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Scientific support to the implementation of a Coordinated Control Plan with a view to establishing the prevalence of fraudulent practices in the marketing of honey

Results of honey authenticity testing by liquid chromatography-isotope ratio mass spectrometry

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Table of contents

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Executive Summary

The European Commission has regularly been informed of the presence on the market, in a potentially significant proportion, of honey that may not meet the composition criteria laid down by Directive 2001/110/EC and/or that is not the result of the production process required by the legal definition of honey.

In 2015 the Directorate-General Health and Food Safety (DG SANTE) of the European Commission launched a coordinated control plan on authenticity of honey where the Joint Research Centre assisted by performing analyses to detect honey adulteration with exogenous sugars (Commission Recommendation C(2015) 1558 final).

The objective of the control plan was to establish the prevalence on the European Union market of: (a) honey mislabelled with regard to its geographical and/or botanical origin and (b) products declared or presented as honey although containing exogenous sugars or sugar products. The sampling strategy should target honey which is more susceptible to having been subjected to the practices that were the purpose of this control plan taking into account available data, including information provided by documentary, identity and preliminary physical checks, and prices.

All EU Member States participated in the control plan with the additional participation of Norway and Switzerland. They collected 2264 honey samples at all stages of the supply chain; the majority (45%) was sampled from retailers.

Commission Recommendation C(2015) 1558 foresaw a tiered approach for the analysis of the collected honey samples. All samples had to be analysed by the Member States to check compliance with certain provisions of the EU Honey Directive, sensory characteristics and pollen profiles (Tier 1). Those that were found compliant in Tier 1 were submitted to sugar analysis (Tier 2) and those that were found still compliant in Tier 2 were subjected to stable carbon isotope analysis by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) and a combination of EA-IRMS and liquid-chromatography coupled to isotope ratio mass spectrometry (EA/LC-IRMS), which are state-of-the-art methods for the detection of added sugars (Tier 3). In case that the LC-IRMS technique was not available in the concerned MS, samples were sent to JRC for analysis.

JRC received 893 samples (40% of all collected samples) that were found compliant by the tests carried out in the Member States, for analysis by EA/LC-IRMS. 14 % of the honey samples checked by EA/LC-IRMS (127 out of 893) did not conform to published benchmark purity criteria indicating that foreign sugars may have been added[1]. The applied analytical method only indicates the presence of foreign sugars; it does not allow quantifying the level of addition.

Around 20 % of honeys either declared as blends of EU honeys (19 out of 96), or unblended honeys bearing a geographical reference related to an EU Member State (53 out of 275) or a third country (11 out of 55) were found to be suspicious of containing

[1] As JRC is not an official laboratory for official controls in the meaning of Article 12 of Regulation (EC) No 882/2004, samples with off-limit values should be considered as a case of suspicion of noncompliance and further investigations should be carried by the competent authorities in order to confirm or to eliminate the suspicion (Commission Recommendation C(2015) 1558).

added sugar. The rate of suspicious honeys was around 10% for blends of EU and non-EU honeys (40 out of 426), blends of non-EU honeys (3 out of 30), and honey of unknown origin (1 out of 11). In this respect references to geographical origin refer to the declarations given on the labels, which were in most cases not verified by analytical methods (e.g. pollen analysis). The number of samples where the EA/LC-IRMS method indicated sugar addition was slightly higher for monofloral in comparison to polyfloral honeys.

The applied technique (analytical method together with the decision criteria) has not been validated in multi-laboratory studies conducted at the international level. It relies on empirically determined benchmark purity criteria, taken from the published literature so that the selection of honeys used to set the benchmark may influence the compliance decision.

Further action is thus necessary to establish the robustness of the results required for evidence in enforcement action.

To improve the capability of authenticating honey, it is recommended to create a compositional database of authentic honeys and of substances which may be added to increase its volume or bulk ("extenders") or which are used as bee feeding products. This would require Member States and possibly third countries to deposit samples representative of their domestic honey production in a centralised sample repository. The selection and sampling of the honey would have to be performed under the supervision of competent authorities. The deposited samples would be analysed by the EA/LC-IRMS method to confirm or amend, if necessary, the isotopic ratio criteria for authentic EU (and possibly third country) honeys and to investigate alternative analytical methods for detecting adulterated honey.

Such comprehensive data would allow a better control of EU honey quality, and protect producers as well as consumers from being misinformed.

1. Introduction

1.1 Honey types

Honey has a long history of human consumption, and is commonly consumed in its unprocessed state (i.e. liquid, crystallised or in the comb). The FAO/WHO Codex Alimentarius issued STAN 12-1981 (revised in 2001), which outlines the provisions related to the naming, chemical properties, level of contaminants, and labelling of honey, among other characteristics [1].

The European Council Directive 2001/110/EC defines honey and establishes minimum quality standards for honey when placed on the market as honey or used as an ingredient in products intended for human consumption [2]. The regulations generally aim to preserve the purity of honey as an unprocessed raw agricultural product, with limited modifications to its chemical composition. The Directive defines honey as the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature. The colour and flavour of honeys differ depending on the nectar source, age, and storage conditions. Honey made primarily from the nectar of one type of flower is called monofloral honey, whereas honey made from several types of flowers is called polyfloral honey. Monofloral honey typically has a high commercial value in the marketplace due to its distinctive flavour. However, most commercially available honey is a blend of honeys differing in floral source and geographic origin.

The composition of honey is rather variable and depends primarily on its floral and geographical source, but certain external factors, such as processing, packaging and storage conditions, also play an important role.

Sugars are the main components of honey. The nectar and honeydew, respectively, are transformed into honey by the bee enzymes diastase (amylases) and invertase (α-glucosidase) during storage and maturation in the beehive. During this process, diastase and invertase catalyse the conversion of the sugars of nectar and honeydew into fructose and glucose, the main constituents of honey. The result is a complex mixture made up of about 70% monosaccharides and 10-15% disaccharides composed of glucose and fructose with the glycosidic bond in different positions and configurations. In addition, there are also minor components consisting of about 25 oligosaccharides.

1.2 Honey market

European apiculture is a niche sector of agricultural production and is dominated by non-professional beekeepers. Overall, EU honey production has been increasing slowly with annual variations depending on climatic conditions. However, keeping this level of production is becoming harder for beekeepers due to the challenges they face in terms of bees' health and environmental constraints.

With a production of around 250 000 tonnes per year in 2015, the EU is the second largest producer of honey after China. Other main honey producers are Turkey with a steady output increase, Ukraine and the United States of America.

