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Recent developments in honey characterization

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1. Introduction

Honey is the natural food produced by honeybees from the nectar or from the secretions of living parts of plants or from the excretions of plant-sucking insects on the living parts of plants. The bees transform the nectar by combining it with own specific substances; then deposit, dehydrate, store and leave it in honeycombs to mature and ripe according to what described by the Codex Alimentarius (2010)[1] and by the European Community (EU) (EU Council, 2002)[2]. A large variety of monofloral and polyfloral honeys are available on the market, presenting large differences in physical, chemical and organoleptic characteristics. The floral origin of honey can be indicated "if the product comes wholly or mainly from the indicated source and possesses the organoleptic, physico-chemical and microscopic characteristics of the source"[2]. Ordinarily a "monofloral" honey has to show more than 45% of the pollen collected from one single plant species[3]; however according to the type of the plant and consequently according to the content of the grains in the pollen, this percentage could increase up to more than 90%, as in the case of chestnut honey or decrease down to 10-20% as for citrus, arbutus, lavender, thymus, and rosemary honey. "Polyfloral" instead refers to honey presenting variable percentage of grains of pollen deriving from different plants[4-6]. Moreover the honeydew honeys are produced by honeybee that collect liquid secreted by plant sap-sucking insects belonging to the genus *Rhynchota*. Honey is appreciated worldwide because of its readily available source of energy and also because of its antibacterial and antioxidant activity[7]. From the chemical point of view honey is a supersaturated sugar solution (saccharides constituted more than 95% of its dry mass [8-10]) mainly made of glucose and fructose. Other saccharides are present in lower amount as well as minor components such as proteins, free amino acids, organic acids, flavonoids, vitamins, minerals, and several volatile compounds are present, contributing to the organoleptic and nutritional properties of honey. Unfortunately, honey adulteration performed by adding of various cheaper sweeteners such as refined cane sugar, beet sugar, HFCS (high fructose corn syrup) and maltose syrup, by feeding bees with sugars or syrups or by mislabeling of both botanical or geographical origins have become very common. Currently the melissopalinological analysis, introduced by Louveaux et al.[11] in 1978, is the reference method recognized by the authorities to address the botanical and the geographical origin of honey. However, for a correct evaluation of the botanical origin, organoleptic and several physicochemical parameters like color, flavor, pH and total acidity, electrical conductivity, optical activity, moisture, sugar profile, proline amount, invertase and diastase activity are required[12,13]. In the last years the pollen analysis, sometimes coupled with physicochemical parameters, organoleptic and/or chemometrics analysis, has been applied to characterize botanical and/or geographical origin of honeys such as *Trifolium* sp. and *Eucalyptus* sp. honeys from the Argentinian Pampean Phytogeographic Province[14], *Euphorbia* honey samples from the Moroccon region of the Ifni Massif Region[15], different Mexican honeys from a subtropical region (Oaxaca)[16], *Mulinum spinosum* (Apiaceae) honeys from Patagonia[17], Polish rape honey (*Brassica napus* L. var. *oleifera* Metzger)[18], Sierra Morena citrus blossom honey (*Citrus* sp.)[19], artisanal honeys produced on the Northwest of Portugal[20], Algerian[21], and Italian honey[22]. The pollen analysis presents in any case some limitations[23]: specialized analysers are requested to recognize the different pollen typologies; the analysis are time-consuming and moreover honey can be filtered and pollen added fraudulently. In this context, pollen analysis represents a valid approach mainly to define the

geographical origin by considering the entire pollen spectrum (presence or absence of pollen as well as type, quantities and association of pollen types) of honeys rather than their botanical origin. Nothwithstanding fraudulent addiction of pollen can invalidate the geographical origin determination as well. Therefore to preserve honey production, to develop high levels of quality standards, to guarantee its authenticity and to protect consumers from commercial speculations, new analytical techniques and new approaches have been developed in the last years. In this context, floral markers of honeys have been recently reviewed by Kaskoniene et al.[24] while the chemical composition, characterization and differentiation of honey according to both botanical and geographical origins have been reviewed by Wang et al.[25], summarizing research studies until 2010. In the present review the recent findings in the honey characterization by using advanced analytical techniques such as nuclear magnetic resonance (NMR), Raman and Infrared spectroscopy (IR), mass spectrometry (MS) also coupled with chromatographic techniques, and other methods are presented covering the period between 2010 and 2015.

2. NMR spectroscopy

NMR is a spectroscopic technique that allows to analyze samples in all physical states, providing detailed information at molecular level. Several classes of chemical compounds can be analyzed in a non-invasive and in highly reproducible way, within a single experiment, with low experimental time and without any sample preparation. The large set of data obtainable by NMR needs to be handled by multivariate statistical protocols. In the last five years, about twenty articles appeared in literature focused on the honey characterization by NMR. Most of them combined the NMR analysis with chemometrics to assess the botanical or the geographical origin of honey taking the advantage of high resolution (HR) NMR spectroscopy, suitably designed for both qualitative and quantitative analysis of samples as well as for structural determinations in solution. Zieliński et al.[26] analyzed the ^{1}H NMR spectra of aqueous extracts of Polish monofloral honeys such as heather (*Calluna vulgaris* L.), buckwheat (*Fagopyrum esclulentum* L.), lime (*Tilia* L.), rape (*Brassica napus* L. var. *napus*), acacia (*Acacia* Mill.), and multifloral honey. PCA (principal component analysis) and OPLS-DA (orthogonal partial least-squares discriminant analysis) let to differentiate samples according to the botanical origin with the only exception of acacia honey that, supported by pollen analysis, revealed the incorrect classification performed by the producers. The proposed markers by authors were phenylacetic acid and dehydrovomifoliol for heater, formic acid and tyrosine for buckwheat, and 4-(1-hydroxy-1-methylethyl) cyclohexane-1,3-dienecarboxylic acid for lime honey. The water extract of Brazilian honeys have been analyzed by Boffo et al.[27] combining ^{1}H NMR spectroscopy and chemometrics obtaining a discrimination among eucalyptus, citrus and wildflower honeys and identifying a higher content of phenylalanine and tyrosine, sucrose, lactic acid in wildflower, citrus and eucalyptus honey respectively. Moreover by the unsupervised PCA analysis, the authors gathered a clear clustering of adulterated honeys that presented higher content in citric acid, ethanol and 5-hydroxymethylfurfural (HMF). The content of this latter compound could be related to honey adulterated with sucrose but it is usually present as a consequence of a high temperature exposure of honey, or too long storage time under non adequate conditions, pH changes etc. Kynurenic acid for sweet chestnut, and α-isophorone and

2,5-dihydroxyphenyl acetic acid for strawberry-tree honey samples (from different region of Europe) have been identified as botanical biomarkers by Donarsky et al.[28] analyzing the aqua honey solutions by ^{1}H NMR and statistical analysis. Additionally, other partially indentified compounds were also suggested as markers for strawberry-tree and Corsican spring Maquis honey. Simova et al.[29] determined by 1D (one dimensional) ^{1}H and ^{13}C NMR and 2D (two dimensional) TOCSY (TOtal Correlation SpectroscopY) experiments the methylene group of quercitol as the specific compound for the identification of oak honeydew honey; this compound resulted completely absent in other honeydew or other honeys analyzed. Quercitol is a deoxyinositol recognized as a good taxonomic marker for the genus of *Quercus* and it presents some health benefits such as the inhibition of glucosidase activity which blocks the metabolism and the absorption of carbohydrates.

Consonni and his group[30,31] performed a botanical and geographical characterization of honeys on the basis of their saccharide content performed on honey. The authors identified 19 saccharides on the basis of their anomeric proton (fructose, glucose, gentiobiose, isomaltose, kojibiose, maltose, maltulose, melibiose, nigerose, palatinose, sucrose, turanose, erlose, isomaltotriose, kestose, maltotriose, melezitose, raffinose, and maltotetraose) by the aid of HSQC (Heteronuclear Single Quantum Coherence) spectra of standard saccharides and by the use of spiking experiments (Fig. 1). The score contribution plots obtained from PCA analysis performed using the mean values for the buckets of the anomeric region for each floral source analyzed (acacia, chestnut, rhododendron, polyfloral and high mountain polyfloral), allowed the identification of saccharides characterizing each honey botanical origin; these results were confirmed by OPLS-DA models. A good discrimination between polyfloral and high mountain polyfloral honeys and between high mountain polyfloral and rhododendron honeys, these latter both collected at high altitude, were also achieved by performing OPLS-DA models on the NMR data. The corresponding S-plot highlighted the characteristic saccharides responsible for the group separation. The saccharide content resulted useful also to discriminate multifloral honey samples from China, Hungary, Italy, and South America and rhododendron and “high mountain multifloral” honeys from the very closely related regions in the northern part of Italy obtaining, also in this case, the characteristic saccharides responsible for the separation.

<Figure 1 >

Schievano et al.[32] investigated the chloroform extracts of 353 honeys of different floral sources (acacia, chestnut, linden, orange, eucalyptus, honeydew, and ploy floral) obtaining a good discrimination among samples according to their botanical origin, by performing *one vs all* O2PLS-DA models and identifying markers for each origin. In particular chrysin and pinocembrin for acacia, γ-LACT-3-PKA for chestnut, 8-hydroxylinalool and caffeine for orange, dehydrovomifoliol for eucalyptus, a diacylglyceril ether for honeydew, and 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-dienecarboxylic acid and 4-(1-methylethenyl)cyclohexa-1,3-dienecarboxylic acid for linden honey. These and other possible botanical markers for unifloral honeys have been chromatographically purified and characterized by 1D and 2D NMR, and ESI-MS (electrospray ionization) by the same group in 2013[33] (Fig. 2).

