ARTICLE IN PRESS

Measurement xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Measurement

journal homepage: www.elsevier.com/locate/measurement

Isotopic and elemental composition of selected types of Italian honey

Luana Bontempo *, Federica Camin, Luca Ziller, Matteo Perini, Giorgio Nicolini, Roberto Larcher

Fondazione Edmund Mach (FEM), Via Mach 1, 38010 San Michele all'Adige, TN, Italy

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Honey
Stable isotope ratio
Elemental composition
Botanical variety
Geographical origin

ABSTRACT

According to EU law, the country of origin in which the honey was produced must be declared on the label and the botanical origin can be indicated. Conventional honey analyses are not always applicable and effective for determining the geographical and botanical origin of honey. In this study 265 honey samples of different botanical origin (polyfloral, citrus, rhododendron, eucalyptus, acacia, chestnut and honeydew) produced throughout Italy in different years were analysed to determine stable isotope ratios (SIRs) using Isotope Ratio Mass Spectrometry and mineral element content using Inductively Coupled Plasma Optical Emission Spectroscopy. The aim was to verify the relationship between these parameters and the geographical origin of honey and the botanical species, as has already successfully taken place for other commodities. The characteristic ranges of variability in terms of SIRs and mineral content in genuine Italian honey samples are presented, as well as their compliance with AOAC limit.

1. Introduction

Honey is legally defined as the natural sweet substance produced by *Apis mellifera* bees from the nectar or from sugary secretions of plants as well as from excretions of plant-sucking insects, which live on the sap of certain trees (European Council Directive 2001/110/EC and subsequent amendments, the last being Directive 2014/63/EU). Both the Codex Alimentarius (Codex Standard for Honey, CODEX STAN 12-1981; 1987 and 2001 revisions) and EU law establish that the country or countries of origin where the honey was produced must be indicated on the label and that this information may be supplemented by reference to the floral or vegetable origin. The reason for this is that, in addition to climatic conditions, the type of plant species and flowers visited by the bees may influence the characteristics of the honey, and therefore both botanical and geographical origin determine honey quality [1]. However, neither EU law nor the Codex Alimentarius provide any information on the analytical method that should be used to determine whether the declarations on the label are true or not. Routine analyses (European Council Directive 2001/110/EC), based on parameters with cut-off limits, have been designed to verify the compliance of the product with basic qualitative characteristics, sometimes also based on certain botanical subgroups. Melissopalynological analysis, based on observation of the pollen in honey and developed to identify the geographical origin in terms of macro-areas, has been used especially to detect botanical origin. However, this technique has some limitations, as the analyst needs considerable experience in pollen identification and subsequent interpretation of the results, and furthermore honey could be fraudulently filtered to remove pollen [2].

At research level, several works have been published suggesting different methods that could be useful for diagnosing the botanical origin of honey, on the basis of

* Corresponding author.
E-mail address: luana.bontempo@fmach.it (L. Bontempo).

http://dx.doi.org/10.1016/j.measurement.2015.11.022
0263-2241/

Please cite this article in press as: L. Bontempo et al., Isotopic and elemental composition of selected types of Italian honey, Measurement (2015), http://dx.doi.org/10.1016/j.measurement.2015.11.022

different analytical approaches and chemometric tools (some examples: [3–5]). On the other hand, to date pollen analysis has remained the only method currently used for identification of geographical origin, as the pollen spectrum reflects the environment where the nectar was collected by bees [2]. In the last years, determination of the stable isotope ratios of bioelements and determination of elemental composition have also been suggested as possible investigation methods. Since 1998, comparison between the isotope ratios of carbon ($\delta^{13}C$) determined in honey and the relative extracted proteins has been used as the official method to detect the fraudulent addition of C4 plant-derived sugars to honey (AOAC 998.12 method). Studies contemplating the use of stable isotope ratios in honey have mainly considered their application to determine compliance with the AOAC method [6–8], as well as characterising specific botanical honeys [6,9–12]. A smaller number of studies have been carried out to determine the geographical origin of honey using the isotopic approach. Specifically, Schellenberg and colleagues [13] determined the stable isotope ratios of four bioelements: carbon, nitrogen, hydrogen and sulphur in honey proteins, to verify the origin of honey from 20 European regions (12 countries), and found that carbon and sulphur provided the maximum discrimination. Kropf et al. [11,12] differentiated honeys from Slovenian macro-regions using carbon and nitrogen isotope ratios. On the other hand, Chesson and colleagues [14] found that honey $\delta^{2}H$ and $\delta^{18}O$ values could change because of water absorption and the exchange of H atoms between sugars and water vapour, suggesting that these parameters are of limited use for geo-location.