On a global scale the EU is the largest importer of honey as the EU production covered only ca. 60% of its consumption in 2015. The three main honey producers in the Union

are Romania, Spain and Germany. Other important producing Member States are Hungary, France, Greece, and Poland. In 2015, the EU imported around 200 000 tonnes of honey, representing in volume around 75% of EU total production. Half of these imports came from China (around 100 000 tonnes). The other two main suppliers were Mexico and Ukraine. Honey imported from third countries is much cheaper than honey produced in the EU. In 2015, the average import unit price for Chinese honey was 1.64 €.kg^{-1} while the average EU price of multi-floral honey sold in bulk at wholesalers was 3.78 €.kg^{-1}. Due to higher production costs EU producers can hardly compete with imported honey.

1.3 Syrups, sweeteners, bee feeding products

Bees need additional feeding at certain moments in the apicultural year, particularly in winter. Another reason is that nectar flows can vary strongly from region to region and according to the season, so that feeding is necessary to maintain breeding activities and to meet food requirements.

The traditional substitute for honey is a sucrose solution. As a rule, sugar and water are mixed in a 3:2 ratio or, less frequently on a 1:1 basis. Different sucrose based bee feeding products exist varying in their composition (e.g. mainly sucrose or sucrose and its building blocks, fructose and glucose) and depending on their uses (e.g. winter feeding, spring stimulation feed or early winter feeding). Next to sucrose a number of sweeteners and sugar syrups are commercially available for feeding bees: syrups made from starch (corn, wheat, rice), sugar cane, sugar beet, agave or syrups of natural origin such as maple. They contain in variable proportion a mixture of several sugars (glucose, fructose, maltose, maltotriose, dextrins, etc) and their price is usually very competitive.

1.4 Honey adulteration

Adulteration by sweeteners is one of the most important authenticity issues. The simplest way to adulterate honey involves the addition of sugar (syrups) directly to honey. Honey adulteration has evolved from the basic addition of sucrose and water to specially produced syrups which mimic the sugar composition of natural honey. For instance, the addition of fructose or industrial glucose could change the fructose /glucose ratio, which has to be 1 – 1.2 in pure honey. Moreover, some other carbohydrate ratios could be used to ascertain honey authenticity [3].

Indirect adulteration by feeding of sugar (syrups) during the main nectar flow period is the second way to adulterate honey; correct beekeeping practice should ensure that sweeteners used to feed bees do not adulterate honey. Indirect adulteration is extremely difficult to detect.

1.5 Analytical methods

A review by Anklam [3] presents a selection of analytical methods reported over the past years for the detection of direct and indirect adulteration in honey. Different chromatographic techniques have been developed for the detection of adulterated honey including thin-layer chromatography [4], gas chromatography−mass spectrometry [5] and high-performance liquid chromatography with electrochemical and evaporative light scattering detection [6, 7]. Markers of honey adulteration include difructose anhydrides (DFAs) [5], polysaccharides [8] and 2-acetylfuran-3-glucopyranoside (AFGP) [9] based on the type of syrup used for the adulteration of honey. However, these methods require complex sample preparation and are highly time-consuming. Alternatively, methods

based on spectroscopy, notably nuclear magnetic resonance (NMR), have been proposed as screening methods [10].

Stable carbon isotope ratio analysis and detection of honey adulteration

Plants can employ three different chemical pathways during photosynthesis. Most plants use an enzyme called ribulose bisphosphate carboxylase/oxygenase (RUBISCO) and are designated as C_3 plants, which constitute about 90% of all plants, including sugar beet, wheat or rice. Alternatively, plants may also use the Hatch-Slack cycle; these plants are designated as C_4 plants and typically originate from hot climates. Maize and sugar cane are C_4 plants. A third group of plants, the CAM plants, has a unique metabolism called the 'Crassulacean Acid Metabolism'. These plants generally use the C_4 pathway, but can also use the C_3 pathway. CAM plants are generally from very arid environments and they include pineapple, cacti and agave.

Isotope ratio mass spectrometry (IRMS) is considered as one of the most powerful analytical techniques for detection of honey adulteration using low cost syrups that often exhibit sugar profiles very similar to authentic honey. It is a specialised technique that enables the precise and accurate measurement of variations in the natural isotopic abundance of stable isotopes of carbon (^{13}C / ^{12}C). Isotopic ratios are measured relative to a working reference gas calibrated using internationally accepted standards and they are reported using the delta notation (δ) and expressed in units per mill (‰). In the case of carbon stable isotope analysis, the delta notation is defined as:

$$\delta^{13}C\ (‰) = [R\ (Sample) / R\ (Standard) - 1] \times 1000$$

where R represents the ratio $^{13}CO_2$ / $^{12}CO_2$ respectively for the sample analysed and the international standard (Vienna Pee Dee Belemnite) used.

C_3 plants exhibit $\delta^{13}C$ values ranging from -23 to -28‰, whereas C_4 plants have isotopic ratios ranging from -9 to -15‰ [11].

The AOAC method 998.12 [12], which is based on elemental analysis – IRMS (EA-IRMS), detects honey adulteration using sugar syrups produced from C_4 plants such as corn and sugar cane. This method can detect honey adulteration using C_4 sugars at levels ≥ 7% but cannot detect adulteration of honey using syrups produced from C_3 plants. Cabañero *et al*. [11] developed a new procedure allowing the determination of the $\delta^{13}C$ isotopic ratios of individual sugars present in honey (i.e. glucose, fructose and sucrose) using liquid chromatography (LC)-IRMS. They demonstrated that isotopic ratio differences between individual sugars could be used as authenticity criteria to detect honey adulteration with C_3 sugars (beet sugar) but also for C_4 sugars (cane sugar, cane syrup, isoglucose syrup and high fructose corn syrup). For instance, detection of exogenous C_3 sugars in honey was possible with detection limits ranging from 5 to 10%. The LC-IRMS method allows to detect adulteration with C_4 exogenous sugars at levels < 7%.

The LC-IRMS approach proposed by Cabañero *et al*. was further improved by Elflein and Raezke [13]. In the Elflein and Raezke method, data obtained using both EA-IRMS (AOAC 998.12) and LC-IRMS were considered to define a set of purity criteria for honey samples. As opposed to the method of Cabañero *et al*., all the sugars present in honey were taken into consideration including di-, tri- and oligosaccharides. Based on the analysis by EA- and LC-IRMS of 451 authentic honey samples, Elflein and Raezke proposed a set of purity criteria to decide whether a honey sample was adulterated by

exogenous sugars but without quantifying the level of sugar (syrup) addition. The probability for an authentic honey to fall outside the limits given in Table 1 is 0.3% (99.7% confidence). These purity criteria are used by the majority of laboratories applying LC-IRMS for checking honey authenticity. To apply the Elflein and Raezke method, the following parameters must be measured using EA-IRMS and LC-IRMS:

- $\delta^{13}C_{fru}$ (isotopic ratio of fructose determined by LC-IRMS);
- $\delta^{13}C_{glu}$ (isotopic ratio of glucose determined by LC-IRMS);
- $\delta^{13}C_{ds}$ (isotopic ratio of disaccharides determined by LC-IRMS);
- $\delta^{13}C_{ts}$ (isotopic ratio of trisaccharides determined by LC-IRMS);
- $\delta^{13}C_{honey}$ (isotopic ratio of the bulk honey determined by EA-IRMS);
- $\delta^{13}C_{protein}$ (isotopic ratio of the protein extracted from honey and determined by EA-IRMS);
- Percent peak area of oligosaccharides in % determined by LC-IRMS.