<Figure 2 >

In 2014 De Oliveira Resende Ribeiro[34] evaluated the possibility to discriminate honeys according to their botanical origin by using the low field nuclear magnetic resonance (LF ^{1}H NMR), a rapid method to investigate water mobility in materials and foods measuring the proton relaxation. Analyzing 80 Brazilian honey samples of eight different botanical origins such as eucalyptus, "assa-lipto", oranges, Barbados cherry, cashew tree, "assa-peixe", "cipó-uva", and polyfloral a correlation between the water distribution and physical and chemical determinations was found. In particular the authors observed a bi-exponential fitting for transverse relaxation time (T_2) thus suggesting two different water populations in all samples corresponding to the relaxation times of 0.6-1.8 ms (T_{21}) and 2.3-5.4 ms (T_{22}). The observed differences suggested that these were influenced by the different botanical origins. As a matter of fact, a direct correlations were observed between T_{21} and T_{22} parameters and pH, water content and water activity. The same approach was applied by the same group to detect the adulteration of honey with HFCS[35]. The authors observed significant correlations between relaxation times and pH, water activity, and moisture content influenced by different concentration of HFCS; in particular they observed that the relaxation times decreased with increased percentages of high fructose syrup in adulterated honey. The detection of honey adulteration by sugar syrups has been investigated by Bertelli et al.[36] by using 1D and 2D HR NMR and chemometrics. The authors analyzed the DMSO extracts of 63 authentic and 63 adulterated honeys prepared with seven different sugar syrups normally used for nutrition of bees. The multivariate statistical analysis performed on both 1D and 2D NMR data led to obtained models with a good predictive capacity as high as 95.2% and as 90.5% respectively (Fig. 3). Nevertheless the 1D experiment has to be preferred because of its simplest and fastest execution with respect to 2D.

<Figure 3 >

Very recently Dinca et al.[37] achieved a geographical and botanical origin discrimination of Romanian honeys, from different regions and floral sources, by means of IRMS (isotope ratio mass spectrometry) and SNIF-NMR (Site Specific Natural Isotope Fractionating Technique-NMR) coupled with chemometrics. SNIF-NMR is an analytical technique capable of detecting the exact site-specific isotope ratio constituting a specific and sophisticated method adopted in food authenticity determination. The authors included, for the first time, the stable isotope ratio of ^{18}O/^{16}O and ^{2}H/^{1}H from honey water to achieve a better geographical discrimination. Data on δ^{13}C, δ^{18}O, and δ^{2}H by IRMS and data on stable isotopes (deuterium and carbon 13) by SNIF-NMR of ethanol extracts of honeys has been measured. Their findings demonstrated that the use of δ^{13}C value as a single parameter was not useful to distinguish honeys on the basis of their floral origin. Conversely, a clear discrimination was obtained by using SNIF-NMR as complementary technique; as the matter of fact the $(D/H)_1$ values resulted to be specific to a given geographical and botanical origin.

Other NMR approaches such as qNMR (quantitative NMR) and DOSY (Diffusion Ordered SpectroscopY) experiment have been explored by two groups[38,39] to analyze manuka honey, the monofloral honey derived from *Leptospermum scoparium*. In particular Donarsky et al.[38] monitored the content of methylglyoxal (MGO); the presence of this naturally occurring di-carbonyl compound is considered responsible for the non-peroxide antibacterial activity exhibited by this honey species. By qNMR the two forms of mono- and di-hydrated MGO were quantified independently and for the first time by summing these values the total MGO concentration without the need of any chromatographic separation was monitored. The comparison of the results obtained quantifying the MGO in commercial manuka honeys by qHNMR and by the previously applied techniques such as OPD (*ortho*- phenylenediamine) derivatisation, LC, MS or UV detection it was observed that those latter techniques may over-estimate the concentration of MGO in manuka honey. Le Gresley et al.[39] demonstrated that DOSY, which allows to separate different compounds present in a mixture on the basis of their diffusion coefficients, according to the size and the shape of the molecules), combined with qNMR could be useful to isolate and to quantify manuka compounds such as MGO down to ppm level.

Finally, NMR spectroscopy combined with MS has been used to characterize the chemical structure of few honey compounds such as the 2-acetylfuran-3-glucopyranoside, proposed as a novel marker to detect adulteration of honey with rice syrup by Xiaofeng et al.[40] while Steinhorn et al.[41] elucidated the structure of the purified arabinogalactan proteins (AGPs) fraction, endowed with immunomodulatory properties, from New Zealand kanuka honey. AGPS resulted mainly constituted by galactose and arabinose linked in a highly-branched structure, typical of type II AGs. The increasing applications of NMR in food characterization and authentication studies, combined with the development of new instruments and technical solutions will increase the possibility to detect frauds ensuring the quality assessment of honey.

3. Raman Spectroscopy

Raman spectroscopy is based on the vibrational transitions within functional groups of chemical compounds. A particular wavelength of an incident laser beam illuminates the sample and the inelastic scattering signals diffused by the sample is further analyzed. Nowadays, Raman spectroscopy, often combined with chemometrics, is increasingly used to evaluate the safety and the quality of foods[42]. Concerning honey, Pierna et al.[43] obtained the discrimination between Corsican and non-Corsican honeys (Italian, Austrian, German, Irish and French from other areas) of different botanical sources, by combining FT-Raman spectroscopy and chemometrics. In particular, the scattering bands of sugars, unknown carbohydrates and proteins contributed to discriminate honeys into the two groups. Özbalci et al.[44] proposed the quantification of glucose, fructose, sucrose and maltose in honey by coupling Raman spectroscopy with chemometrics methods such as PCA, PLS (Partial Least Squares) and ANN (artificial neural network). By performing PCA on the complete Raman spectra of 40 model solutions prepared by mixing different percentage of glucose, fructose, sucrose and maltose for a total content of 20% of sugars in each solution, a clear clustering among samples was achieved according to the higher sugar content present in the solution. Models/trained networks created using calibration data set and tested by a validation

data set led to obtain correlation coefficients between actual and predicted values for the four sugars with both PLS and ANN models in the range of 0.949 and 0.978. Finally Shuifang et al.[45] applied Raman spectroscopy to distinguish authentic from adulterated honeys with HFCS and/or maltose syrup and to detect these adulterants in honey. By applying PLS-LDA (Partial Least Squares-Linear Discriminant Analysis) on Raman data a good discrimination between authentic and adulterated honeys was achieved. Three sets of samples constituted by authentic and adulterated honeys by HFCS (accuracy 91.1%), or by maltose syrup (accuracy 97.8%), or both of them (accuracy 75.6%) were considered. The better performance obtained by considering the set of samples constituted by authentic and adulterated honey with maltose syrup was explained by the fact that maltose resulted less represented in authentic honey while it is the main saccharide (≥50%) in maltose syrup. Conversely, glucose and fructose content is about 70% in authentic honey resulting close to the adulterated honey with HFCS, whose glucose and fructose content is ≥ 92%. A good accuracy (84.4%) was also achieved by PLS-LDA model considering a set of samples constituted only by adulterated honeys with HFCS or maltose syrup. Very recently Corvucci et al.[46] obtained good results in the botanical discrimination of honeys of different floral sources and origins by combined FT-microRaman spectroscopy and multivariate statistical analysis (PCA). The authors observed that to improve the quality of signal and consequentially the discrimination among samples, FT-Raman spectra should be registered with fluorescence correction.

4. Infrared spectroscopy

In 2011 Cozzolino et al.[47] reviewed several articles concerning the use of infrared spectroscopy in the quality control of honey. Successively Svečnjak et al.[48] applied standard methods and Fourier-transform infrared (FT-IR) spectroscopy to analyze 144 Croatian honeys of nine different unifloral source (black locust, sweet chestnut, lime, sage, heath, rosemary, lavender, mandarin, and strawberry tree) in order to confirm claimed botanical origin. The results obtained by standardized methods confirmed the floral origin for each type of honey analyzed. Moreover significant clustering of samples according to their botanical origin was achieved by performing PCA on selected IR spectral regions (from 1200 to 700 cm^{-1}). FT-IR and electronic nose (e-nose) of pure and adulterated honeys have been analyzed by Subari et al.[49]. In particular ten pure Tualang honeys from Indonesia were analyzed together with the same honeys each adulterated at various concentrations (20%, 40%, 60%, and 80%) with beet or cane sugar. PCA and LDA were performed on FT-IR, e-nose, and on the fusion of the FT-IR and e-nose data; LDA performed on FT-IR data led to obtained higher classification accuracy (88.0%) than e-nose data (76.5%). Nevertheless higher classification accuracies were achieved by using fusion data; in particular Stepwise LDA led to achieve better results than Direct LDA obtaining 92.2% and 88.7% of classification accuracy for fusion data normalized at low-level and intermediate-level respectively. Attenuated Total Reflectance Fourier-transform infrared (FT-IR ATR) spectroscopy was compared with the standard methodologies for physico-chemical parameters determination for eighteen *Melipona subnitida* honey samples by Bicudo de Almeida-Muradian et al.[50]. Significant differences were found only for HMF, ash and electrical conductivity. In addiction, the authors evaluate the effect of temperature on the quality honey parameters by storing honey at room temperature, in the fridge (4°C) and in

the freezer (-18°C). They observed that honeys stored in the fridge were not statistically different from honeys stored in the freezer, except for the free acidity while samples stored at room temperature presented most of the physic-chemical parameters significantly different from those observed for honeys kept in other conditions resulting the best way to preserve honey, above all for colour even if HMF values clearly increased significantly at higher temperature. The same technique was used to perform a preliminary analysis on Brazilian unifloral honey from the northeast region together with palynological, HMF, colour and sensorial analysis[51] and by Lenhardt et al.[52] to analyze 130 Serbian unifloral honey (acacia, linden, and sunflower) with the aim of botanical origin determination. PCA performed on spectral data (between 3718 and 631 $^{cm-1}$) led to obtain a good clustering of samples according to their floral source. The PCA's principal components were successively used in the SVM-DA classification model obtaining a medium classification rate of model equal to 98.6%. Finally very recently FT-MIR spectroscopy has been used by Sultanbawa et al.[53] to detect methylglyoxal content and antibacterial activity in Australian honeys. A linear relationship between methylglyoxal content (in the range of 279-1755 mg/kg) in *L. polygalifolium* (jelly bush) honeys and the bacterial inhibition for *Escherichia Coli* (R^2=0.80) and *Staphylococcus aureus* (R^2=0.64) was observed. Moreover, combining FT-MIR data with PLS regression, a good prediction (R^2=0.75) of methylglyoxal content in honey was obtained.

