A Review of the Analytical Methods used in the Quality Control of Honey

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ABSTRACT

Honey is a natural sweet substance produced by bees (*Apis mellifera)*. In this work, the main parameters used in routine quality control of honey and the most commonly used analytical methods for their determination were reviewed. Honey can be adulterated with cheaper sweeteners or in an indirect way feeding the bees with sugars. Therefore, methods for detecting and quantifying adulteration are necessary. Chromatographic techniques are widely used in honey analysis. More recently, techniques such as Raman, near-infrared, mid-infrared, and nuclear magnetic resonance spectroscopy in combination with chemometric data processing have been proposed. However, spectroscopy does not allow the determination of enzyme activities, one criteria of great importance for the honey trade. Methylglyoxal is an interesting compound for its antibacterial properties. Methods for its determination were also reviewed.

Keywords: honey; quality control; adulteration; analytical methods; hydroxymethylfurfural; methylglyoxal.

INTRODUCTION

Honey is probably one of the most complex natural foods. It is the only sweetener that does not require processing for its human consumption [1]. Bees (*Apis mellifera*) make honey using nectar of plants (blossom honey), secretions of plants, or excretions of plant-sucking insects on plants (honeydew honey). Bees collect and transform the nectar or honeydew and deposit, dehydrate, and store in the hives to allow the honey to ripen and mature (EU Directive 110/2001) [2]. Honey can be adulterated with cheaper sweeteners or in an indirect way feeding the bees with sugars. Therefore, methods for detecting and quantifying adulteration are necessary.

Blossom honey can be classified as 2 types: a) unifloral or monofloral honey and b) multifloral honey. Unifloral honey has a higher market value due to limited production and availability. Due to the existence of protected designation of origin (PDO) and protected geographical indication (PGI), honey classification can also has economic importance [3].

The carbohydrates of honey are formed by the action of several enzymes on nectar sugars. Therefore, honey is a complex mixture mainly composed of carbohydrates (70-80 % w/w), water (10-20 % w/w), and a large number of minor components [4]. Carbohydrates comprise about 95 % w/w by dry weight of honey. The major carbohydrates are glucose and fructose, 65-80 % (w/w) of the total soluble solids, and disaccharides of glucose and fructose having the glycosidic bond in different positions and configurations [5].

Using gas chromatography (GC), 16 oligosaccharides in honey have been found, including 11 disaccharides (turanose, sucrose, maltose, isomaltose, kojibiose, cellobiose palatinose, gentiobiose, laminaribiose, neotrehalose, and nigerose) and 5 trisaccharides (erlose, panose, isopanose, maltotriose, and theanderose) [6]. Other trisaccharides include

melezitose, isomaltotriose, raffinose, kestoses, and isomelezitose, which have been identified by chromatographic methods. Tetrasaccharides in honey include isomaltotetraose, maltotetraose, stachyose, nystose, fructosyl-isomelezitose, α-4′-glucosyl-erlose, and α-6′-glucosyl-erlose [7].

The minor components in honey include, among others, organic acids, proteins, enzymes, and hormones. Organic acids are responsible for the taste of honey. The main proteins are globulin and albumin which come from pharyngeal glands of bees. The enzymes come from the glands of bees being the most important diastase (amylase), invertase, and glucose oxidase. The origins of the hormones (such as acetylcholine and its precursor, choline) are plants or bees themselves. Other minor compounds are flavonoids, phenolic acids, anthocyanins, vitamins, essential oils, pigments, sterols, phospholipids, and minerals. The mineral substances include those occurring in the largest quantities such as Ca, Mg, K, and P, and those in minor amounts such as Fe, Cu, Zn, Co, Si, S, Mn, F, Mo, Cr, and I.

Honey has a high-energy value and many manufactured foods are elaborated with honey as an ingredient. Furthermore, its oligosaccharides seem to present potential prebiotic activity (prebiotic index: 3.38-4.24) in that they increase the populations of *Bifidobacteria* and *Lactobacilli* [8]. Sugars are also responsible for such properties as viscosity, hygroscopy, and granulation [9]. Honey has antibacterial and anti-inflammatory properties that help healing of wounds and burns and treatment of gastric ulcer. Additionally, honey has significant antioxidant activity [10].