The mineral composition of honey has been investigated more extensively to characterise both botanical variety and geographical origin [15–23]. It is well-known that mineral content is strictly related to the pedological characteristics of the areas in which the plants grow, but also to the botanical species (see different ash content and electrical conductivity prescribed in European Council Directive 2001/110/EC) as different plants can absorb the various mineral elements in a different way [24,25].

To date, to our knowledge, the combined use of isotopic and elemental composition approaches has never been applied to characterise honey. Moreover isotopic and mineral data for Italian honey have not been extensively studied. In this study, multifloral honeys, honeydew and five groups of unifloral honeys (acacia, chestnut, citrus, eucalyptus and rhododendron) produced in different Italian regions, were collected and analysed to determine the characteristic ranges of variability of stable isotope ratios and mineral content in genuine Italian samples, also checking the compliance of carbon ratios with the official AOAC limit. Furthermore, the influence of the botanical species and geographical origin on isotopic and mineral content was investigated.

2. Material and methods

2.1. Sampling

The study was carried out on 265 genuine honey samples from seven different botanical sources (multifloral $N = 112$, acacia $N = 60$, chestnut $N = 37$, citrus $N = 18$, rhododendron $N = 15$, eucalyptus $N = 13$, honeydew $N = 10$) produced from 1999 to 2005 in different Italian regions at all Italian latitudes.

The botanical origin of the samples was verified through the classic sensory, microscopic and physicochemical parameters [26].

2.2. Preparation for stable isotope ratio analysis

To determine the $\delta^{13}C$ and $(D/H)_l$ (the deuterium/hydrogen – $^{2}H/^{1}H$ isotope ratio of the methyl group of the ethanol molecule) of ethanol, the honey was fermented by adding a yeast strain (*Saccharomyces bayanus* or *Saccharomyces cerevisiae*, about 1–3 g/l) (AOAC 995.17 and 2004.01). The fermented honey was then distilled using a specially designed system, able to prevent any isotopic fractionation. The alcoholic distillate was collected with a yield of more than 98% and an ethanol content of more than 94% w/w, guaranteeing non-significant isotope fractionation [27].

The official AOAC method (AOAC 998.12) according to White and Winters [28,29] for the precipitation of honey proteins was carried out to obtain the $\delta^{13}C_{proteins}$ value to which the $\delta^{13}C$ of whole honey must be compared. $\delta^{15}N$ was also determined on the proteins extracted.

2.3. Preparation for analysis of elemental composition

Before analysis of the mineral content using Inductively Coupled Plasma Optical Emission Spectroscopy, (ICP–OES) all the samples were digested at high temperature and pressure in a microwave oven (Mars5 – CEM, Matthews, USA) in closed vessels. About 0.5 g of honey were weighed into a quartz vessel, with the addition of 5 ml of HNO_3 67% Suprapure (Carlo Erba Reagenti, Rodano, Italy), 4 ml of MQ water (18.2 MΩ, Millipore, Bedford, MA, USA) and 1 ml of Yttrium 500 μg/l (Merck, Darmastadt, Germany) to correct the volume. After the treatment, following suitable cooling of the vessels, the samples were carefully transferred into 15 ml vials, previously washed with HNO_3 5% Suprapure and finally brought to a volume of 13 ml with MQ water.

2.4. Stable isotope ratio analysis

$\delta^{13}C_{ethanol}$ was measured in the ethanol obtained from the fermentation and distillation of honeys according to the AOAC 2004.01 method, using an isotope ratio mass spectrometer (VG Isogas or Delta V Thermo Scientific) connected to an elemental analyser (NA 1500, Carlo Erba Strumentazione, Milan, Italy or EA Flash 2000, Thermo Scientific). Around 1.6 mg of freeze-dried extracted proteins was weighed and placed in tin capsules to measure $\delta^{13}C_{proteins}$ and $\delta^{15}N_{proteins}$ in one run and 0.8 mg to measure the $\delta^{13}C$ of bulk honey. The analyses were carried out using a Delta Plus XP isotope ratio mass spectrometer (ThermoFinnigan) equipped with a Flash EA 1112 elemental analyser (ThermoFinnigan). The isotope ratios were expressed in δ against V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$ and against Air for $\delta^{15}N$ according to the following formula:

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