Near-infrared (NIR) spectroscopy combined with chemometrics has been used by Xiangrong et al.[54] to detect adulterants in honey, such as sweetener materials. In particular sixty-eight authentic Chinese honeys of six different floral sources and sixty-seven adulterated honeys obtained by adding solutions of glucose and fructose in different ratio have been analyzed. The NIR spectra were compressed by using both wavelet transformation (WT) and PCA analysis, resulting the first more effective in variables selection. Moreover the least square support vector machine (LS-SVM) resulted to be the best powerful model in classifying pure and adulterated honeys, leading to an accuracy of 95.1% for test set samples. The same approach has been adopted by Lanzhen et al.[55] to classify Chinese honeys according to their floral origin. Five different floral sources were analyzed including acacia (*Robinia* pseudoacacia L.), jujube (*Ziziphus* jujube Mill. Var. inermis (*Bunge*) Rehd.), vitex (*Vitex* negundo var. Heterophylla Rehd.), rape (*Brassica* campestris L.) and linden (*Tilia* amurensis Rupr.). The non-linear supervised method BP-ANN (back propagation artificial neural network) resulted to be more suitable for honey classification than the linear MD-DA (Mahalanobis-distance discriminant analysis) obtaining correct classification rates of linden, acacia, vitex, rape, and jujube of 97.1%, 94.3%, 80.0%, 97.1%, and 85.7% in calibration and 100%, 93.3%, 80.0%, 100%, and 73.3% in validation respectively.

5. Mass spectrometry

MS is a very well suited detector for high accurate mass measurements. It is usually coupled with chromatographic techniques and according to recent developments, like hybrid mass spectrometers and other ionization techniques, new frontiers in food characterization and authentication have been explored. In this respect, different approaches covering honey characterization, like botanical, geographical and adulteration detection, could be classified into two phases, liquid and volatile.

Low abundant compounds are usually targeted by mass spectrometry: protein and amino acids (usually present in a range of 0.1-0.,5%), have been recently reviewed by Suan Chua et al.[56,57] . The characterization of volatiles usually imply the analysis of the so called "headspace"; the corresponding concentration of the identified compounds could be further evaluated using kinetic parameters. In the view of the characterization of minor organic components, a dispersive liquid-liquid microextraction technique (DLLME) has been recently proposed as an optimized procedure, leading to a reduced consumption of solvent and other benefits like higher peak intensities for most of the volatile components and detection of highly polar and water soluble compounds, usually scarcely concentrated with respect to HS-SPME (headspace solid phase micro extraction extraction)[58].

The extracted phase could be investigated after a chromatographic separation. Recent MS development and applications in food based on metabolomics studies have been reviewed by Ibanez et al.[59] in 2013.

5.1. LC/MS

Honeys constitutes a very complex matrix, mainly constituted by saccharides but in relatively small amounts, other important compounds could be detected and used as potential markers, like flavonoids, amino acids and terpenes. In other cases these small abundant compounds could add important nutritional characteristics to honey. Honey antioxidant capacity is one of the most attracting parameters when human nutrition and health are concerned. Antioxidant activity and phenolic profile has been characterized on polyfloral Serbian honeys by UPLC-LTQ Orbitrap MS, (ultrahigh performance liquid chromatography-Linear Trap Quadrupole) identifying 24 flavonoids, 10 phenolic compounds and two abscisic acids[60,61]. The regional origin characterization of the two main regions Vojvodina and Zlatibor based on the phenolic profile was also successfully explored, obtaining models with predictive capability for the two areas from the rest of Serbia region. The content of major phenolic and flavonoids were investigated also in Czech honeys from various locations by HPLC-DAD (diode array detection) and GC-MS[62]. The results of such investigations enriched the knowledge on the content of bioactive compounds as a good source of natural antioxidants effective in reducing the risk of occurrence of heart disease and other inflammatory processes. Flavonoids have been investigated in unifloral honeys by HPLC-CEAD (Coulorimetric Electrode Array Detection) after extraction by a nonionic polymer resin and further separation by reverse phase chromatograpgy. By using this process, quercetin, naringenin, hesperidin, luteolin, kaempferol, isorhamnetin and galangin were detected and quantified in honeys of different origin and varieties, confirming floral source as the primary reason for large variations in the flavonoid content. Notwithstanding the successful determination, the authors underlined that only flavonoid content resulted not sufficient in the view of an affordable floral characterization[63]. Flavonoids and their corresponding glycosilated derivatives (e.g. kaempferol, quercetin and isorhamnetin) could be adopted as complementary biomarkers in the floral characterization of Argentinian *Diplotaxix tenuifolia* honeys by HPLC-PAD-MS/MS analysis (HPLC-Pulsed Amperometric Detector-MS/MS) and also to evaluate the degree of maturation of honey[64]. In the view of botanical classification, flavonoids and two isomers of abscisic acid content (proposed as

marker for heater honey) have been evaluated by LC-DAD-ESI/MS analysis for honeys of different locations in Slovenia[65]. It has been already reported that few botanic species could be recognized by specific flavonoids content, used as typical marker: tricetin, myricetin, quercetin, luteolin and kaempferol for eucalyptus, hesperetin for citrus, kaempferol for rosemary, quercetin for sunflower. In the present study, no specific markers have been identified for the botanical classification explored with LDA analysis. Both isomers of abscisic acid and homogentisic acid, evaluated by HPLC-MS/MS analysis, were found to be possible markers for Sardinian strawberry tree honey (*Arbutus unedo* L.) (Fig. 4). Notwithstanding abscisic acids were found in other floral honeys, in Sardinian samples they were found to be present in much larger amount and with a constant ratio of the two isomers of about 1:1[66].

<Figure 4 >

Typical Cuban monofloral honeys were investigated for their total antioxidant capacity (TAC) by HPLC-DAD-ESI-MS/MS. Eight phenolic acids and six flavonoids, with their glycosylated forms, have been recognized. Interestingly, also in Cuban honey, the total phenolic and flavonoid content determined by Folin-Ciocalteu method resulted higher than the phenolic compounds quantified by HPLC analysis thus confirming that the TAC is the result of a synergic activity of phenolic compounds and other reducing molecules[67].

Water-soluble vitamin content (B2, B3, B5, B9 and C) has been successfully quantified at very low level by RP-HPLC, obtaining a LOD ranging from 0.1 mg/kg up to1.75 mg/kg and the LOQ equal to three times LOD, with very good linearity in large concentration intervals. These investigations were performed on honeys of different botanical origins from Sardinia and from Northern part of Italy[68].

Sugar profiles of 50 mono- and polyfloral honeys from different regions of Algeria have been obtained by HPLC analysis; in particular two monosaccharides and nine oligosaccharides have been detected and identified. Principal component and factorial analysis have been applied to chromatographic data for samples differentiation; in this way only Apiaceae honeys could be separated from other botanical species[69].

SPE has been successfully used by Sergiel et al.[70] for pre-concentration and isolation of selected chemical compounds, further analyzed by HPLC-ESI-MS/MS, namely phenolic derivatives in Polish honeys with the aim of botanical assessment.

An optimized analytical method for amino acids investigation in honey has been applied on Estonian honeys with the aim of evaluating a relationship between their content and both botanical and geographical origin. The method proposed by Rebane et al.[71], consists of a sample preparation (SPE extraction) followed by a chemical derivatization (by a DEEMM reagent, diethyl ethoxymethylene-malonate) and chromatographic analysis. The use of this reagent allowed both UV and MS detection. Amino acids content resulted also useful in characterizing rhododendron and honeydew honey from Turkey by LC-API-MS analysis (LC-Atmospheric Pressure Ionization-MS), combined with PCA and HCA (Hierarchical Clustering Analysis). The result obtained by Silici et

al.[72], indicated free amino acids as good indicators for botanical origin discrimination between these two species, in fully agreement with melissopalynological analysis.

5.2. GC/MS

Concerning the investigation of the volatile fraction of honey, several examples are reported in the literature about the use of GC/MS analysis for both floral or geographical characterization. Phenolic compounds, terpenoids and aliphatic compounds are mostly detected compounds in honey samples.

Taking the advantage of the performance of gas chromatography and the quadrupole time of fly (QTOF) mass spectrometry, volatile and semi-volatile fractions could be detected and accurate mass capability of this system enabled discrimination of compounds with the same nominal masses but having different empirical formulae, with a mass window of 0.005Da (error below 1mDA), particularly powerful in the identification of new compounds[58].

American honeys of different floral sources and locations (Indiana and Ohio states) were investigated by Agila et al.[73]. The results suggested a different volatile composition of honey with the same botanical source but different origin. In this study, H_3O+, NO+ and O_2+ were adopted as selected source for positive ionization in the ion flow tube (SIFT)-MS spectrometer (Selected Ion Flow Tube-MS). The same authors evaluated the effect of the addition of HFCS up to 40% to the same American honeys previously investigated[74].

Greek unifloral honey of different botanical sources have been successfully discriminated by performing PCA and OPLS-DA on the aroma fraction obtained by SPE extraction with a divynilbenzene-carboxen-polydimethylsoloxane fiber (DVB/PDMS), which provides the broadest range of extracted volatiles from the headspace[75].

An interesting study connecting flower chemistry and the collected unifloral honey has been presented very recently by Aronne et al.[76] . *Robinia* honeys of different origins were investigated by SPME-GC-MS analysis. The comparison between honey and flower profiles of different tissues (stamens, petals, calyxes and nectaries) highlighted compounds derived directly from the flowers, such as hotrienol and β-pinene.

Manuka and kanuka honeys as well as Australian jelly bush honey were analyzed by UHPLC-PAD-MS/MS for the non-volatile fraction while HS-SPME-GC/MS was used for the volatile fraction (Fig. 5). These samples were successfully differentiated among them on the basis of their non-volatile profile by chemometrics (PCA), highlighting the characteristic substances for each honey and allowing the classification of samples in three clusters[77].

