdtman (1960), adapted for honey samples by Louveaux *et al.* (1978) was used. A minimum of 1200 grains of pollen was identified, as recommended by Vergeron (1964). The method is based on treatment of the pollen sediment, after the honey samples have been centrifuged, with a mixture of sulfuric acid and acetic anhydride (1:9) to eliminate both the living contents of the pollen grains and any other remains that might hinder correct observation of the exin. Together with other characters, it is this layer of the pollen that permits identification of pollen types. Following this artificial fossilization and several washes, the microscope specimens were mounted and sealed for study. The pollinic spectra of some of these honeys have been described by Barrios *et al.* (1994) and Gonzalez *et al.* (1994).

Quantitative analysis

After a known dilution had been made up with 50 g of unacetolyzed honey, a THOMA cell-counting chamber was used to count the grains of pollen and honey-dew elements. The pollinic spectra of some of these honeys have been described by Barrios *et al.* (1994) and Gonzalez *et al.* (1994).

Electrical conductivity

After calibrating the conductimeter, the electrical conductivity of a honey solution at 20% dry matter was measured at 20°C (Boletin Oficial del Estado, BOE, 1986).

Sensory analysis

For this, the method of Gonnet (1991) was followed. The method consists of three successive steps: visual, olfactory and gustative, detecting the specific characteristics of colour, aroma and taste, which serve to differentiate among the different monoflor honeys and honey-dew honey.

Determination of $\delta^{13}C$

These values are determined by isotope ratio mass spectrometry after complete sample combustion to carbon

dioxide as prescribed by the official Association of Official Analytical Chemists (AOAC) method (978.17) (1990). The method specifies the combustion system of Craig (1953), which uses a tubular furnace with gas recirculation. More recently, Sofer (1980) has described the use of combustion in sealed quartz tubes. Here, sealed-tube combustion was used for all samples. The samples to be analyzed (less than 10 mg) were placed in quartz tubes, adding 2 g of copper oxide (previously purified in an electric oven for 1 h at 900°C). Tubes were prepared for combustion by evacuation on a vacuum line (0.04 mbar) and sealing with a torch. Combustion was performed at 850°C over 3 h, after which the tubes were allowed to cool to room temperature inside the oven. Following combustion, the tubes were cracked open to extract pure CO_2 on the vacuum line using acetone/CO_2 cryogenic traps to retain the water vapour (at −80°C) and another liquid nitrogen trap in which the CO_2 was condensed (at −196°C), removing the uncondensable gasses. The CO_2 generated was measured on mass isotope spectrometer (VG ISOGAS model SIRA Series II isotope ratio spectrometer connected to an IBM PS/2, 59 computer). The results were expressed in $\delta^{13}C$, ‰ against the reference values of the PDB (Pee Dee Belemnite) scale (The National Bureau of Standards supplies NBS-21 graphite with a $\delta_{PDB}{}^{13}C$ of 0–28.10).

Following this, mixtures of honey and cane sugar of known composition were prepared, adding increasing amounts of cane sugar ($\delta^{13}C = -11.4$‰) to 1 g samples of honey. The samples were prepared by heating in a water bath and were homogenized with a magnetic stirrer. The results obtained, shown in Fig. 1, show that in general the addition of 0.1 g of sugar enriches the isotope ratio by 1%, from which it may be inferred that for each type of honey amounts ranging between 0.1 and 0.3 g of sugar g^{-1} of honey would be necessary for the sample to be considered 'doubtful' from the isotopic point of view.

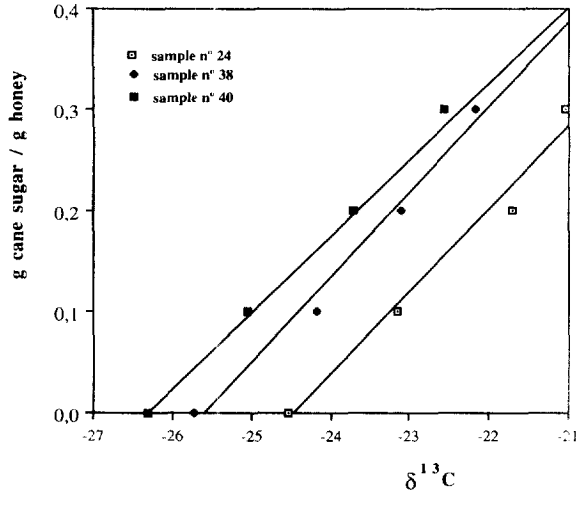

Fig. 1. Variation in $\delta^{13}C$ in honey due to the addition of cane sugar.