Therefore, quality control is necessary to verify the presence of health-beneficial properties of the honey. In this work, the main parameters used in the routine quality control of honey and the analytical methods for its determination are reviewed. Analytical methods to determine carbohydrate composition and detect and quantify

adulteration in honey were reviewed in depth as well as those for methylglyoxal (MGO), an interesting compound known for its antibacterial properties.

ANALYTICAL METHODS FOR QUALITY PARAMETERS

Honey quality is evaluated using its more significant physical, chemical, and biochemical parameters. Such parameters have been classified according to their contribution to honey quality (Table 1). Composition criteria of honey in accordance with the European Legislation cited above are shown in Table 2. The International Honey Commission (IHC, http://www.ihc-platform.net) has recommended methods to use in the routine quality control of honey. In the following sections, the contribution of each parameter to honey quality as well as recent advances in spectroscopic methods for its determination is presented.

Chemical parameters. Moisture is related to the honey capability to remain stable and to resist spoilage by yeast fermentation. The moisture content of honey generally depends on its botanical origin, climatic conditions, and degree of maturity of the honey as well as processing techniques and storage conditions [11,12]. Moisture has an influence largely on sensory characteristics of the product, especially its flavor and granulation [13].

Moisture, together with the main sugars and other physicochemical parameters of honey, has successfully been determined by near-infrared (NIR) and mid-infrared (MIR) spectroscopy. The advantage of using infrared (IR) spectroscopy is that the determination of several analytes in samples can be realized simultaneously. For light-scattering samples such as honey, NIR transflectance spectroscopy is considered a better analytical technique than NIR transmittance spectroscopy. This is because in the

102 transflectance mode backscattered radiation is also collected and measured. Therefore, a
103 more reliable value of absorbance is obtained [14].

104 The moisture measurements in the NIR region were performed in the
105 transmittance [15], transflectance [16], or reflectance mode [17]. Good prediction accuracy was
106 also obtained by Fourier transform near-infrared spectroscopy (FT-NIR) in the
107 transflection mode [18]. Coefficients of determination (r^2) of the models obtained by NIR
108 (transmittance or transflectance) and FT-NIR (transflection) spectroscopy ranged
109 between 0.96 and 1. Prediction standard errors (SEP) and standard errors of cross-
110 validation (SECV) were 0.16-0.3 % (w/w) and 0.08-0.3 % (w/w), respectively [15,16,18]. A
111 study using NIR in reflectance mode reported a SECV of 3.1 % (w/w) and a value of r^2
112 in cross-validation of 0.94 [17].

113 In the MIR region, Fourier transform infrared spectroscopy with attenuated total
114 reflectance (FTIR-ATR) was also evaluated for determining moisture in honey. Good
115 prediction accuracy was obtained, similar to that obtained by NIR, with values of r^2,
116 SEP, and SECV of 0.989, 0.24 % (w/w), and 0.46 % (w/w), respectively [19].

117 The pH is related to the stability and the shelf-life of honey and it can be used to
118 indicate microbial contamination [11]. IR spectroscopy has been evaluated to determine
119 pH and acid parameters in honey. The prediction accuracy obtained by NIR
120 spectroscopy was poor and unreliable [15,18]. However, pH and free acidity were
121 determined with acceptable accuracy by FTIR-ATR spectroscopy [19]. For pH, the r^2 and
122 SEP values were 0.868 and 0.16, respectively. For free acidity, r^2 and SEP were 0.958
123 and 2 meq/kg, respectively.

124 The glucose/moisture ratio is an indicator used for predicting honey
125 crystallization. When glucose/moisture is 1.7 or lower, honey will not crystallize. The
126 fructose/glucose ratio has also been recommended and, when this ratio is higher than

1.3, honey remains liquid or crystallize slowly [18,20]. Fructose/glucose and glucose/moisture ratios are useful for the classification of unifloral honeys [21]. The fructose/glucose and glucose/moisture ratios can be determined by FT-NIR and FTIR-ATR spectroscopy [18,19]. Better prediction accuracy was obtained using the latter. For the fructose/glucose ratio, the r^2 and SEP values obtained were 0.975 and 0.03, respectively, and for the glucose/moisture ratio, the r^2 and SEP values were 0.942 and 0.06, respectively. Using FT-NIR spectroscopy, for the fructose/glucose ratio, r^2 and SEP were 0.820 and 0.09, respectively. For the glucose/moisture ratio, r^2 and SEP were 0.849 and 0.12, respectively.