<Figure 5 >

The volatile fraction of thistle Italian honeys aimed with the assessment of its botanical origin, have been investigated with two different extraction procedures: an optimized HS-SPME (head space solid phase microextraction) better suited for less volatile compounds and DHS (dynamic head space) better suited for high volatile compounds. Sixteen compounds have been selected

among the volatile profiles and submitted to statistical analysis by means of one way ANOVA, allowing the characterization of this thistle honey[78].

A study performed on western Mediterranean honeys combined pollen analysis and volatile fraction investigated by GC-MS in the view of characterizing the typical autumnal products[79].

Chestnut honey from different areas in Spain were investigated by Castro-Vazquez et al.[80]. The effect of the geographical origin was pursed comparing the sensory characteristics and the volatile composition. PCA analysis performed on the whole data allowed successful differentiation of north-east, north-west and south-east areas of Spain.

Three types of honeys (Tahonal, Dzidzilché and Haabin) from the Yucatan peninsula have been classified according to their botanical origins by using HS-SPME-GC/MS analysis with DVB/PDMS fibers. The PCA analysis performed on selected peaks out of the 70 peaks derived from the volatile fraction data showed the possibility of a successful differentiation of these honeys[81]. The use of DVB/PDMS fibers, evaluated among five commercially available fibers, allowed the best results in terms of the highest number of detected compounds. In this respect, 35 samples of honeydew honeys from Slovakia have been investigated by GC-GC-TOF/MS technique, giving rise up to 300 compounds detected, belonging to different chemical classes (hydrocarbons, alcohols, aldehydes, Ketones, terpens and aromatic derivatives). Only four of them, namely 2,3 butanediol, 3-hydroxy-2-butanone, acetic acid and methyl ester of 2-hydroxibenzoic acid were established as markers of honeydew honey[82].

The rare *Acer* spp. honey from Croatia has been investigated by Jerkovic et al.[83] Ultrasonic solvent extraction (USE) has been performed to obtain the volatile components, further analyzed by GC/MS (Fig. 6). Syringaldehyde, previously found in maple sap and syrup, resulted the marker for this rare honey sample. The same extraction procedure was further performed by the same group[84] to characterize the Sardinian unifloral Sulla (*Hedysarum coronarium* L.) honey. Notwithstanding the composition of the volatile fraction of this honey resulted quite distinctive respect to other honeys approached with GC/MS, no specific markers of the botanical origin were found.

<Figure 6 >

Two different extraction techniques such as HS-SPME and USE (Ultrasonic Solvent Extraction) followed by GC/MS analysis were employed in the study of *Prunus mahaleb* L. honey aimed to a possible botanical characterization[85]. Vomifoliol and cumarin resulted characteristic compounds for *P mahaleb* honey, suggesting their potentiality as biomarkers for botanical origin. The same analytical approach has been used by the same group[86] for establishing the botanical origin of *Asphodelus microcarpus* Salzm. and Viv. Honey. Methyl syringate (methyl 4-hydroxy-3,5-dimethoxybenzoate) resulted as a potential marker of asphodel honey.

An optimized SPME extraction procedure based on different parameters setting like temperatures and times used in a single assay, has been proposed by Bianchin et al.[87], for screening of volatile compounds in honey. In particular explored ranges for temperature was 0-60°C, extraction time 10-18 min, salt percentage 0-100% and water volume 0.5-5ml. The optimum percentage of salt versus extraction time was observed for more than 80% and for 60 min respectively, with the

amount of water of 2 ml. Because of optimal conditions of extraction will not be adequate for all volatiles, more than one temperature were needed for the optimization of extraction efficiency and fractions obtained with variable temperature were divided into three groups: most volatile compounds (GP1), intermediate compounds (GP2) and less volatile compounds (GP3). The commercial fiber CAR/DVB/PDMS was chosen and the best results for the extraction procedures were obtained at 60°C for 36 min, followed by 18 min of extraction at 40°C and a final extraction at 0°C for 6 min, with a total extraction time of 60 min. Another SPME extraction optimization has been proposed by Ceballos et al.[88], carried out by response surface methodology applied on Cuban monofloral honeys. In this case, the authors evaluated optimal extraction conditions like temperature, fiber type, salt addition, sample size for pre- and extraction time as well as desorption time. In the optimized procedures 6 g of honey were dissolved in 3ml of Milli-Q water with 1.4 g of NaCl, and preconditioned at 60°C for 20 min, while the head space SPME was obtained after magnetic stirring for 30 min at 60°C. At the end of the extraction procedure, the fiber was removed and inserted into the injection port of the GC for thermal desorption at 250°C for 4 min. The best results was obtained with PDMS/DVB fiber with two desorption times (2 and 4 min).

The major compounds extracted by USE from samples of cornflower honey (*Centaurea cyanus* L.) from Poland were characterized by means of GC-MS and GC-FID resulting in C_{13} and C_9 nor-isoprenoids as useful markers of this botanical origin. HPLC-DAD analysis also revealed lumichrome, riboflavin and phenyllactic acid as typical compounds for this honey type. Additionally, the authors[89] evaluated antioxidant and antiradical capacity as well as colour capacity of this honey.

Langford et al.[90] evaluated in their exploratory study, the performance of Ion Flow Tube Mass Spectrometry (SIFT-MS) in monitoring the VOC (volatile organic compounds) at low concentration level (ppt) to determine the aroma compounds in New Zealand monofloral honeys, without sample preparation. The aroma profiles successfully allowed the discrimination among samples according to their botanical origin (beech honeydew, clover, kamahi, manuka, rata, rewarewa, tawari, thyme and viper bugloss) (Fig. 7).

<Figure 7 >

The Buckwheat honey, a particular honey characterized by a dark purple color, strong animal and malty aroma, has been characterized in terms of composition and properties. Volatiles have been characterized by SPME-GC/MS, consisting in large amount of furfural derivatives and other compounds detected in most of honeys[91].

Unifloral Estonian honeys such as raspberry, rape, heather, alder buckthorn and blossoms of the four corresponding flowers were investigated to check for odour active compounds present in both honeys and blossoms by SPME GC/MS analysis[92]. HCA was used as data treatment, highlighting clustering of honeys from the same botanical origin, even though no exclusive compounds were found for a specific honey/blossom combination.

Rhododendron and pine honeys from the Black Sea region in Turkey and Mugla Marmaris disctrict in southwest Turkey respectively, were characterized by their volatile fractions by SPME-GC/MS

analysis. Within the total of 72 volatile compounds identified for rhododendron honey, 1,2 benzenedicarboxylic acids, tributhyl phosphate, stearic acid, propanoic acid, benzene, ethylphenylacetate, and benzophenone were recognized as floral specific molecules[93]. In pine honey volatile fraction, a total of 42 compounds were identified and nonanal, benzene, 4-hexen-3-ol, alpha-pinene,and 2-heptanone were recognized to be specific floral origin markers of pine honey[94].

SPME followed by comprehensive two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer (SPME-GCxGC-TOF/MS) was used to characterize volatile organic compounds in rape, sunflower, acacia, lime, raspberry, and phacelia honeys from Slovakia. In particular lime honey was found to be the richest in the volatile profile, with more than 900 compounds detected. In general two classes of compounds could be classified: those in common with all honey types and others floral specific, specifically terpenes or aldehydes[95]. The same group investigated the role of enantiomer distribution of chiral volatiles in botanical origin determination, like linalool, linalool oxides, hotrienol, largely abundant, but also 4-terpineol, α-terpineol and all isomers of lilac aldehydes, present in lower abundance. Significant differences in concentrations were observed among rapeseed, orance, acacia, linden, chestnut and sunflower honeys of different geographical origin[96]. Similarly, a recent study on the enantiomers of linalool and its oxides analyzed by DVB-CAR-PDMS-GC/MS, demonstrated that they were less influenced by processes in obtaining and storing honeys samples, thus proposing them as marker for a rapid use in floral origin determination in Sicilian orange honeys[97].

A verification of geographical origin of honeys was investigated by different pattern recognition techniques performed on volatile profiles obtained by SPME-GCxGC-TOF/MS analysis, irrespective of volatile fraction was mainly used in floral characterization. Honey samples were collected within the EU-TRACE project. LDA SIMCA (Soft Independent Modeling of Class Analogies), discriminant partial least square (DPLS) support vector machines (SVM), the newest Pearson VII universal kernel (PUK) were used to discriminate Corsican and non-Corsican samples. LDA, DPLS and SVM were successful in detect mislabeled Corsican honeys[98].

GC-FID and GC-MS analysis have been performed on unifloral sour cherry honey (*Prunus cerasus* L.) for the first time, in combination with other physico chemical determinations. Headspace SPME and USE were previously applied to obtain volatile fraction. The dominant component of USE extract was found to be vomifoliol and additionally moderate content of polyphenols was also found[99].

The distribution of enantiomers of chiral volatiles organic compounds were used to characterize rapeseed, acacia, sunflower basswood and raspberry Slovakian honeys by SPME GC-MS analysis. Mono dimensional GC with chiral stationary phase provided chiral separation of the enantiomers and two dimensional GC was required to determine correct isomeric ratios. In particular, linalool, *cis* and *trans* furanoid linalool oxides, hotrienal and four isomers of lilac aldehydes were determined. Acacia honey resulted characterized by differences in the ratio of lilac aldehyde isomer B and hotrienol, while trans-furanoid linalool was found characteristic for sunflower honeys[100].

Volatile compounds of honey from Nuble province (Chile) were obtained by SPME and carboxen polymethylsiloxane fiber (CSR/PDMS) followed by GC-MS analysis. Different classes of compounds

were identified and among those, ethanol, acetic acid, 1hydroxy-2-propane, 3-hydroxy-2-butane and furfural were found in highest percentage[101].