$\Delta\delta^{13}C_{max}$ is the maximum difference observed between all possible isotopic ratios measured ($\Delta\delta^{13}C_{fru\text{-}ds}$ / $\Delta\delta^{13}C_{fru\text{-}ts}$ / $\Delta\delta^{13}C_{fru\text{-}protein}$ / $\Delta\delta^{13}C_{glu\text{-}ds}$ / $\Delta\delta^{13}C_{glu\text{-}ts}$ / $\Delta\delta^{13}C_{glu\text{-}protein}$ / $\Delta\delta^{13}C_{ds\text{-}ts}$ / $\Delta\delta^{13}C_{ds\text{-}protein}$ / $\Delta\delta^{13}C_{ts\text{-}protein}$). $\Delta\delta^{13}C_{protein\text{-}honey}$ is the difference between the isotopic ratios of the bulk and the protein honey. Finally, $\Delta\delta^{13}C_{fru\text{-}glu}$ is the difference between the isotopic ratios of fructose and glucose. Elflein and Raezke assumed that oligosaccharides are not normally present in authentic honeys at high concentrations and are therefore indicative of the presence of exogenous sugars (Table 1).

Purity parameter for honey	**Proposed limit**
$\Delta\delta^{13}C_{fru\text{-}glu}$	± 1.0‰
$\Delta\delta^{13}C_{max}$	± 2.1‰
Percent peak area oligosaccharides	< 0.7%

Table 1. Purity criteria (99.7% confidence level) defined by Elflein and Raezke [13] to determine the potential adulteration of honey with exogenous sugars.

2. Objectives

The Commission has regularly been informed of the presence on the market, in a potentially significant proportion, of honey that may not meet the composition criteria laid down by Directive 2001/110/EC [2] and/or that is not the result of the production process as given in the legal definition of honey. In order to establish the prevalence of fraudulent practices in the marketing of honey DG SANTE launched a coordinated control plan on authenticity of honey to detect the following fraudulent practices:

- honey mislabelled with regard to its geographical and/or botanical origin;
- products declared or presented as honey although containing exogenous sugars or sugar products.

To support the implementation of the plan, JRC was requested to suggest a systematic approach to detect extension/substitution of honey with sweeteners, feeding of sweeteners to bees and removal of pollen. More specifically, JRC should:

- Provide guidance concerning the technical parameters for the appropriate collection, storage and transport of samples (preliminary homogenisation in case of bulk packaging, amount of substance to be saved for LC-IRMS, specifications for containers, temperature and other physical conditions etc.);
- Set-up and in-house validate an LC-IRMS method, whose operating and evaluation principles are described in Cabañero *et al*. and Elflein and Raezke;
- Make available to laboratories identified by the competent authorities of the Member States sending testing results and/or honey samples for analysis appropriate templates for transmitting relevant information related to the nature of the samples and testing results already carried out in the Member States;
- Receive, register and store the samples under appropriate conditions until analysis and retain them for an additional 12 month period for re-analysis if required;
- Check and if necessary clarify the information accompanying the samples, including the results of the tests already carried out by the Member State;
- Analyse the samples received from the Member States by the in-house validated LC-IRMS method and interpret the results on the basis of authenticity criteria described in the above-mentioned scientific publications;
- Communicate to the entities having sent the sample the results of LC-IRMS test with the appropriate interpretation. The result should be transmitted once they are established, and in any case within 5 months following the reception of the sample;
- Create a database to collate and organise the obtained LC-IRMS data in combination with the testing results submitted by the Member States;
- On the basis of the results collected, produce a comparative assessment of efficacy of the different methods to detect adulteration with added sugar;
- Set-up an appropriate quality control scheme including internal quality controls (quality control samples and control charts) and external quality controls (a certain number of honey samples will be analysed by external contract laboratories);
- If necessary, propose next steps for validating the EA/LC-IRMS method for honey and further data management at EU level;

- Recommend modalities for the establishment of an EU wide analytical databank for honey.

3. Honey samples

3.1 Sampling by the MS

All EU Member States (MS) participated in the honey control plan, along with Norway and Switzerland.

According to Commission Recommendation C(2015)1558 honey should be sampled by the Member States from various points of the production and supply chain (Table 2). The sampling strategy should target honey which is more susceptible to have been subjected to the practices that were the purpose of this control plan taking into account available data, including information provided by documentary, identity and preliminary physical checks, and prices.

Source type	Samples collected	
Border inspection	35	1.5%
Distributor	157	6.9%
Importer	63	2.8%
Packaging companies	134	5.9%
Processor	81	3.6%
Producer	152	6.7%
Retailer	1010	44.6%
Storage companies	60	2.7%
Wholesaler	81	3.6%
Unknown	*491*	*21.7%*
Total	2264	100.0%

Table 2. Number of samples collected along the production and supply chain (according to information supplied by the Member States).

To cover the various geographical origins of honeys, sampling of honey was divided into three parts as defined in Commission Recommendation C(2015) 1558:

Part A: Samples collected in a MS and originating from the same MS. This should represent 20% of the samples collected.

Part B: 40% of the honey samples should have a declared origin outside the MS where the samples were collected (EU or not EU), not including blends.

Part C: 40% of the honey samples should be either a blend of EU honeys, or a blend of non-EU honeys, or a blend of EU and non-EU honeys.

Furthermore, the Recommendation foresaw a tiered approach for the analysis of the collected honey samples. All samples of part A and B had to be analysed by the Member States to check compliance with certain provisions of the EU Honey Directive, sensory characteristics and pollen profiles (Tier 1). One third of the A and B samples that were found compliant in Tier 1 and all part C samples had to be submitted to sugar analysis in the Member States (Tier 2) and those that were found still compliant in Tier 2 were

subjected to stable carbon isotope analysis by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) and a combination of EA-IRMS and liquid-chromatography coupled to isotope ratio mass spectrometry (Tier 3). In case that liquid-chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) was not available in the Member State, the samples were sent for testing to JRC.