Ash content is an indicator of the mineral content. It is considered as a quality criterion for honey origin. The blossom honeys have lower ash contents than the honeydew ones (IHC). Generally, the blossom honeys have an ash content ≤ 0.6 % (w/w) while the honeydew honeys, blends of honeydew and blossom honeys, or chesnut honey ≤ 1.2 % (w/w) [4].

Proline is the main amino acid of honey and it is associated with its antioxidant properties [22]. The amount of proline is used as a criterion of honey ripeness and it can also be used to detect adulteration with sugar. The level of proline decreases significantly by adulterating the honey [23]. European legislation does not set a minimum value for the proline concentration in honey. However, honey with proline at less than 180 mg/kg is considered to be either non-ripe or adulterated [24].

The analytical methods proposed by the IHC and the Association of Official Analytical Chemists (AOAC, http://www.aoac.org) are derived from the original Ough photometric method [25]. The IHC method introduces some significant changes that lengthen the time of analysis. Concerning the AOAC method, the absorbance of a honey sample without reagents is subtracted of that of the sample of honey. A comparison

between the 3 methods was recently performed by analyzing honey samples of several botanical origins. No statistically significant differences were found between the Ough and IHC methods for all honey types investigated. Therefore, the extra treatment stages proposed by the IHC method are pointless. On the other hand, the AOAC method provided greater accuracy and time-saving. Quality parameters of the AOAC method were studied. No statistically significant differences between the slopes of addition and calibration lines were found and linearity range was confirmed up to 1800 mg/L. By contrast, the matrix effect was important for the limits of detection (LOD) and quantification (LOQ). The LOQs values were 20 and 61 mg/L using extern calibration and standard additions method, respectively. Analytical recoveries ranged from 92 to 111 %, and precision was better than that reported by the IHC [26]. The spectroscopic techniques, FT-NIR and FTIR-ATR, were evaluated for determining proline. However, prediction accuracy was poor and unreliable due to the low concentrations present in honey [18,19].

Sucrose is an important sugar from a regulatory point of view. The analysis of sucrose content has been mainly used to control adulteration with commercial sucrose. Genuine honeys contain only about 5 % sucrose [27]. A high amount of this sugar is usually due to an early harvest of honey. Therefore, sucrose is not completely transformed into glucose and fructose by the enzyme invertase [28]. High levels of sucrose may also be due to inadequate maturation of the honey [29] or to the fact that the beekeeper over-fed the bees with sugar during spring [30]. On the other hand, during honey storage the sucrose level can decrease by action of the invertase [30].

The measurement of water-insoluble content is used to detect an amount of impurities higher than the permitted maximum value in honeys. Hydroxymethylfurfural (HMF) is widely used as an indicator of honey quality deterioration produced by

177 excessive heating or inadequate storage conditions. The HMF content in fresh honeys is
178 low. However a high concentration of this compound is present in old honeys, honey
179 that has been heated, stored in non-adequate conditions, or adulterated with invert sugar
180 or syrup [31]. Extremely high amounts of HMF (>500 mg/kg) due to adulteration with
181 invert syrup have been reported [32].

182 The interest in the HMF determination in food is also related to its toxicity.
183 Ingestion, inhalation, or skin absorption are the three ways of exposure to HMF [33]. HMF
184 and its derivatives (5-chloromethyl and 5-sulfidemethylfurfural) have been reported to
185 have genotoxic, cytotoxic, mutagenic, and carcinogenic effects [34-41]. However, other
186 studies have suggested that HMF probably does not pose a serious risk to human health
187 [40].

188 Besides HMF, other furanic aldehydes such as 2-furaldehyde (furfural or 2-F)
189 and furan-3-carboxaldehyde (3-furaldehyde or 3-F) can be present in honey, although in
190 smaller quantities. Furanic acids such as furan-2-carboxylic acid (2-furoic acid or 2-FA)
191 and furan-3-carboxylic acid (3-furoic acid or 3-FA) have also been found in honey.