5.3. Stable isotopes and multi-element analysis

Geographical characterization of food products is an important challenge, and lots of efforts have been focused on this aim by several research groups worldwide. Among all proposed analytical techniques multi-elemental and trace analysis appears to be a quite promising approach notwithstanding the intrinsically large number of elements that need to be detected. Different aspects of elemental analysis have been recently reviewed by Pohl et al.[102] and they will not be treated here. Chemometrics methods are always required for data evaluation as well as a large number of samples to validate statistical models able to classify samples according to their origins. In trace element analysis, different spectrometry techniques have been proposed in food analysis, like flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GF-AAS), inductive plasma atomic emission (ICP-AES) and inductive coupled plasma mass spectrometry (ICP-MS). In these last years ICP-MS experienced a large consensus among different laboratories because of its several advantages against the other techniques, like multi-element measurements endowed with very low detection limits. Fifty-seven Brasilian honeys were investigated by ICP-MS analysis, detecting forty-two chemical elements (toxic and essentials); data mining has been performed by different treatments, using SVM, multilayer perceptron (MLP) and random forest (RF). A subset consisting of only five elements (Pb, Tl, Pt, Ho and Er) resulted sufficient to provide a good geographical origin classification of honey samples[103].
Isotope ratio mass spectrometry (IR-MS) was employed in the view of detecting sugar addition to authentic honey samples[104]. The authors investigated the in-house validation of IR-MS measuring the $\delta^{13}C$ values of whole honey and its protein fraction, by evaluation of linearity, repeatability, accuracy, limit of detection (LOD=0.11%), limit of quantification (LOQ=0.38%) and the recovery of the technique (98.57%). In order to check the validity of the approach, 13 different brands of honey were collected from Turkish market and analyzed; only one sample resulted adulterated.
Alternatively, botanical origin classification has been investigated by ICP-MS using mineral elements on 163 Chinese honeys of different botanical and geographical origins[105]. PCA, PLS-DA and back propagation artificial neural network (BP-ANN) analysis performed on mineral data of 12 elements resulted in a better performance for the BP-ANN model, tested on 42 independent set of honeys: linden, vitex and rape honeys were predicted with an accuracy as high as 100%, one acacia honey was badly predicted and rape honeys were accurately predicted (92.3%) (Fig. 8).

<Figure 8>

Rape, buckwheat and honeydew honeys from different regions in Poland were investigated by ICP-MS analysis measuring 13 elements[106]. Multivariate analysis like CA (cluster analysis) and PCA were used to highlight possible determinant elements in geographical discrimination. The authors obtained a reduced number of elements, namely K, Al, Ni and Cd, as strongly associated with principal component, despite the fact they did not achieved a clear discrimination, most likely due

to the effect of different botanical origins. This preliminary study, was followed by a broader investigation two years later by the same authors. In this last study 140 honey samples of the same previous botanical origin, from 16 different origins were approached. Different pattern recognition techniques like LDA and C&RT (Calibration and Regression Tree) were used in order to obtain a samples classification according to their origins. A perfect discrimination according to the botanical origin was achieved by LDA model, while only a variable classification was obtained for rape, buckwheat and honeydew honeys according to their geographical origins[107].

Organic acids in 140 French honeys (acacia, chestnut, rapeseed, lavender, fir, linden and sunflower) have been also proposed for botanical origin discrimination by the use of $^{13}C/^{12}C$ ratio. Qualitative analysis of 14 organic acids (quinic, pyroglutamic, gluconic, lactic, propionic, formic, butyric, pyruvic, galacturonic, gluatamic, citramalic, citric, iso-citric anc *cis*-aconitic) was first carried out by ion chromatography with electrochemical detector followed by IRMS analysis of $^{13}C/^{12}C$ isotopic ratio. Gluconic acid resulted dominant in honey of all botanical origins. Moreover, fir honey can be easily characterized by its highest content of galacturonic acid, as well as higher content in propionic, pyruvic and citric content, this latter useful in distinguishing fir honey from acacia, rapeseed, lavender and linden. Additionally, sunflower and chestnut honeys contained the higher level of citrate; this organic acid could also be a reliable parameter for the differentiation between floral and honeydew honey. Pyroglutamic could be used to distinguish chestnut from the other monofloral honeys, with the only exception for linden, which showed a very large concentration variability of this acid. Isocitric could be used for distinguishing fir honey from rapeseed, lavender and sunflower. By PCA analysis of raw data, by using only monocarboxylic acids as gluconic, pyroglutamic and pyruvic, and tricarboxylic acids as citric and *cis*-aconitic, a good discrimination among the different monofloral varieties has been achieved[108]. A low number of trace elements (Na, Mg, P, K, Ca, Mn, Zn, Rb, Sr and Ba) were investigated by ICP-MS in rape honey and its corresponding flowers and stems with the aim of tracing rape flower for geographical and botanical origins of honey instead of rape honey[109]. The distribution of these elements appeared different within the investigated matrixes. First of all K, P, Ca, Na and Mg resulted higher respect to the other elements, with K the top value. Additionally, K, P and Ca resulted higher in rape flower and stem, in particular with P, Ca, Mn, Zn and Rb content slightly higher in rape flower than in stem.

6. Other techniques

6.1. Electrophoresis

In 2011 Beckmann et al.[110] investigated the possibility to distinguish filtered and unfiltered honey by the electrophoresis of enzyme fractions. The practice of honey filtration is nowadays allowed, according to both the European Council's Honey Directive 2001/110/EC and the Codex Alimentarius Honey Standard; the filtered honey must be specifically labeled as "filtered honey". The filtration is performed by producers to remove small impurities and crystallization nuclei such as pollen, glucose crystals or foreign particles. After the filtration process, the botanical and geographical origin and consequently the authenticity of honey by means of melissopalynological analysis is no longer possible due to the total removal of pollen. Moreover considering that the

price of honey depends also by the floral source and/or the provenance, the frauds practices of mixing unfiltered more expensive honey with cheaper filtered honey increased. The authors performed on forty-two samples of different botanical and geographical origin, several comparative tests such as pollen spectrum, enzyme activities of diastase and sucrase (or invertase), HMF, pH, protein content, sugar profile, free acids, flavonoids, phenolic acids, electrical conductivity, UV- and IR-absorption on filtered and unfiltered honey. Among all these parameters only HMF, pollen, and enzyme activities presented differences between filtered and unfiltered honey. HMF resulted higher in filtered samples heated before filtration process; pollen, as expected, was absent in filtered ones while the activity of diastase and sucrase decreased after filtration while the protein content didn't change. Anyway the decrease of diastase activity was not sufficient to distinguish filtered from unfiltered honey due to the natural variation of this parameter while reduction of sucrase activity was higher and useful to the purpose. Sucrase fractions of unfiltered honey, isolated by gel chromatography and analyzed by gel electrophoresis, resulted dominated by two protein bands of 40 and 65 kDa. In filtered honey the protein band at 65 kDa was almost absent while the band at 40 kDa was slightly changed. Moreover electrophoresis investigations of blends of unfiltered and filtered honeys at different ratios showed a decrease of the intensity of the 65 kDa with increasing content of filtered honey (Fig. 9). The quantitative densitometry analysis of these two protein bands allowed to detect addictions of filtered in unfiltered honey until 15%.

<Figure 9 >

Rizelio et al.[111] designed a rapid method for determining the content of cations in honey samples by using capillary electrophoresis. This technique allowed the separation and the quantification of K^+, Na^+, Ca^{2+}, Mg^{2+} and Mn^{2+} cations. In particular forty honeys produced in different regions of the state of Santa Catarina in the southern of Brazil were analyzed. By performing PCA it was possible to correlate the cations content of honeys with their provenance observing that honey samples from coastal regions presented the highest amount of cations, most likely due to the influence of salinity from the sea.

6.2. Fluorescence Spectroscopy

In 2014 de Oliveira Resende Ribeiro et al.[112] detected up to twelve elements (K, Ca, Ti, Cr, Mn, Fe, Ni, Cu, Zn, Se, Br, and Sr) in 160 Brazilian honeys from different regions in Rio de Janeiro State (Barra Mansa, Teresópolis, northern and southern Nova Friburgo) by using Total Reflection X-Ray Fluorescence (TXRF). TXRF is a variant of energy-dispersive X-ray spectrometry which allows to detect several elements simultaneously with a low detection limit, small volume of sample and being time-saving method. Samples from Teresópolis presented higher levels of both essential and non-essential elements with the only exception for Ni, with respect to honeys from other regions (Fig.10). K and Ca were detected in higher concentration in all samples while Ni, Cu, Zn, Se, and Sr were in lower concentrations in all samples, indicating a low level of contamination in all regions thus including the Barra Mansa, that showed the lowest overall contamination levels, even though

this site is very close to the largest steel mill in Latin America. In this last case honey could be considered as a bio-indicator of no contaminants of that environment.

<Figure 10 >

More recently Lenhardt et al.[113] proposed a method based on fluorescence spectroscopy EEM (excitation emission matrix) coupled with PARAFAC (parallel factor analysis) and PLS-DA to classify Serbian honeys on the basis of their floral source (acacia, sunflower, linden, meadow, and artificial honey) and to distinguish natural honeys from artificial ones obtained by feeding honeybees with sucrose solutions. Two dimensional fluorescence spectra were measured by recording emission from 270 to 640 nm with the excitation in the range of 240-500 nm. PARAFAC model allowed the determination of the number of fluorophores in honey, the excitation and emission spectra of each fluorophore, and their concentration. In particular the emissions from phenolic compounds and Maillard reaction products showed the largest difference among honeys of different botanical origin. The largest difference in the fluorescence was clearly observed between real and flake honey leading to a PLS-DA model, performed on PARAFAC scores, able to detect flake honey with a sensitivity and specificity of 100%. Good results were also obtained with PLS-DA to classify honey according to their botanical origin with errors of 0.5%, 10%, and about 20% for linden, acacia and both sunflower and meadow respectively.