Detection of beet sugar adulteration

To detect the addition of beet sugar, a method based on treatment of the oligosaccharide fraction contained in the honey samples with galactose oxidase was used, White *et al.* (1986). Separation of monosaccharides, disaccharides and oligosaccharides was accomplished by adsorption chromatography in a column of activated carbon, Doner *et al.* (1979), prepared according to the official AOAC method 979.22 (1990). Galactose oxidase oxidizes the free hydroxyl group of carbon 6 of galactose and its derivatives and can therefore be used to measure galactose, mellibiose, raffinose and other oligo-

Table 1. $\delta^{13}C_{PDB}$ values found for the samples of honey

Sample n°	Floral type	$\delta^{13}C_{PDB}$
1	*Robinia pseudoacacia* L.(C)	−21.75
2	Forest	−22.33
3	Forest	−22.82
4	Forest(C)	−23.31
5	Honey dew(C)	−23.10
6	*Citrus* sp.(C)	−23.47
7	Forest	−23.72
8	*Eucaliptus* sp. (C)	−23.86
9	*Helianthus annuus* L	−23.89
10	Forest	−24.16
11	Honey dew	−24.22
12	Honey dew (C)	−24.23
13	Honey dew (C)	−24.28
14	Multifloral (C)	−24.35
15	Honey dew	−24.37
16	Honey dew	−24.46
17	*Lavandula latifolia* Medicus (C)	−24.49
18	Forest	−24.52
19	Multifloral (C)	−24.55
20	Forest	−24.58
21	Multifloral	−24.59
22	*Castanea sativa* Miller (C)	−24.64
23	*Castanea sativa* Miller	−24.64
24	Forest	−24.71
25	Honey dew	−24.73
26	Forest	−24.78
27	*Erica* sp. (C)	−24.86
28	Forest	−24.86
29	Multifloral (C)	−24.97
30	Honey dew	−24.97
31	Forest	−25.00
32	Forest (I)	−25.00
33	*Eucaliptus* sp.	−25.14
34	*Rosmarinus officinalis* (C)	−25.47
35	Honey dew	−25.57
36	Multifloral	−25.60
37	Forest	−25.63
38	Forest	−25.73
39	*Arbutus unedo* L. (C)	−25.74
40	Forest	−26.30
41	*Echium* sp.	−26.42
42	*Echium* sp.	−27.51
43	*Lavandula stoechas* L.	−26.67
44	*Lavandula stoechas* L.	−27.26
45	*Echium* sp.	−27.52
46	*Eucaliptus* sp (I)	−24.30
47	Forest (I)	−25.14
48	Multifloral (I)	−25.41
49	Forest (I)	−26.46

and polysaccharides with a galactose terminal. The enzyme oxidizes the hydroxyl group to give an aldehyde group plus hydrogen peroxide, which in turn, in the presence of peroxidase, oxidizes a chromogen group whose colour is measured spectrophotrometrically. In the present case, the chromogen employed was orthocresol, which affords a linear relationship between absorbance and the galactose concentration.

To detect the addition of beet sugar, the method based on treatment of the oligosaccharide fraction contained in the honey with galactose oxidase was used. This procedure was applied to the polysaccharide fraction in samples of beet sugar, honey, and mixtures of both, with the finding that the amount of galactose forming part of the polysaccharides (BG) was 0.06 g 100 g^{-1} of beet sugar. To check the reliability of the method, increasing amounts of beet sugar containing 0.060 g of BG 100 g^{-1} sample were added to 1 g of honey from a reference hive (sample 48). The results show that recovery of the amounts of BG added was good.

RESULTS AND DISCUSSION

Pollen analysis and electrical conductivity

Quantitative pollen analyses: values ranged between the 35 000 grains of pollen 10 g^{-1} honey of sample 34, classified as rosemary (*Rosmarinus officinalis* L), a commercially exploited honey, to the 285 000 grains 10 g^{-1} honey of sample 46, classified as eucalyptus (*Eucaliptus* sp.) (research hive), which are included in classes I, II and III of Maurizio (1939).

The samples shown in Table 1 as honey-dew contain numerous elements typical of this and their electrical conductivity is higher than 9×10^{-4} Sm cm^{-1}. Samples 5, 11, 12, 13, 15, 16, 25, 30, 35 correspond to honey-dew honeys (*Quercus* sp.). The samples featured as 'forest' have many elements typical of honey-dew but their electrical conductivities are lower than 9×10^{-4} Sm cm^{-1}. Samples 2, 3, 4, 7, 10, 18, 20, 24, 26, 28, 31, 32, 37, 38, 40, 47, 49 correspond to honeys classified as 'forest' The samples appearing as multiflor do not feature any major pollen type; namely, samples 14, 19, 21, 29, 36, 48.