192 Analytical techniques used to determine HMF and other furanic aldehydes and
193 acids in honey are shown in Table 3. The analytical technique regularly used to
194 determine HMF is high-performance liquid chromatography (HPLC). This technique
195 avoids interferences and allows the simultaneous analysis of compounds. The
196 disadvantage of the GC is that it requires a previous derivatization of HMF. The IHC
197 proposed a HPLC method [42] for determining HMF. Honey is simply dissolved in water
198 and HMF is determined by reverse phase HPLC (RP-HPLC) with ultraviolet (UV)
199 detector (285 nm). Elution in isocratic mode with 90 % (v/v) methanol as mobile phase
200 is used. The IHC also proposed 2 spectrophotometric methods known as White method
201 [43] and Winkler method [44]. Differences between the 3 methods were only found at very

low levels of concentration (about 5 mg/kg) without interest for assessing honey quality (IHC).

The White and HPLC methods have recently been compared by several researchers for its application to samples of honey with a very low HMF content (<4 mg/kg) [45]. Quality parameters of both methods were determined. For the HPLC method, the LOD and LOQ were affected by matrix effect. Using the standard additions method, the LOD and LOQ were 0.27 and 0.83 mg/L, respectively. For the White method, the values of LOD and LOQ were similar, 0.22 and 0.67 mg/L, respectively. 43 honey samples were analyzed with both methods. For samples with HMF content between 1 and 4 mg/kg, no statistically significant differences were observed. However, the precision was higher with the HPLC method (3.5 %) than with the White method (6.4 %). Therefore, the HPLC method would be more adequate for this range of concentrations. For samples with a HMF content much lower (<1 mg/kg), the results were inaccurate with either method.

Homogentistic acid (HA) is a phenolic acid (2,5-dihydroxyphenylacetic acid) that has been identified in strawberry-tree (*Arbutus unedo*) honey by mass spectrometry (MS) and nuclear magnetic resonance (NMR). Homogentistic acid has been suggested as a marker for this variety of honey [46]. In strawberry-tree honey, interference of HA in the determination of HMF by the HPLC method proposed by the IHC has been reported. HA completely prevented the quantification of HMF due to overlapping of their corresponding chromatographic peaks. In order to prevent this interference, a new RP-HPLC method using gradient elution (solvent A: 0.01 mol/L H_2SO_4, solvent B: methanol) and detection at 291 nm was proposed. The separation was completed in 15 min, a time 20 % less than that of the IHC method. The LOD and LOQ of the method proposed were 0.2 mg/L (1.9 mg/kg) and 0.4 mg/L (4.0 mg/kg), respectively. For HMF

levels between 5 and 140 mg/kg honey, the repeatability and reproducibility were better than about 2 and 3 %, respectively. Therefore, this method is much more precise than the HPLC method proposed by the IHC for this range of concentrations. The method presented also good accuracy and a high linearity interval (2-800 mg/kg) [47].

Some methods based on IR spectroscopy have been studied to determine HMF. Poor prediction accuracies were obtained because the low concentrations of HMF in honeys [15,17-19]. A method by RP-HPLC for the simultaneous determination of HMF, 2-furaldehyde, 3-furaldehyde, 2-furoic acid, 3-furoic acid, and methyl anthranilate (MA) in honey has been reported [31]. MA is the 2-aminobenzoic acid methyl ester. This compound has been proposed as a marker for citrus honey. In order to prevent overlapping interferences and pre-concentrate the analytes, a previous solid-phase extraction on polymeric cartridges was used. The compounds were separated by gradient elution (solvent A: 1 % (v/v) acetic acid–acetonitrile (97:3), solvent B: 50 % (v/v) acetonitrile). Detection at 250 nm was used. Precision, accuracy, LOD, LOQ, and linearity range for each component were reported. For HMF, the LOD and LOQ were 0.04 and 0.13 mg/L, respectively, and the linearity range was confirmed up to 50 mg/L. Precision (RSD %) was 2.47 %. This method has significant advantages over the method developed for HMF by Spano [47]. It is more sensitive, allowing also the simultaneous determination of other furanic aldehydes and acids as well as MA with great sensitivity (LOD: 0.01-0.08 mg/L). On the other hand, the precision of both methods is similar.