6.3. Electronic tongue and nose

Electronic tongue (e-tongue) and electronic nose (e-nose) mimic the human taste and smell sensors and their communication with the human brain[114]. The e-tongue systems use different sensors sensible to acids, salts, sugars, bitter compounds etc.. The e-nose systems are constituted by sensors interacting with the volatile compounds of the analyzed matrix. Comparing the results obtained by these e-devices with the organoleptic profile of a reference sample, a digital fingerprinting of the food matrix is obtainable. Both e-tongue and e-nose send signal to a computer and the complex data sets obtained have to be handled by multivariate statistical protocols such as PCA, LDA, HCA, PLS etc or for, non-linear responses, ANN are usually employed. Concerning honey Major et al.[115] a commercial electronic tongue comprised of seven potentiometrics sensors coupled with an Ag/AgCl reference electrode was applied to perform a botanical discrimination and physicochemical profile of acacia, chestnut and honeydew honeys. By applying ANN (artificial neural network) modeling a 100% of correct classification of samples according to their floral source was achieved. The measurement of the physicochemical parameters, whose reference values were determined by traditional methods, by ANN resulted in a correlation between observed and predicted values of 0.999 for electrical conductivity, 0.997 for acidity, 0.994 for water content, 0.988 for invert sugar content, and 0.979 for total sugar content. The same successfully result in the botanical origin determination of honey was achieved by Escriche et al.[116] by using electronic tongue made with seven different electrodes as potentiometric sensors: pure metal (Au, Ag and Cu) and four with metal compound electrodes (Ag_2O, AgCl, Ag_2CO_3 and Cu_2O). PCA and ANN models revealed that potentiometric electrodes,

useful to classified honey according to the botanical origin, didn't seem capable to discriminate among raw, liquefied and pasteurized honey. Comparing the results obtained by the traditional method to monitor the physicochemical parameters, a good correlation by performing PLS analysis was achieved above all for color-Pfund (r^2=0.958), luminosity (r^2=0.935), and diastase activity (r^2=0.926). Weaker correlation were obtained by considering volatile compounds though this improved by considering characteristic compound in each monofloral honey. Very recently Lingxia et al.[117] used instead the electronic nose (e-nose) to identify the botanical and geographical origin (14 different floral sources were considered) of Chinese and Australian honeys and, for the first time, to measure the glucose, fructose, HMF, amylase activity (AA), and acidity of honey in order to determine its quality. The authors explored different statistical protocols but the LS-SVM (least squares support vector machine) models showed better ability for discrimination of both botanical and geographical origin of honey with 100% of accuracy. Moreover three selected sensor algorithms were used to analyze the e-nose fingerprints of honey and quantitative prediction models were built obtaining good accuracy when compared with reference quality parameters.

Honey is a very complex matrix to be investigated. Several chemical classes of compounds are present in a very large range of concentration. Large number of research groups worldwide focus their attention and studies to improve the knowledge of honey characterization; it appears clear that new developments of technological improvements would help in putting lights on this complex problem. In the meantime, it is also clear that the officially recognized methodologies are no more sufficient to answer to the more demanding questions concerning honey authenticity. Complicated and time demanding samples preparation, requirement of specialized persons in pollen analysis significantly slow down analysis response, becoming nowadays non more affordable. The emerging new techniques are opening new frontiers in honey characterization and the more promising approach seems to be the multidisciplinary one, focused on the detection of multiple components, with the aid of chemometrics. Apicultural industries, as well as small producers, will benefit by the advantages of more sophisticated techniques that allow more rigorous controls heightening the level of quality and safety of honey and derivatives.