Country	Total samples collected	Samples sent to JRC
Austria	102	59
Belgium	101	60
Bulgaria	10	5
Croatia	70	42
Cyprus	19	11
Czech Republic	90	3
Denmark	46	11
Estonia	20	12
Finland	46	24
France	149	88
Germany	138	27
Greece	97	56
Hungary	74	49
Ireland	70	40
Italy	110	91
Latvia	50	33
Lithuania	50	30
Luxembourg	20	12
Malta	36	12
Netherlands	107	30
Norway	63	10
Poland	100	60
Portugal	70	40
Romania	81	49
Slovakia	62	38
Slovenia	50	30
Spain	163	10
Sweden[2]	27	27
Switzerland	96	88
United Kingdom	147	22
TOTAL	2264	1069

Table 3. Number of samples collected per Member State and sent to JRC.

[2] These samples were provided after the publication of the preliminary results (https://ec.europa.eu/food/safety/official_controls/food_fraud/honey/tests_en).

Table 3 shows the number of samples collected per country and the proportion sent to JRC for analysis by LC-IRMS.

3.2 Sample reception

Member States' competent authorities sent 1069 samples to JRC-Geel (Belgium) and all samples submitted were tested by LC-IRMS. Next to 893 samples, which were compliant according to Tier 1, 2 and EA-IRMS analyses carried out in the Member States, they also sent 138 non-compliant samples for verification. For 38 samples no meta-data (origin and results of Tier 1, 2, and EA-IRMS testing in the Member States) were provided by the Member States and were, therefore, not included in the data analysis (Table 4).

Samples	Number
Collected by MS (plus Norway and Switzerland)	2264
Sent to JRC and analysed by LC-IRMS	1069
of which without meta-data	38
non-compliant by applying the tests of Tiers 1 and 2 and EA-IRMS in the Member States	138
compliant by applying the tests of Tiers 1 and 2 and EA-IRMS in the Member States	893

Table 4. Number of honey samples collected in the MS (plus Norway and Switzerland) and number of samples received and analysed by JRC

A 20-gram aliquot was withdrawn from each honey sample and subjected to LC-IRMS analysis. To ensure uniformity in case of bi-phasic honey (liquid and crystalized forms both present), a plastic cylinder was used to take a core sample from the top to the bottom of the container. Aliquots for analysis were contained in amber glass jars and protection from light.

Finally, the original samples were stored at room temperature in the dark.

3.3 Data management

MS were asked to report the metadata of their samples and analysis results in Excel® sheets containing all the fields mentioned in the Commission Recommendation. A guideline was also sent to help the collaborators filling in the templates.

Once completed, the resulting files were uploaded by the MS on the collaborative platform of the European Commission CIRCABC. Each MS had a reserved folder, invisible to the other MS (except JRC and DG SANTE), to deposit the results of their analysis.

After data collection and inspection for completeness, JRC aggregated all the MS files. Data was formatted (translation into English, decimals, capital letter …) and standardized (restricted list of vocabulary, consistent abbreviation …) to ensure uniformity throughout the whole dataset.

Some values were cross-checked for consistency (geographic part corresponding to the country of origin, botanical origin matching the name on the label, etc). These verifications only concerned the description of the samples. The analytical results obtained by the MS were not challenged except for obvious typing errors, mistakes on the use of units or extreme outliers. In case of ambiguity or missing important values, JRC asked for feedback of the responsible MS.

The dataset, checked for consistency, was supplemented with the results of the EA-IRMS and LC-IRMS analyses carried out at JRC and used for statistical analysis.

Datasets collected from the MS were transferred into an MS Access database, which improves data robustness and future data usability.

4. Analytical methods

According to Commission Recommendation C(2015)1558, samples that were compliant with the purity criteria of Tier 1 (organoleptic analysis, electrical conductivity, diastase activity and pollen analysis) and had a compliant sugar profile in the Tier 2 testing, had to be subjected to EA/LC-IRMS if the method was available in the MS (Tier 3).

When LC-IRMS was not available, the samples had to be analysed by the MS using EA-IRMS (AOAC official method 998.12). In case that the obtained results did not indicate addition of C_4 based sugar syrups, samples were sent to JRC for testing by LC-IRMS accompanied with the results of the tests already carried out (templates were provided by JRC via CIRCABC).

When LC-IRMS was carried out in the MS, samples with off-limit values should be sent to JRC-Geel for the honey sample repository and any further analysis if needed.

Some MS also sent part of their non-compliant samples based on EA-IRMS analysis, or sent samples without EA-IRMS results.

4.1. EA-IRMS

For the measurement of bulk and protein honey isotopic ratios, EA-IRMS was used following the experimental conditions of AOAC method 998.12. Analysis was carried out using a NCS 2500 elemental analyser (Thermoquest Italia S.p.A., Milan, Italy) coupled to a Thermo Fisher Scientific Delta Plus XP IRMS operated using ISODAT NT 2.0 software.

The IRMS was calibrated using a two-point linear normalisation with two certified reference standards NBS-19 ($\delta^{13}C_{VPDB}$ = +1.95 ‰) and LS-VEC ($\delta^{13}C_{VPDB}$ = -46.6 ‰), as described by Paul *et al*. [14]. Two working reference standards were analysed daily to monitor the stability of the reference gas value and prevent any drifts from the initial calibration of the IRMS. These were the certified reference material BCR-657 (glucose) exhibiting $\delta^{13}C_{VPDB}$ of -10.76 ± 0.04 ‰ obtained from JRC-Geel and a certified reference protein standard (casein) exhibiting $\delta^{13}C_{VPDB}$ of -26.98 ± 0.13 ‰ obtained from Elemental Microanalysis (Okehampton, UK). In addition, a quality control honey sample was analysed within each sequence of analysis. For the in-house quality control honey, the bulk honey $^{13}C_{VPDB}$ value was -25.16 ± 0.12 ‰ based on the analysis of 50 samples analysed between August 2015 and March 2016. These values were used to establish Shewhart quality control charts for the on-going control of method stability and precision.

4.2. LC-IRMS

LC-IRMS analyses were performed using a Dionex Ultimate 3000 HPLC system equipped with a pump, an auto-sampler and a column compartment for temperature control. The HPLC was linked to a ThermoFisher Scientific LC-ISOLINK interface and analyses of CO_2 isotopic ratios were carried out using a ThermoFisher Scientific Delta V Advantage IRMS operated using ISODAT 3.0 software.