Another method to determine all these compounds, except MA, in honey has been developed [48] as a modified method of that for HMF described by Spano [47]. Elution in gradient mode with 0.1 mol/L H_2SO_4 (solvent A) and methanol (solvent B) was used. For HMF, the LOD and LOQ values reported were 0.003 and 0.010 mg/L. These were

252 lower than those obtained by Nozal [31]. A wider linear range of concentrations was also
253 obtained (0.01-100 mg/L). The precision and accuracy of the method were very good.
254 The repeatability and reproducibility values were 0.99 and 1.47 %, respectively.
255 Average analytical recovery was 97 %. For the other compounds, low LODs and LOQs,
256 wide linear intervals of concentration, and good accuracy and precision were also
257 obtained.
258
259 **Biochemical parameters: Enzyme activity.** Diastase and invertase activities
260 are also used as a marker for the freshness of honeys because they decrease in old or
261 heated honeys.
262 The IHC recommends 2 methods to determine honey diastase activity: Schade
263 method and Phadebas method. The Schade method (traditional) is based on the original
264 work of Schade [49], modified by White Jr and Pairent [50] and Zurcher and Hadorn [51].
265 Diastase activity in Schade units is directly determined using starch as a substrate. The
266 Phadebas method measures the absorbance of a honey solution after the enzymatic
267 hydrolysis of an artificial substrate (cross-linked starch), which is directly proportional
268 to the diastase activity [52]. A method similar to the Phadebas method is the Amylazyme
269 method which employs cross-linked amylose as a substrate (the linear fraction of starch)
270 [53]. The Phadebas and Amylazyme methods use substrates commercially available in
271 tablet format (Phadebas test and Amylazyme tablets) and they are easier to apply than
272 the Schade method. The IHC has also recommended a photometric method for the
273 determination of invertase activity [54].
274 Recently, a direct potentiometric method to determine diastase activity was
275 developed. The method involves the formation of a starch-triiodide complex with
276 characteristic colors. The starch is hydrolyzed by the enzyme producing the release of

277 triiodide iones which are measured using a platinum redox electrode. This method
278 presented a good correlation with both standard methods. It is much simpler and faster
279 and requires low-cost instrumentation. Furthermore, it did not require additional
280 dilutions and volume adjustments. Therefore, the errors associated with this procedure
281 are lower than those of the Schade method [55].

282 **Physical parameters.** Electric conductivity (EC) is a good criterion for
283 differentiate the botanical origin of honey (nectar or honeydew). The EC values of
284 blossom honeys are lower than those of honeydew honeys (Table 2). Blossom honey
285 and blends of these honeys have EC values ≤ 0.8 mS/cm. However, there are exceptions
286 to this rule which include eucalyptus, strawberry tree *(Arbutus unedo)*, bell heather
287 *(Erica)*, ling heather *(Calluna vulgaris)*, lime (*Tilia* spp.), tea tree *(Melaleuca spp.*), and
288 manuka or jelly bush *(leptospermum)* honeys. Honeydew honey and chestnut honey,
289 and blends with blossom honey (except with those indicated above), have values ≥0.8
290 mS/cm. The EC values have also been used for the classification of unifloral honeys [21].

291 IR spectroscopy has been evaluated to determine the EC of honey. The
292 prediction accuracy obtained by FTIR-ATR spectroscopy was good (r^2: 0.979, SEP:
293 0.05 mS/cm) [19]. However, it was lower using NIR reflectance spectroscopy (r^2: 0.88,
294 SECV: 0.01 mS/cm) [17] and FT-NIR spectroscopy in transflection mode (r^2: 0.870, SEP:
295 0.14 mS/cm) [18].

296 Color is one of the most variable features of honey and depends mainly on the
297 botanical origin. Other factors that affect the color of the honey are its ash content,
298 temperature, and length of storage, as well as the presence of antioxidant pigments such
299 as flavonoids and carotenoids [56]. Generally, honeydew honeys are darker than the
300 blossom honeys. Some blossom honeys such as chestnut and heather have also dark
301 color.