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Figures

Fig. 1

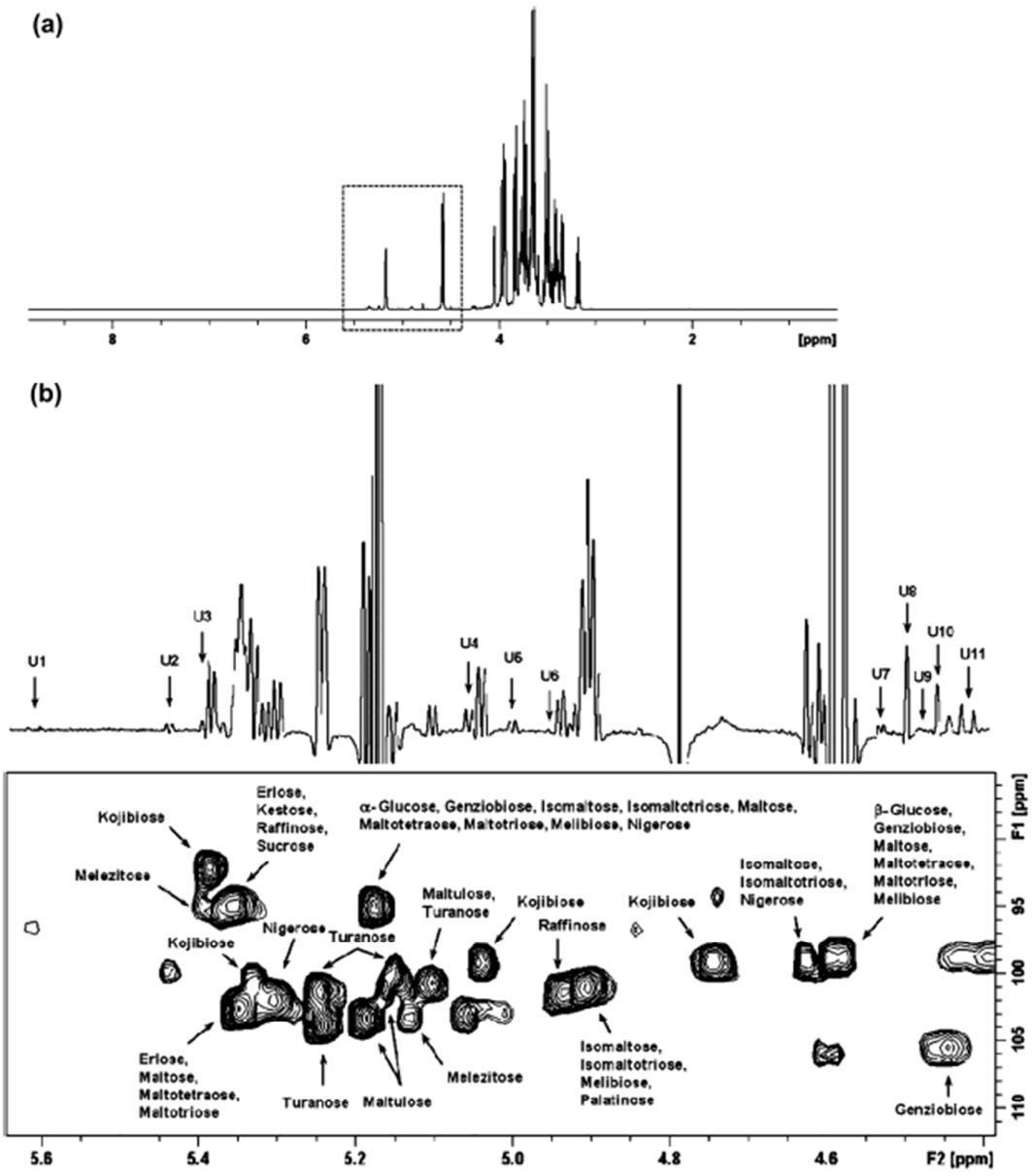


Fig. 2

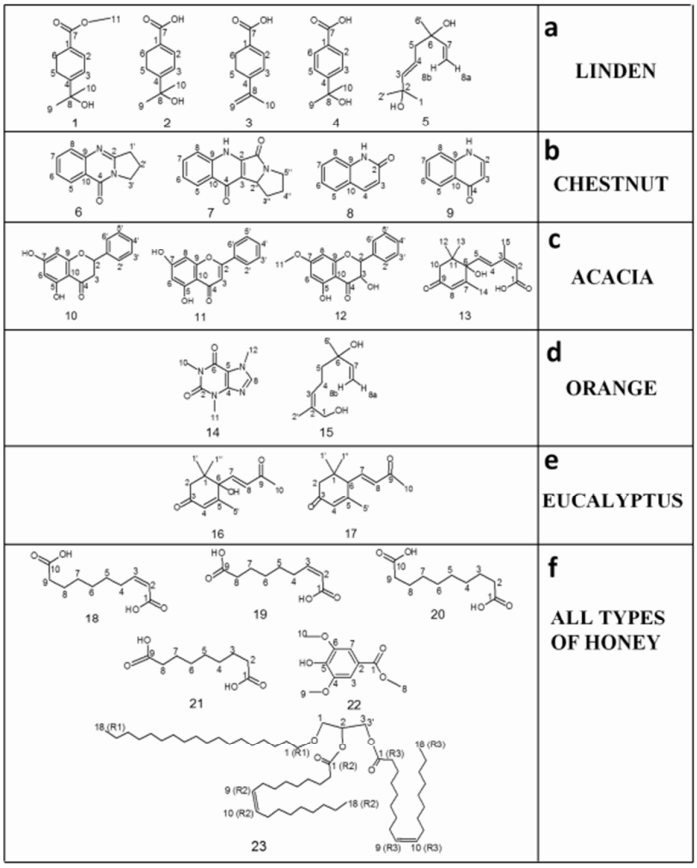


Fig. 3

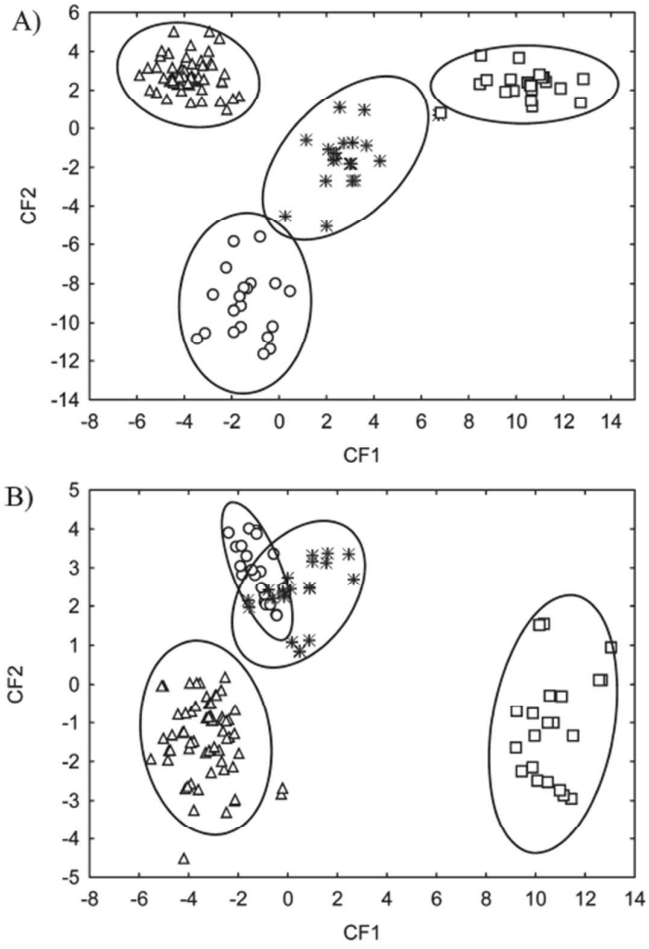


Fig. 4

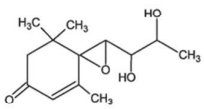
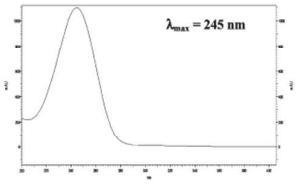
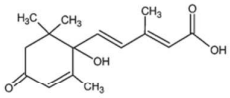
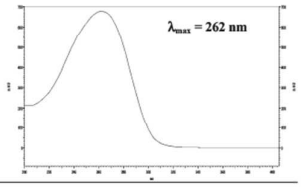
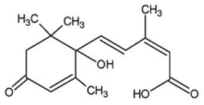
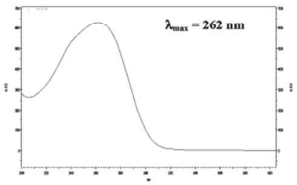
Name	MW	Formula	UV spectra
Unedone (**2**)	240.1434		
(±)2-*trans*, 4-*trans* abscisic acid (**3**)	264.3162		
(±)2-*cis*, 4-*trans* abscisic acid (**4**)	264.3162		

Fig. 5

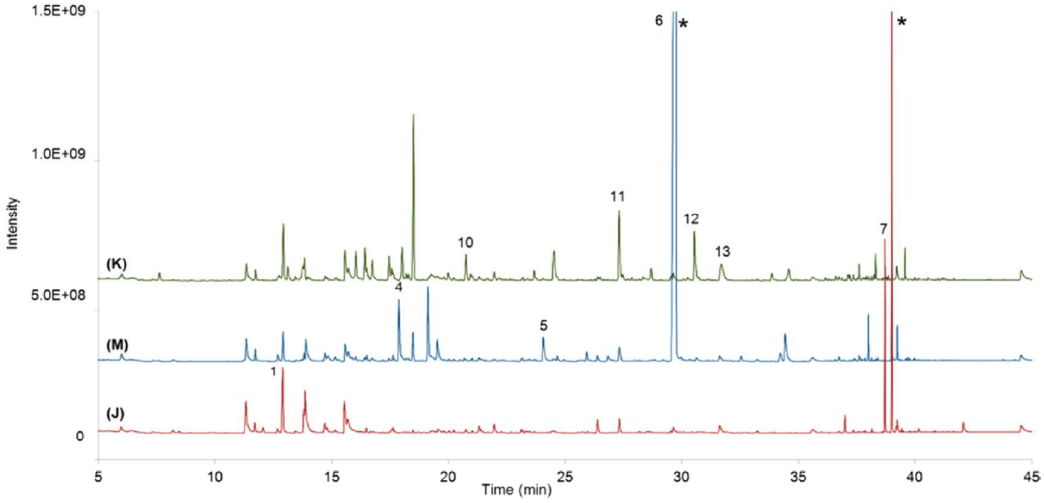


Fig. 6

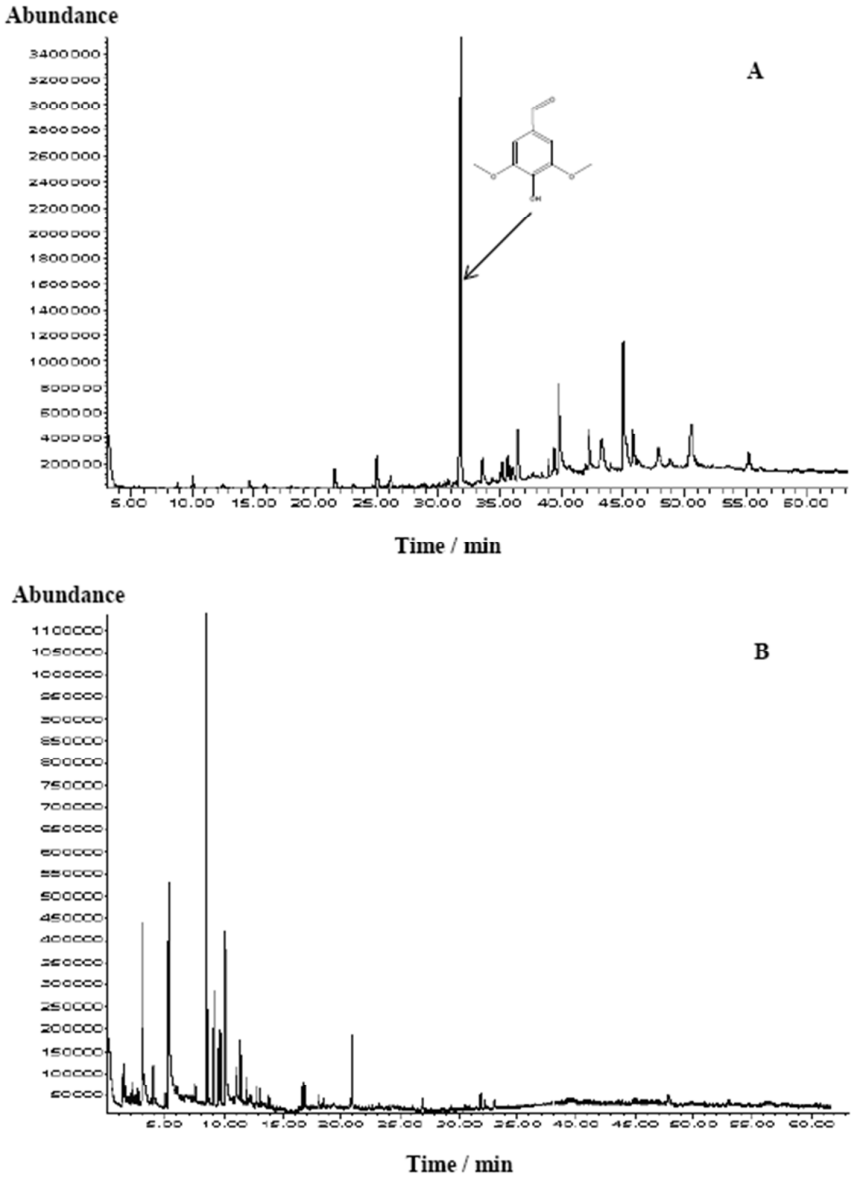
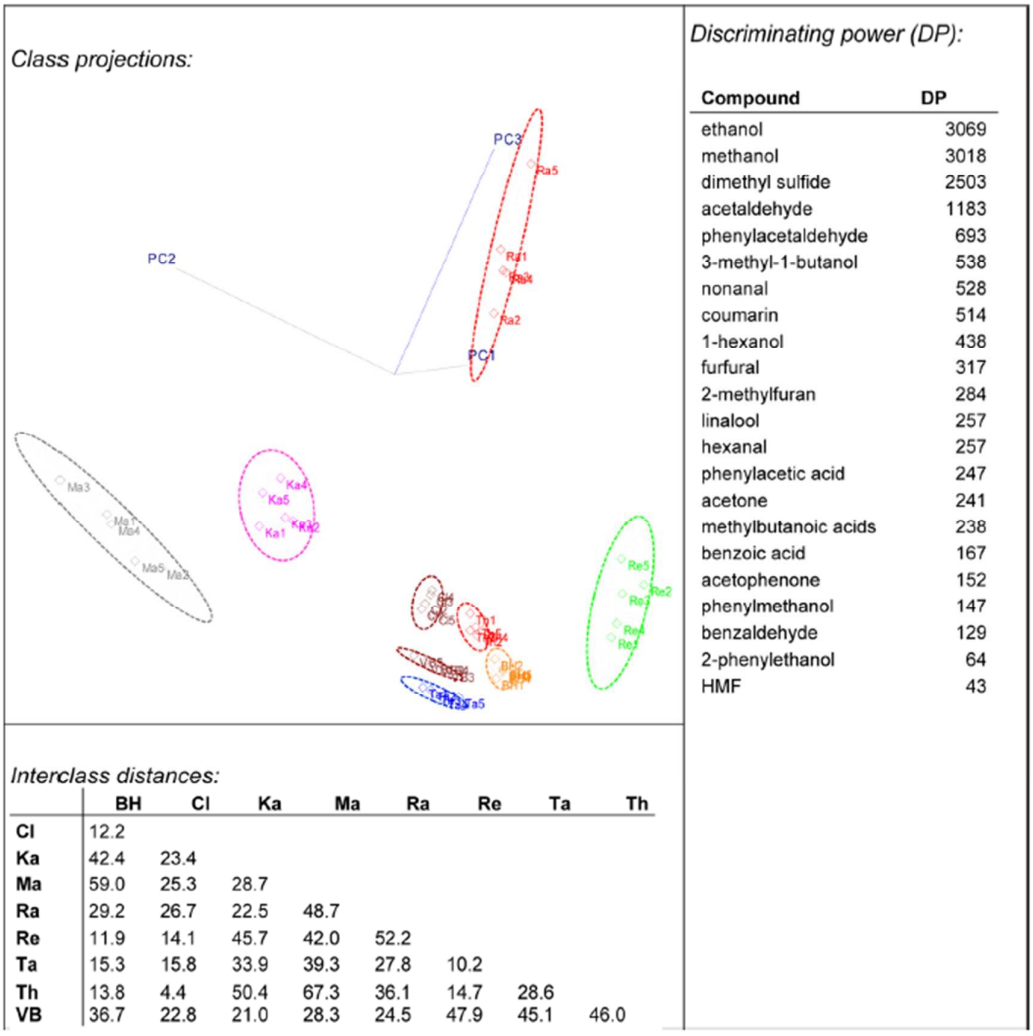