Honey samples were prepared by diluting honey with Ultrapure water (Fluka Analytical) to a concentration of 4 $mg.mL^{-1}$. After dilution, samples were filtered through an Acrodisc 25 mm syringe filter equipped with a 1-µm PTFE membrane from Pall Life Sciences (Ann Arbor, USA). The HPLC column was a 300 x 7.8 mm Phenomenex Rezex RCM Monosacharride (Phenomenex, Utrecht, NL). A flow rate of 0.3 $mL.min^{-1}$ and a temperature of 70 °C were used during analysis. Ultrapure water from Fluka Analytical

was used as an eluent. The LC-ISOLINK could be used in two different modes. In HPLC mode, compound-specific isotope analysis was carried out and the direct injection mode was used for calibration of the IRMS. In HPLC mode, sugars in the sample were separated on the HPLC column. The oxidation reagent consisted of a solution of potassium peroxodisulfate $K_2S_2O_8$ at a concentration of 160 $g.L^{-1}$ and the acid reagent was a solution of phosphoric acid (H_3PO_4) at a concentration of 147 $g.L^{-1}$. Both reagents were pumped separately by two-head pumps. Prior analysis, both the mobile phase and the two reagent solutions were thoroughly degassed under vacuum.

The IRMS was calibrated using a two-point linear normalisation with two certified reference standards IAEA-601 benzoic acid ($\delta^{13}C_{VPDB}$ = -28.81 ± 0.04‰) and IAEA-CH6 sucrose ($\delta^{13}C_{VPDB}$ = -10.45 ± 0.07‰). Calibration was carried out using the direct injection mode on the LC-ISOLINK interface using three distinct analytical runs in which each standard solution was injected six times consecutively. The certified reference material BCR-657 (glucose) exhibiting $\delta^{13}C_{VPDB}$ of -10.76 ± 0.04‰ was analysed daily to monitor the stability of the reference gas value and prevent any drifts from the initial calibration of the IRMS. In addition, a quality control honey sample was analysed within each sequence of analysis. That sample was identical to the quality control honey used for EA-IRMS. For the in-house quality control honey, the $^{13}C_{VPDB}$ values for fructose, glucose, disaccharides and trisaccharides are summarised in Table 5.

Parameter[1]	**Mean (‰)**	**SD (‰)**
$\delta^{13}C_{fru}$	-25.10	0.11
$\delta^{13}C_{glu}$	-24.57	0.12
$\delta^{13}C_{ds}$	-26.51	0.18
$\delta^{13}C_{ts}$	-26.85	0.23
os % area	0.26%	0.66%

[1] $\delta^{13}C_{fru}$ - isotopic ratio of fructose; $\delta^{13}C_{glu}$ - isotopic ratio of glucose; $\delta^{13}C_{ds}$ - isotopic ratio of disaccharides; $\delta^{13}C_{ts}$ - isotopic ratio of trisaccharides; os % area - % area of the oligosaccharide peak.

Table 5. Mean and standard deviation (SD) obtained for a honey quality control sample by LC-IRMS analysis of 108 samples over a duration of nine months.

The standard deviations obtained for glucose and fructose were within the same range as the standard deviation obtained using the EA-IRMS method (ca. SD = ± 0.12‰). For di- and trisaccharides, the standard deviations obtained by LC-IRMS were somewhat higher owing to the fact that these peaks were detected as a sum of several compounds; however, they were still within an acceptable range. These values were used to establish Shewhart quality control charts for the on-going control of the LC-IRMS method in terms of stability and precision.

The individual data points of the Quality Control (QC) sample for fructose, glucose, disaccharides and trisaccharides were usually within ± 1 SD, and only a few deviations were observed during the entire duration of the project. These deviations were investigated and the samples re-analysed when necessary.

Within each batch of samples, the certified reference material BCR-657 (glucose) was also analysed to monitor daily the stability of the reference gas value. The calculated

reference gas value was -38.36 ± 0.19‰ based on the analysis of 70 replicates between November 2015 and May 2016.

Measurement uncertainty of the LC-IRMS method was estimated using the intra-laboratory reproducibility standard deviation (Table 4). The expanded uncertainty (*U*) of the $\Delta\delta^{13}C$ values used for compliance testing (Table 1) was calculated according to:

$$U = 2 * \sqrt{SD^2(a) + SD^2(b)}$$

$SD^2(a)$ and $SD^2(b)$ being the intra-laboratory reproducibility variances of the sugars used for calculating the differences.

5. Results

5.1 EA/LC-IRMS method implementation and evaluation of detection capability

A series of adulteration experiments were carried out to test the sensitivity of the LC-IRMS method for detection of addition of C_3, C_4 and mixtures of C_3 / C_4 exogenous sugars. For C_4 sugars, a honey sample which complied with all the purity criteria of the method proposed by Elflein and Raezke was selected. It exhibited a $\Delta\delta^{13}C_{max}$ value of 1.36‰ and a $\delta^{13}C_{protein}$ value of -25.12‰.

This honey was also found compliant using the AOAC 998.12 method. For adulteration, high fructose corn syrup (HFCS-42) obtained from Cargill (Mechelen, BE) was added at 1%, 3%, 5%, 10%, 15%, 20%, 30% and 50%. It did not contain oligosaccharides.

HFCS-42 could be detected at very low concentrations (between 1% and 5%) because the $\Delta\delta^{13}C_{max}$ parameter was already above the limit of 2.1 at 1% HFCS-42 added to honey.

For the C_3 and mixtures of C_3/C_4 experiments, a honey which complied with all the purity criteria of the method proposed by Elflein and Raezke and which also complied with the AOAC method 998.12 was selected. It was a lavender honey exhibiting a $\Delta\delta_{13}C_{max}$ value of 1.58‰ and a $\delta^{13}C_{protein}$ value of -26.46‰. In this sample, no oligosaccharides were detected by LC-IRMS. The C_3 adulteration experiment was conducted by adding 1%, 3%, 5%, 10%, 15%, 20%, 30% and 50% of rice syrup. For the experiment with the mixture of C_3/C_4 sugars, equal weights of HFCS-42 and rice syrup were thoroughly mixed. The C_3/C_4 adulteration experiment was conducted by adding 1%, 3%, 5%, 10%, 15%, 20%, 30% and 50% of the mixture to the pure lavender honey sample.

Adulteration with rice syrup (C_3 plant) could only be detected using the percent area of the oligosaccharide peak parameter. The $\Delta\delta_{13}C_{max}$ parameter did not significantly exceed the limit of ±2.1 even at 50% adulteration with rice syrup. The results indicated that it was possible to detect adulteration with rice syrup at concentrations > 3% using the oligosaccharide percent peak area parameter which was > 0.7% at this level of adulteration.