The most used methods to determine the color of honey are based on optical comparison using simple color grading after Pfund [57] or Lovibond [21]. Color characteristics can be assessed by the CIE L* a* b* *tristimulus* method. The L* refers to lightness and a* and b* refer to redness-greenness and yellowness-blueness, respectively. The lighter honeys holds an L* value > 50 whereas dark honeys showed a value < 50 [58]. Color grading (mm Pfund scale) has also been determined by NIR reflectance spectroscopy (r^2: 0.97, SECV: 4.7 mm Pfund) [17].

Honey holds the property of rotating the plane of polarized light. Specific rotation depends mainly on types and relative amounts of their sugars. The method recommended by the IHC for its measure is based on published methods [21,59]. Polarimetric properties of honey (direct polarization, polarization after inversion, polarization due to non-monosaccharides, and specific rotation in dry matter) have been successfully calibrated using NIR spectroscopy [60].

The determination of the specific rotation can be used for the differentiation between honeydew honeys and blossom honeys. Most of the honeydew honeys are dextrorotatory, whereas nectar honeys are levorotatory. Furthermore, the specific rotation can also be useful for the classification of unifloral blossom honeys [21].

ANALYTICAL METHODS FOR CARBOHYDRATE COMPOSITION OF HONEY

Honey is composed primarily of carbohydrates. Therefore, this section is dedicated to the review of analytical methods for determining carbohydrates in honey. Furthermore, some of the quality parameters of honey are related to glucose, fructose,

and sucrose contents. Analytical methods to detect and quantify adulteration of honey with commercial sweeteners are also reviewed.

Determination of carbohydrates in honey. Instrumental analytical methods for determining sugars in honey are usually based on chromatographic and spectroscopic techniques as shown in Table 4.

Several chromatographic techniques have been used: HPLC with refractive index detector (HPLC-RID), evaporative light scattering detection (HPLC-ELSD) or diode array detector (HPLC-DAD), anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), gas chromatography with flame ionization detector (GC-FID), and gas chromatography–mass spectrometry (GC-MS).

The main problems for oligosaccharides determination arises from their low concentrations in honeys as well the lack of commercial standards. Furthermore, the presence of structurally similar carbohydrates makes their chromatographic resolution very difficult. The large contents of monosaccharides and the relatively high amounts of disaccharides in honey also make it difficult to separate oligosaccharides with an acceptable resolution. Therefore, they should be separated by a chromatographic run or a previous extraction step.

HPAEC-PAD is a valuable technique for oligosaccharides determination. Sugars are very weak acids (pKa: 12-13) being partially or totally ionized at high pH (12-14). Therefore, they can be separated by anion-exchange chromatography. Oligosaccharides profiles obtained with this technique have been used to detect adulteration of honey with sugar syrups. Before the chromatographic separation of oligosaccharides, honey samples were pre-treated with activated charcoal which removes mono- and disaccharides and retains the fraction of oligosaccharides [61]. HPAEC-PAD has good

sensitivity, comparable to that of GC-FID [62]. Furthermore, PAD proved to be more selective and sensitive than the RI and ELS detections offering the possibility of using gradient elution [63]. However, this detection system is not as usual in analytical laboratories as RI and UV detectors. The UV detector is more sensitive that the RI detector but less than PAD.

HPLC-DAD has the disadvantage of requiring derivatization of carbohydrates time-consuming. A method using this technique has been applied to characterize the di- and tri- saccharides profiles of 5 samples of honey from different botanical origins collected in different regions of Province of Buenos Aires (Argentina). The carbohydrates were isolated from monosaccharides-rich matrix using a solid-phase extraction procedure with porous graphitic carbon cartridges, the extraction being quantitative. Then, they were derivatized with the chromophoric reagent 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid at 70 ºC for 60 min. Subsequently, the sugars were separated and eluted at 40 min by RP-HPLC and detected at 271 nm. LODs (16-24 pmol) were calculated for 4 sugars and correspond to a 0.005 % (w/w) honey sample. This apparent increase in sensitivity was due to the pre-concentration during the solid phase extraction step [62].

Better resolution was provided using GC-MS that HPAEC-PAD for honey oligosaccharides analysis [27]. The main limitation of the GC if that a previous derivatization is needed in order to obtain volatile carbohydrate compounds, a step that may be long, difficult, and time consuming. Furthermore, very complex chromatograms with a high degree of overlap can be obtained due to the large number of carbohydrate isomers produced during the derivatization reaction. The trimethylsilyl oximes obtained from reducing sugars produced only two chromatographic peaks and those from nonreducing sugars one [64-66].