The samples classified as monoflor show the corresponding pollen types to a significant extent. In these cases for classification purposes different percentages of pollen typical of each honey were considered. This type of classification is not legislated but tends to be followed by researchers. Thus, the following monoflor samples were considered (Table 2). In all cases, the information given on the labels of the commercial (C) samples coincided with the analytical results.

Table 2. Honey classification for polinic spectrum

Honey classification	% of characteristic pollen	n° sample
Robinia pseudoacacia L.	More than 50	1
Citrus sp	15	6
Eucaliptus sp.	More than 90	8, 33, 46
Helianthus annuus L	More than 60	9
Lavandula latifolia Medicus	25	17
Castanea sativa Miller	80	22, 23
Erica sp	More than 60	27
Rosmarinus officinalis	15	34
Arbutus unedo L.	More than 60	39
Echium sp.	More than 60	41, 42, 45
Lavandula stoechas L.	20	43, 44

Table 3. Probability that the $\delta^{13}C$ of a sample of authentic honey will be more negative than a set limit

Probability (n° of samples, %)	Limit $\delta^{13}C$ (‰)
5 of 6; 84.1	$\bar{x} + s = -23.57$
43 of 44; 97.72	$\bar{x} + 2s = -22.33$
769 of 770; 99.87	$\bar{x} + 3s = -21.10$
24,999 of 25,000; 99.996	$\bar{x} + 4s = -19.86$

Sensory analyses

The results show that all samples conserve the sensory properties typical of their botanical origin. However, the commercialized honeys were darker and had a caramel (burnt sugar) taste, possibly due to the heating they are subjected to for bottling purposes.

Determination of $\delta^{13}C$

Table 1 shows the $\delta^{13}C$ values of the honeys studied. From the results it may be concluded that the dominant plant type corresponds to type C_3 plants. Owing to the broad range of $\delta^{13}C$ values found for the samples, the degree of uncertainty of this technique, White and Doner (1978), was used to check the existence of cane sugar. There is some degree of uncertainty in the use of this technique to determine the existence of cane sugar owing to the broad range of values found for the honey. The upper limit (the most negative value) of $\delta^{13}C$ for

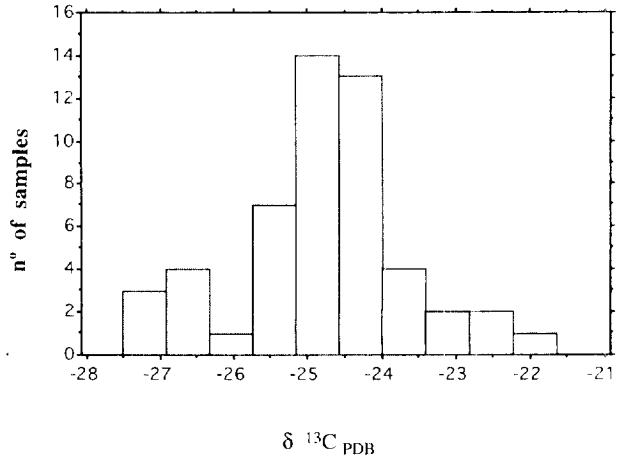

Fig. 2. Frequency distribution for $\delta^{13}C$ values.

Table 4. $\delta^{13}C$ values for sugars and glycerine

Sample	$\delta^{13}C$ (‰)
Cane sugar	−11.40
Beet sugar	−23.95
Fructose	−24.94
Glucose	−25.05
Sucrose	−22.96
Glycerine	−23.64

Table 5. Amount of galactose forming part of polysaccharrides (BG)

Sample n°	g BG 100 g^{-1}
2	0.090
3	0.086
5	0.069
7	0.044
9	0.087
15	0.087
16	0.061
18	0.047
20	0.058
21	0.052
25	0.054
28	0.079
30	0.059
31	0.051
32	0.067
33	0.046
35	0.061
36	0.053
41	0.064
42	0.050
43	0.047
45	0.047
46	0.055
47	0.048
48	0.049
49	0.050

honey can be established with a percentage of probability by applying statistical criteria. In this case, the mean value plus a multiple of the standard deviation was used (Table 3). There is no point in using the mean value minus the standard deviation since the existence of adulterations that produce impoverishments in ^{13}C (shift towards more negative values) is unknown. Figure 2 shows the frequency distribution of the values.