A 10% addition of a mixture (50/50) of HFCS-42 (C_4) and rice syrup (C_3) could be detected by using the oligosaccharide percent peak area parameter, which exceeded 0.7%. The $\Delta\delta_{13}C_{max}$ parameter was only exceeded by adding more than 20% of a mixture (50/50) of HFCS-42 and rice syrup. These results showed that the most important Elflein and Raezke purity criterion to detect adulteration with rice syrup was the oligosaccharide peak area.

Analysis of the data also suggested that if two or more parameters of the Elflein and Raezke method were non-compliant, this would most probably indicate adulteration with a mixture of C_3 and C_4 sugars. In our experiment, both the $\Delta\delta_{13}C_{max}$ and the percent peak area of the oligosaccharide parameters were non-compliant when equal quantities of C_3 and C_4 sugars were used for adulteration at levels above > 20%.

5.2 Comparison of results obtained by the MS and JRC

Four countries shared LC-IRMS results with the JRC for external quality control purposes. Norway, Finland, and the Czech Republic submitted results obtained by the Elflein and Raezke method for a total of 35 samples. Spain used the method of Cabañero and shared

the results for 10 samples. The latter method uses the $\delta^{13}C$ values of fructose, glucose, and sucrose but not of trisaccharides.

To illustrate the inter-laboratory reproducibility of the LC-IRMS method, a correlation was computed considering the $\delta^{13}C$ results of fructose and glucose (common to both methods) for 45 samples. Disaccharide values were available in both JRC and MS for 35 samples. Too few samples (7) had a $\delta^{13}C$ of trisaccharides measured by the MS to allow reliable calculations.

The correlation between the measured parameters is shown in Figure 1. R^2 values ranging between 0.8 and 0.9 confirmed the comparability of data produced by JRC and MS laboratories.

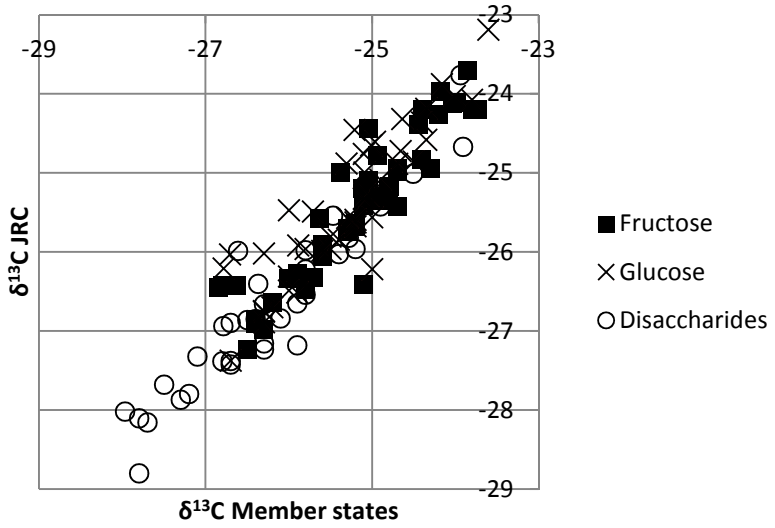

Figure 1. Correlation between JRC and Member States related to the LC-IRMS measurements for δ13C of fructose, glucose, and disaccharides.

For 29 out of 35 samples MS and JRC came to the same conclusion regarding compliance (Table). The JRC judged six honeys as non-compliant according to the Elflein and Raezke criteria, whereas the respective MS did not. Out of these six, three failed because of a low isotopic ratio for the trisaccharides (value not measured by the MS). One was mistakenly compliant by the MS and did not actually comply with the $\Delta\delta^{13}C_{max}$ criterion after recalculation. One failed because of the oligosaccharide area criterion (not calculated by the MS), and the last one failed due to the $\delta^{13}C$ fru-glu criterion. This last discrepancy was the only major disagreement between the two laboratories.

Comparing the JRC results (Elflein and Raezke method) with the Cabañero method, the results were concordant except for one sample. It could be explained by the difference in the $\delta^{13}C$ glucose values between the two laboratories. For that particular sample, the deviation was the largest among the 10 samples (difference of 0.68‰).

Elflein and Raezke method (n = 35)				Cabañero method (n = 10)			
	JRC LC-IRMS				JRC LC-IRMS		
		C	NC			C	NC
MS LC-IRMS	C	26	6	MS LC-IRMS	C	4	0
	NC	0	3		NC	1	5

[1] C: compliant honey; NC: Non-compliant honey.

Table 6. Comparison of the conformity assessments by Member states (MS) and JRC for the Elflein and Raezke, and Cabañero methods.

5.3 Compliance of the honey samples

The Elflein and Raezke (2008) as well as the Cabañero et al. (2006) methods are based on the principle that the differences in the $\delta^{13}C$ values ($\Delta\delta^{13}C_{max}$) of the individual sugars of authentic honey are distributed around a value close to zero. Based on the analysis of 451 authentic honey samples Elflein and Raezke calculated purity criteria (confidence level 99.7%) which flag a honey sample as non-compliant if one of the $\Delta\delta^{13}C$ falls outside the cut-off limit (Figure 2). In the example given in Figure 2, the result in black colour is beyond reasonable doubt outside the acceptable range of values, whereas the result in red colour, though outside the cut-off limit, is compliant, since its associated uncertainty extends inside the acceptable range.

For 208 out of the 893 (23.3%) compliant honey samples submitted by the Member States at least one of the $\Delta\delta^{13}C$ values fell outside the purity criteria of Elflein and Raezke (2008). Applying the generally accepted principle that measurement uncertainty has to be taken into account for decision making, 127 (14.2%) samples were beyond doubt out of the acceptable range and therefore regarded as suspicious of being non-compliant.

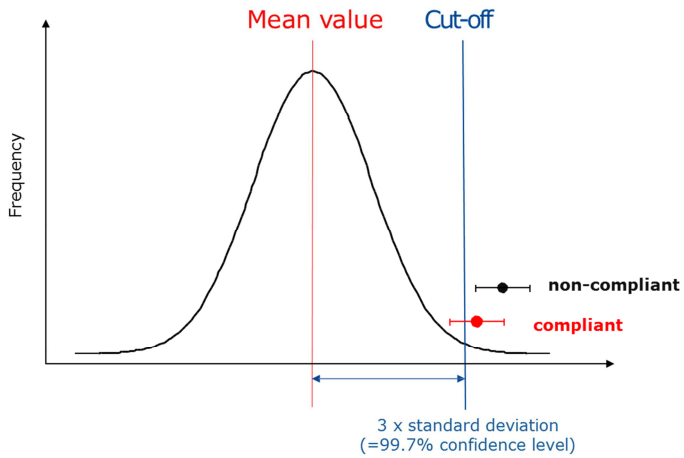

Figure 2. Rule for deciding whether a honey sample is suspected of containing added sugar (syrup) representing the distribution of a benchmark purity criterion. Error bars represent the uncertainty of the estimated value.