The IHC has recommended several chromatographic methods to determine sugars in honey. One of the methods involves HPLC-RID [67]. It uses a chromatographic column of amine-modified silica gel and 80 % (v/v) acetonitrile as the mobile phase. Column oven and detector must be at 30 ºC. Another method uses HPAEC-PAD. Honey samples are simply diluted with ultra-pure water, being sugars separated with an anionic exchange column and eluted with a sodium hydroxide solution.

Two methods using GC-FID have been recommended: Pierce-Portallier method [68] and I.N.A. method [69]. In the former, any sugars containing free aldehyde or ketone groups, such as glucose and fructose, are derivatized to their corresponding oximes and then silylated. In the second method, sugars are directly silylated. The silylated derivatives obtained are separated and quantified by GC. Mannitol is used as the internal standard.

Sugar composition in honey has been widely studied using chromatography. For instance, amounts of oligosaccharides in 70 samples of Brazilian floral honeys from different geographical regions have been determined using HPLC-RID [70]. The oligosaccharides identified included disaccharides (sucrose, maltose, nigerose, turanose, isomaltose, and melibiose) and trisaccharides (panose, maltotriose, raffinose, and melezitose). The major disaccharide found in all samples was maltose. Its mean content ranged from 1.58 to 3.77 % (w/w) with an average value of 3.05 % (w/w). The lowest amounts of oligosaccharides corresponded to melibiose (0.05-0.15 % (w/w) and panose (0.03-0.08 % (w/w). The presence of melezitose in the floral honeys was probably due to contamination with honeydew while the origin of raffinose was not clear. Raffinose could arise from nectar or honeydew contamination [71].

Sugar composition in Algerian floral honeys from different botanical origins has been also studied [72]. Glucose, fructose, and 9 oligosaccharides were quantified in 50

401 samples using HPAEC-PAD. The separation was completed in 40 min. using gradient
402 elution (solvent A: ultra-pure water; solvent B: 0.2 M NaOH). Maltose and sucrose
403 were the major disaccharides found. The mean values ranged from 0.47-3.42 % (w/w)
404 and from 0.48-5.26 % (w/w) for maltose and sucrose, respectively. Maltose was also the
405 main disaccharide in Brazilian honeys. Melibiose was not detected in all samples.

406 HPAEC-PAD has also been used to determine sugars in 63 samples of
407 Portuguese floral honeys [73]. A good separation was obtained using isocratic elution with
408 a 50 mM NaOH solution for 25 min. LOQs of the analytical method were determined:
409 0.7, 1.2, 1.7, 0.8, 1.3, 1.2, and 0.9 % (w/w) for trehalose, glucose, fructose, sucrose,
410 melezitose, turanose, and maltose, respectively. Repeatability of the method was also
411 evaluated: 0.25, 0.40, 0.16, 0.13, 0.25, and 0.28 % (w/w) for glucose, fructose, sucrose,
412 melezitose, turanose, and maltose, respectively. The separation took place in less than
413 20 min. Maltose and turanose were the most important disaccharides present in the
414 samples in term of quantity. Values up to 2 % (w/w) (mean value 1.47 %) and up to 3 %
415 (w/w) (mean value 2.65 %) were found for maltose and turanose, respectively.
416 Trehalose was the minor disaccharide with amounts below LOQ for most of the
417 samples. Low amounts of sucrose were found; below LOQ for approximately 50 % of
418 the samples. Values of the melezitose up to 6.26 % (w/w) and a mean value of 2.19 %
419 (w/w) were found. These values were higher than those found by others [70,72].