According to these results, it may be concluded that: (a) values less negative than −19.86‰, are conclusive evidence of adulteration with corn syrup or cane sugar, (b) values between −19.86 and −22.30‰ are not conclusive and require checking by the TLC test, and (c) values more negative than −23.4‰ would characterize pure honey. Accordingly, samples 1,2 would be susceptible to further checking with TLC, White (1987); the rest may be considered as pure (96%).

Measurements of $\delta^{13}C$ were also taken in samples of cane sugar, beet sugar, other sugars and glycerine to check whether, using the stable isotope technique, it is possible to detect adulterations with any of these products. As shown in Table 4, only cane sugar has values well differentiated from those obtained for honey (Table 1) owing to the fact that it comes from a C_4 plant, as mentioned above. The other sugars and glycerine have values very similar to those of honey and hence any adulteration by one of these substances would pass undetected with the technique employed.

Detection of the addition of beet sugar

The procedure was then applied to 26 samples of honey previously analyzed with the stable isotopes technique. The results, shown in Table 5, with the same numbering as in Table 1, and following the criterion of White *et al.* (1986) show that samples with values higher than 0.08 g of BG 100 g^{-1} of sample may have been adulterated with beet sugar (15%).

Samples 3, 9 and 15 had contents slightly higher than the maximum according to the criterion of White. We believe that the range of this criterion is excessively broad ($\pm$0.020 g BG 100 g^{-1} sample) to decide whether a sample has been adulterated with beet sugar.

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REFERENCES

AOAC (1990). (15th edn), sec 978.17. Association of Official Analytical Chemists, Arlington, V.A., USA.

AOAC (1990). (15th edn), sec 979.22. Association of Official Analytical Chemists, Arlington, V.A., USA.

Barrios, J., González, B., Sánchez, J. (1994). Analisis polínico de mieles de la Cuencia del rio Alagón (Salamanca). In *Polen y esporas: contribución a su conocimiento*, ed. I. La Serna. Universidad de La Laguna, Tenerife. pp. 229–239.

BOE (1983). del 13 de Agosto, pp. 22383–22386.

BOE (1986). 18 Junio, pp. 22195–22202.

Craig, H. (1953). The geochemistry of the stable carbon isotope. *Geochem. Cosmochim. Acta*, **3**, 53–92.

Doner, L. W. & White Jr, J. W. (1977). Carbon-13/carbon-12 ratio is relatively uniform among honeys. *Science*, **197**, 891.

Doner, L. W., White Jr, J. W. & Kushnir, I. (1979). Gas-liquid chromatographic test for honey adulteration by high-fructose corn sirup. *J. Assoc. Off. Anal. Chem.*, **62**, 186–189.

Erdtman, G. (1960). The acetolysis method. A revised description. *Svensk. Bot. Tidskr*, **54**, 561–564.

Gonnet, M. & Vache, G. (1991). *Le Goût du miel*. Ed. U.N.A.F., Paris.

González, B., Barrios, J. & Sánchez, J. (1994). Analisis polínico de mieles salmantinas. In *Polen y esporas: contribución a su conocimiento*, ed. I. La Serna. Universidad de La Laguna, Tenerife, pp. 259–269.

Louveaux, J., Maurizio, M. & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World*, **59**(4), 139–157.

Maurizio, A. (1939). Untersuchungen zur quantitativen pollenanalyses des honing. *Mitt. Geb. Lebensmittelelunters*, **30**, 27–69.

Sofer, Z. (1980). Preparation of carbon dioxide for stable carbon isotope analysis of petroleum fractions. *Anal. Chem.*, **52**, 1389–1391.

Vergeron, P. H. (1964). Interpretation statistique des results en matiere pollinique des miels. *Ann. Abeille*, **7**(4), 349–364.

White Jr, J. W. (1987). Wiley Award Address. *J. Assoc. Off. Anal. Chem.*, **70**, 181–189.

White Jr, J. W. & Doner, L. W. (1978). Mass spectrometric detection of high-fructose corn sirup in honey by use of $^{13}C/^{12}C$ ratio: collaborative study. *J. Assoc. Off. Anal. Chem.*, **61**, 746–750.

White Jr, J. W., Meloy, R., Probst, J. & Huser, W. (1986). Detection of beet sugar adulteration of honey. *J. Assoc. Off. Anal. Chem.*, **69**, 652–654.

Zeigler, H., Maurizio, A. & Stichler, W (1979). Die charakterisierung von honigen nach ihrem gehalt an pollen und an stabilen isotopen. *Apidologie*, **10**, 301–311.