Table 7 shows the prevalence of suspicion of non-compliant honeys. For blended honeys the suspicion of non-compliance was higher for blends of EU honeys than blends of EU and non-EU honeys. Unblended honeys of EU and non-EU origin exhibited similar levels of non-compliance. It should be noted that the geographical origin of a honey was inferred from the label declaration, which was only verified in a few cases by other analytical methods.

Origin	**Samples**	**Suspicion of non-compliance**	
	(n)	**(n)**	**(%)**
Blend of EU honeys	96	19	19.8
Blend of EU and non-EU honeys	426	40	9.4
Blend of non-EU honeys	30	3	10.0
Single EU Member State	275	53	19.3
Single non-EU country	55	11	20.0
Unknown	*11*	*1*	*9.1*
TOTAL	893	127	14.2

Table 7. Prevalence of suspicion of non-compliant honeys depending on their declared origin (n, number of samples).

Table 8 provides the prevalence of non-compliance at different stages of the supply chain.

Category	**Samples**	**Suspicion of non-compliance**	
	(n)	**(n)**	**(%)**
Border	4	0	0
Distributor	106	8	7.6
Importer	21	2	9.5
Packager	29	4	13.8
Processor	36	3	8.3
Producer	51	5	9.8
Retailer	563	92	16.3
Storage	22	3	13.6
Wholesaler	56	10	17.9
Unknown	*5*	*0*	*0*
TOTAL	893	127	14.2

Table 8. Prevalence of suspicion of non-compliance of honeys at different points of the supply chain (n, number of samples).

The rate of non-compliance of honeys was slightly higher for honey declared as monofloral in relation to polyfloral (Table 9).

Category	Samples (n)	Suspicion of non-compliance	
		(n)	(%)
Monofloral	238	40	16.8
Polyfloral	468	60	12.8
Unknown	*184*	*27*	*14.7*
TOTAL	893	127	14.2

Table 9. Prevalence of suspicion of non-compliance of honeys in relation to their declared floral origin (according to information provided by MS; n, number of samples).

A suspicion of non-compliance of 36% was found for honeydew honey compared to 12% for blossom honey pointing out the susceptibility to fraud of honeydew honey most likely using sugar syrups from C_3 plants (Table 10).

Category	Samples (n)	Suspicion of non-compliance	
		(n)	(%)
Blossom	563	70	12.4
Honeydew	58	21	36.2
Mixture	30	4	13.3
Unknown	*242*	*32*	*13.2*
TOTAL	893	127	14.2

Table 10. Prevalence of suspicion of non-compliance of honeys determined in relation to their declared source (according to information provided by MS; n, number of samples).

For several honey samples the non-compliance with the $\Delta\delta^{13}C_{max}$ criterion can be explained by the observation that the isotopic ratios within the trisaccharide peak were not uniform.

From Figure 3 it becomes evident that in the left flank of the trisaccharide peak the isotopic ratio was more positive compared to the right flank of the peak. It is well known that individual trisaccharides cannot be separated with the type of HPLC column used; however, only this type is compatible with the LC-IRMS technique. When the total area of the trisaccharide peak was used for calculating the $\delta^{13}C$ ratio, this resulted in a more positive isotopic ratio and consequently a high $\Delta\delta^{13}C_{max}$ value outside ± 2.1‰. For some samples, the reverse situation was also observed.

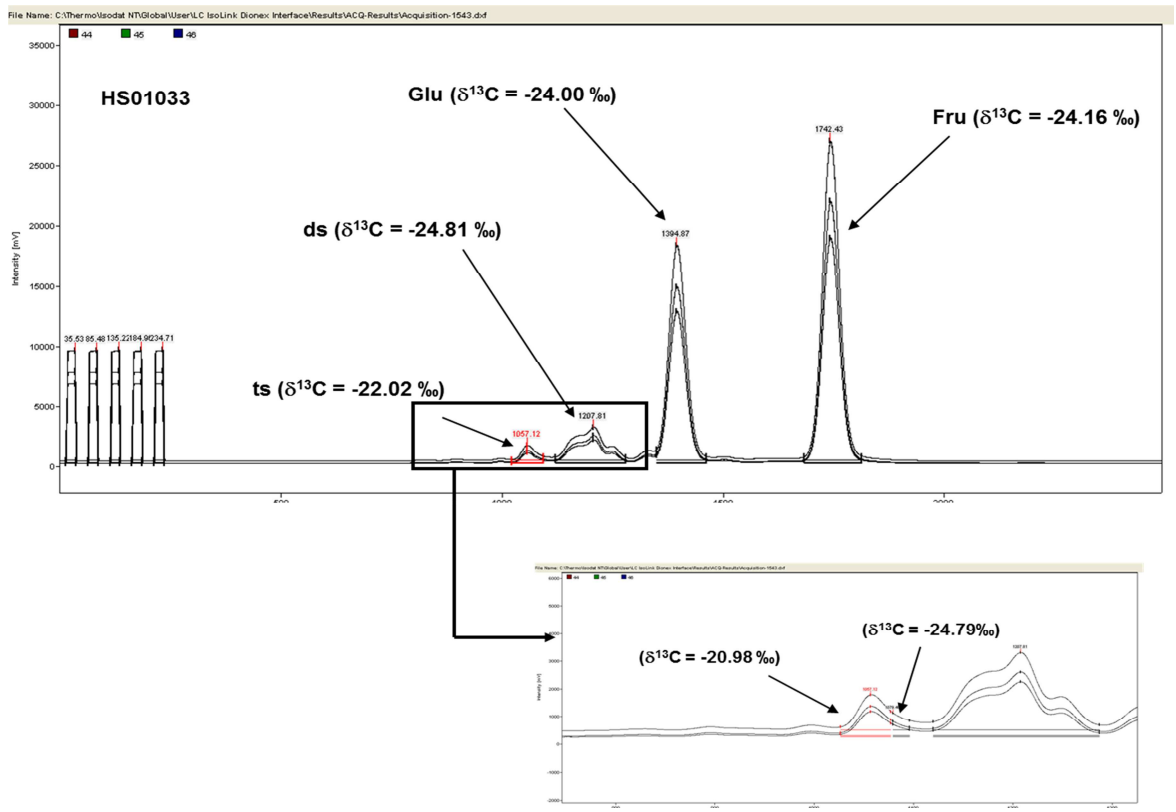

Figure 3. Example of difficulties encountered for estimating of the trisaccharide peak in some honey samples exhibiting a high $\Delta\delta^{13}C_{max}$ value.