Fig. 7



Discriminating power (DP):

Compound	DP
ethanol	3069
methanol	3018
dimethyl sulfide	2503
acetaldehyde	1183
phenylacetaldehyde	693
3-methyl-1-butanol	538
nonanal	528
coumarin	514
1-hexanol	438
furfural	317
2-methylfuran	284
linalool	257
hexanal	257
phenylacetic acid	247
acetone	241
methylbutanoic acids	238
benzoic acid	167
acetophenone	152
phenylmethanol	147
benzaldehyde	129
2-phenylethanol	64
HMF	43

Interclass distances:

	BH	Cl	Ka	Ma	Ra	Re	Ta	Th
Cl	12.2							
Ka	42.4	23.4						
Ma	59.0	25.3	28.7					
Ra	29.2	26.7	22.5	48.7				
Re	11.9	14.1	45.7	42.0	52.2			
Ta	15.3	15.8	33.9	39.3	27.8	10.2		
Th	13.8	4.4	50.4	67.3	36.1	14.7	28.6	
VB	36.7	22.8	21.0	28.3	24.5	47.9	45.1	46.0

Fig. 8

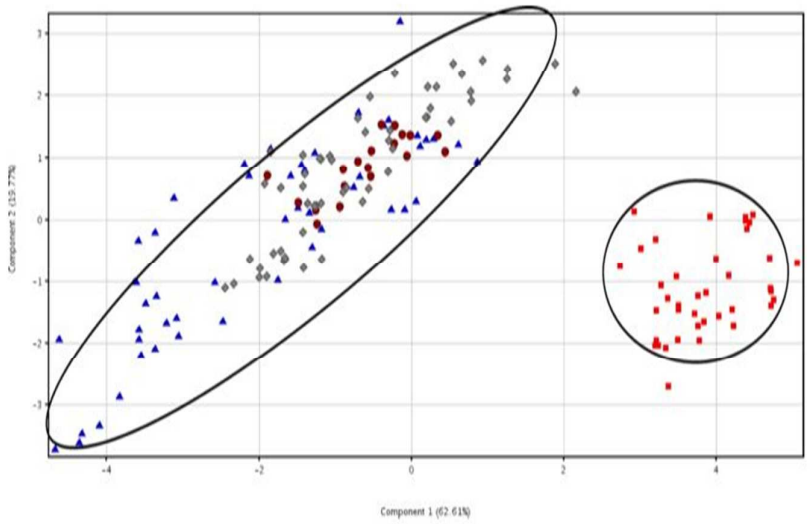


Fig. 9

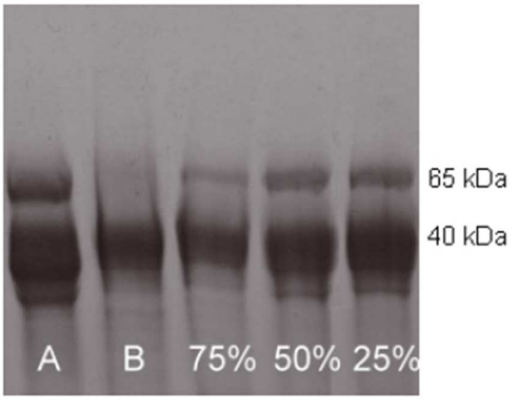


Fig. 10

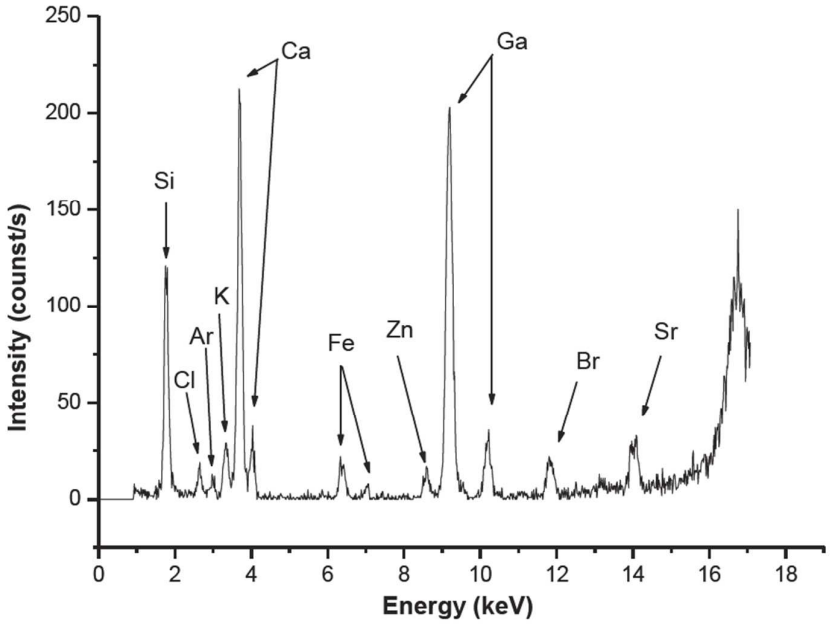


Figure Captions

Fig. 1. (A) ^{1}H NMR spectrum of the aqueous extract of a chestnut honey sample, with the selection of the anomeric region. (B) Anomeric region of 1D ^{1}H NMR spectrum processed with a Gaussian function (LB = −5 Hz and GB = 0.2) and the corresponding HSQC expansion with assignment of saccharides. Unassigned resonances are indicated by the letter U. Reprinted with permission from R. Consonni et al., *J. Agric. Food Chem.*, 2012, 60, 4526-4534. Copyright (2012) American Chemical Society.

Fig. 2. Structures of the identified compounds. Structures of the identified compounds. **1.** 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-dienecarboxylic acid methyl ester, **2.** 4-(1-hydroxy-1-methylethyl)-cyclohexa-1,3-dienecarboxylic acid, **3.** 4-(1-methylethenyl)-cyclohexa-1,3-dienecarboxylic acid, **4.** 4-(1-hydroxy-1-methylethyl)benzoic acid, **5.** (E)-2,6-dimethyl-3,7-octadiene-2,6-diol, **6.** deoxyvasicinone, **7.** γ-lactam derivative of 3-(2′-pyrrolidinyl)-kynurenic acid; γ-LACT-3-PKA, **8.** 2-quinolone, **9.** 4-quinolone, **10.** pinocembrin, **11.** chrysin, **12.** alpinone, **13.** (Z,E)-abscisic acid, **14.** caffeine, **15.** (E)-2,6-dimethylocta-2,7-diene-1,6-diol; 8-hydroxylinalool, **16** dehydrovomifoliol, **17.** 3-oxo-α-ionone, **18.** (E)-2-decenedioic acid, **19.** (E)-2-nonenedioic acid, **20.** decanedioic acid, **21.** nonanedioic acid, **22.** methyl syringate, **23.** diacylglyceryl ether. Reprinted with permission from. E. Schievano et al., *J. Agric. Food Chem.*, 2013, 61, 1747-1755. Copyright (2013) American Chemical Society.

Fig. 3. (A) Score plot of the first two canonical functions for the data set obtained by DMSO-d_6 ^{1}H NMR spectra. (B) Score plot of the first two canonical functions for the data set obtained by DMSO-d_6 ^{1}H-^{13}C HMBC [10% adulterated honeys (○), 20% adulterated honeys (∗), 40% adulterated honeys chestnut honeys (□), authentic honeys (△)]. The confidence ellipses coefficient is set to 95%. Reprinted with permission from D. Bertelli et al., *J. Agric. Food Chem.*, 2010, 58, 8495-8501. Copyright (2010) American Chemical Society.

Fig. 4. Chemical characteristics of the norisoprenoid compounds detected in *A. unedo* L. nectar and honey samples. Reprinted with permission from Tuberoso et al. *J. Agric. Food Chem.*, 2010, 58, 384-389. Copyright (2010) ACS.

Fig. 5. HS-SPME-GC/MS profiles: comparison of pure kanuka honey (K), pure manuka honey (M), and pure jelly bush honey (J); (1) cislinalool oxide, (4) 2-methylbenzofuran, (5) 2′-hydroxyacetophenone, (6) 2′-methoxyacetophenone, (7) 3,4,5-trimethylphenol, (10) 2,6,6-trimethyl- 2-cyclohexene-1,4-dione, (11) phenethyl alcohol, (12) p-anisaldehyde, (13) unknown; * peak cut.. Reprinted with permission from Beitlich et al. *J. Agric. Food Chem.*, 2014, 62, 6435-6444. Copyright (2010) ACS.

Fig. 6. Representative TIC chromatograms of *Acer* spp. honey dichloromethane extract obtained by USE (A) and headspace obtained by HS-SPME (B). Reprinted with permission from I. Jerković et al. *Molecules*, 2010, 15, 4572-4582, open access.

Fig. 7. SIMCA multivariate analysis of SIFT-MS SIM data from New Zealand monofloral honeys. All compounds in the method are shown. The key to the honey types shown in the interclass distances is given in Table 1. See the text for further details. Reprinted with permission from Langford et al. J. Agric. *Food Chem*. 2012, 60, 6806-6815. Copyright (2014) ACS.

Fig. 8. Two first-component scores of honeys from different botanical origins: linden (■), vitex (▲), rape (●), and acacia (◆) honey. Reprinted with permission from Chen et al. J*. Agric. Food Chem*., 2014, 62, 2443-2448. Copyright (2010) ACS.

Fig. 9. Electrophoresis of unfiltered clover honey (A), filtered polyfloral honey (B) and mixtures of filtered honey into unfiltered honey in amounts of 25, 50 and 75%. Reprinted with permission from Beckmann et al., *Apidologie*, 2011, 42, 59-66. Copyright (2011) Springer.

Fig. 10. Spectrum of sample from Teresópolis honey. Reprinted with permission from. R. de Oliveira Resende Ribeiro et al., *J. Food Sci.*, 2014, 79, T738-T742. Copyright (2014) Wiley.

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