Another issue related to the integration of trisaccharide peaks was linked to the peak intensity threshold below which the isotopic ratio of the trisaccharide peak should be ignored when using the LC-IRMS method. At low concentrations the isotopic ratios of the trisaccharides were clearly affected. On the basis of a serial dilution of a standard solution of maltotriose, it was decided that any trisaccharide peak exhibiting a peak area below 10 (arbitrary units) should be ignored. However, several honey samples had peak areas only marginally above the limit of 10, and they exhibited relatively high isotopic ratios in comparison with glucose, fructose or disaccharides. As a result, these samples had high $\Delta\delta^{13}C_{max}$ values and fell outside the purity criteria according to Elflein and Raezke.

Consequently, there is a clear need to define rules for the integration of the trisaccharide peak in the method proposed by Elflein and Raezke.

6. Conclusions

1) The physico-chemical methods applied to check the quality criteria of honeys as laid down in Directive 110/2001/EC are of limited value to detect and prove admixtures of foreign sugars to honey. The reason is that honey is a natural product showing large compositional variations depending on the geographical origin, the botanical type and environmental factors which complicate the definition of exact product specifications to allow a distinction from non-authentic products. Additionally, retail honeys are often commercial blends of various geographical and botanical origins which make it even more difficult to set boundaries.

2) Addition of sugars from C_4 plants (sugarcane, maize) can be reliably detected by the EA-IRMS method with a sensitivity of 7%. The knowledge that sugar syrups made from maize starch, which are readily available at very competitive prices, can be easily detected by EA-IRMS should have a deterrent effect on fraudsters from adding those extenders to honey.

3) Adulteration with C_4 and C_3 sugars can be detected by applying EA/LC-IRMS for the determination of $\delta^{13}C$ values of fructose, glucose, and sucrose in honey, and calculating the differences ($\Delta\,\delta^{13}C$) between these values including the one of protein. The sensitivity of the method is 1% for detecting adulteration with C_4 sugars and 10% for C_3 sugars.

4) The tests performed by EA/LC-IRMS indicated that 14.2% of the 893 honeys analysed within were found suspicious of containing added sugar syrups according to the purity criteria for genuine honey published by Elflein and Raezke [13]. Those purity criteria have been empirically determined and are based on the analysis of 451 authentic honey samples. Although they are frequently used for checking the authenticity of honey, they have not been formally endorsed by competent authorities, standard developing organisations or trade associations.

5) EA/LC-IRMS has an increased sensitivity and ability to detect different kinds of sugar additions not revealed by other techniques. In this respect, di- and trisaccharides, despite being minor sugars in honeys, are important as marker molecules for the detection of sugar addition. To improve the robustness of the EA/LC-IRMS clear rules for evaluating complex chromatograms including the integration of the trisaccharide peak need to be elaborated and tested, preferably by an inter-laboratory study.

6) The detection limits of the EA/LC-IRMS method were empirically evaluated by spiking experiments; however, the detection limits can be higher, particularly when honeys and syrups (C_3 sugars or a mixture C_4/C_3 sugars) used for blending are carefully selected so as to have similar isotopic patterns. In such cases, isotopic screening has to be complemented by alternative analytical methods which are more specific and more sensitive for these types of adulteration. Among the options are the methods which detect oligo- and polysaccharides which do not occur naturally in flower or honeydew honeys.

7. Recommendations

To improve the reliability of the techniques used to determine the authenticity of honey, a number of recommendations are listed below, which are directed to different decision makers.

- Harmonization of analytical methods

Harmonised methods exist for certain provisions of Directive 110/2001/EC. Further harmonisation is still needed, in particular with a view to validate the EA/LC-IRMS method by an international collaborative study. Once accomplished, the International Honey Commission, or another Standard Developing Organisation, such as the European Committee for Standardization, could be approached to endorse the method or accept it for formal standardisation.

- Biobank of honeys, sugar syrups and bee feeding products

In any project to fight food fraud, access to authentic materials covering all its natural variations is essential. To reduce costs and avoid duplication MS are invited to share via a centralised "biobank" samples of their domestic honey production but also sugar syrups and bee feeding products. Modalities of such a biobank should be defined among a network of experts and agreed by the authorities of the MS.

Such a centralised repository of authentic honey samples will form the basis for the development of purity criteria of EU honeys using the EA/LC-IRMS method or alternative methods for testing honey authenticity which may emerge in the future. The biobank needs to reflect the variety of honeys produced in the Member States and the specimens deposited in the biobank shall be produced under the supervision of the national competent authorities so as to ascertain their genuineness. Not only the setting up but also the maintenance of the biobank, which is needed to keep it up to date and take account of scientific developments, will require considerable resources.

- European honey reference database

The specimens of the biobank shall be analysed by the validated EC/LC-IRMS or any other suitable method to estimate purity criteria and their natural variability. All the analytical data and the sampling history of the honey specimens in the biobank should be stored in a centralised honey reference database. As sourcing of authentic honey samples from third countries will be difficult, the developed purity criteria will, strictly speaking, be only applicable to EU honeys. Nevertheless, it is reasonable to assume that traders exporting honey to the EU will voluntarily apply the EU purity criteria to avoid disputes.

Such a database will have many advantages like its legal value recognised by the national authorities for authenticating EU honey and in case of dispute resolution.

The governance of the operation of the biobank and the associated database should be exercised by a network of experts together with representatives from the involved EC services.

- Validation of emerging analytical methods

To date, no universal method exists that is able to determine all the different types of honey adulterants with sufficient sensitivity and robustness. As a consequence, several complementary methods have to be applied in order to perform a reliable assessment of honey authenticity. The availability of authentic samples from the biobank will greatly facilitate the development process. The effectiveness of those alternative methods should be evaluated by the network of experts and afterwards be harmonised and validated.

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List of abbreviations

AFGP	2-acetylfuran-3-glucopyranoside
AOAC	Association of Official Analytical Chemists
CAM	Crassulacean Acid Metabolism
DFAs	Difructose Anhydrides
EA-IRMS	Elemental Analyser - Isotope Ratio Mass Spectrometry
EU	European Union
GC-MS	Gas Chromatography - Mass Spectrometry
HFCS	High Fructose Corn Syrup
HPLC	High Performance Liquid Chromatography
IRMS	Isotope Ratio Mass Spectrometry
LC-IRMS	Liquid Chromatography - Isotope Ratio Mass Spectrometry
MS	Member State
QC	Quality Control
RUBISCO	Ribulose Bisphosphate Carboxylase Oxygenase
SD	Standard Deviation
TLC	Thin Layer Chromatography
VPDB	Vienna Pee Dee Belemnite

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