420 Amounts of sugars in Spanish floral honeys by GC-MS have also been reported
421 [74]. 109 samples of honey were analyzed. Sugars were derivatized to their corresponding
422 oximes with 2.5 % hydroxylamine chloride in pyridine at 75 ºC for 30 min. Then the
423 samples were persilylated (45 ºC for 30 min) using hexamethyldisilazane and
424 trifluoroacetic acid and subsequent centrifuged. The chromatographic method used was
425 that described by Sanz [75]. Two capillary columns containing different stationary phases

allowed the analysis of fructose and glucose as well as a high number of disaccharides and trisaccharides. The separation was completed in less than 1 h. The mean amounts obtained for fructose and glucose were 37.1 and 30.0 % (w/w), respectively. The main disaccharides were turanose (2.16% w/w), maltulose (1.92 % w/w), isomaltose (1.35 % w/w), maltose (1.54 % w/w), kojibiose (1.42 % w/w), and trehalulose (1.14 % w/w) which were present in all samples and with a very high variability. The sucrose content was very low in most of the samples. α , β -Trehalose (0.23-0.79 % w/w) was present in all examined samples whereas α,α-trehalose only in 5. The total concentration of trisaccharides was also highly variable in the samples (0.37-5.31 % w/w). Raffinose co-eluted with 1-kestose. Erlose (0.01-3.0 % w/w), raffinose+1-kestose (0.02–0.81 % w/w), and melezitose (0-0.81 % w/w) were the most abundant trisaccharides.

The chromatographic methods present some disadvantages. They are expensive and time-consuming. The industry needs fast, accurate, easy-to-use, and low-cost analytical methods. Therefore, methods based on IR and Raman spectroscopy have been developed to analyze honey. They are rapid and inexpensive, not needing sample preparation or expensive reagents. In addition, in comparison to some chromatographic methods, the spectroscopy has the advantage of being non-destructive.

IR spectroscopy is a technique of great importance in the analysis of foods, especially when it is used in combination with multivariate analysis. IR spectroscopy has been applied to honey analysis: quality control, detection and quantification of adulteration, and authentication of botanical or geographical origin of honey samples.

MIR spectroscopy presents advantages over NIR spectroscopy. MIR spectroscopy is 10-100 times more sensitive than NIR spectroscopy and spectra in the MIR region (2500–25000 nm) contain more information than those obtained in the NIR region (750-2500 nm). The absorption of the major sugar in honey takes place in the

451 region between 1500 and 750 cm^{-1}. Another advantage of MIR spectroscopy is its better
452 resolution. Traditionally, sample handling was a problem in MIR spectroscopy.
453 Nowadays attenuated total reflectance (ATR) crystals have simplified the handling [76].

454 NIR spectroscopy allowed the determination of major compounds in honey.
455 Some minor constituents could also be determined. Fructose, glucose, sucrose, maltose,
456 and moisture have been determined in commercial honey samples by NIR spectroscopy
457 in transmittance mode [15]. The r^2 values between the predicted and reference
458 concentrations were 1.0, 0.97, 0.91, 0.86, and 0.93 for moisture, fructose, glucose,
459 sucrose, and maltose, respectively. SEP values were 0.16, 0.42, 0.34, 0.34, and 0.27 %
460 (w/w) for moisture, fructose, glucose, sucrose, and maltose, respectively. Sharper peaks
461 and better resolution were obtained using transmittance spectra rather than reflectance
462 spectra. Furthermore, the performance of the calibration was 30–70 % better. The
463 optical path length of the cuvette affected significantly the transmittance spectra of
464 samples. An optical path of 1 mm produced the least saturated spectra in the range
465 1300-2500 nm. Therefore, lower SECV values were obtained for all compounds
466 analyzed. NIR transflectance spectroscopy has also been used for the determination of
467 fructose and glucose [16]. The r^2 values obtained were 0.98 and 0.95 for fructose and
468 glucose, respectively. The SEP values were 0.35 and 0.70 % (w/w) for fructose and
469 glucose, respectively. The results obtained were similar to those obtained by Qiu [15].

470 FT-NIR spectroscopy in transflection mode has also been assessed for its
471 application to the simultaneous determination of sugars and main quality
472 physicochemical parameters in honeys. 421 samples of honey from different botanical
473 origins and countries were used in order to develop the calibration model. The most of
474 the samples were collected in Switzerland and Germany [18]. The prediction accuracies
475 obtained were quite acceptable for fructose, glucose, sucrose, and total monosaccharide

476 content (sum of fructose and glucose), as well as for electric conductivity and the
477 fructose/glucose and glucose/moisture ratios. For moisture, the precision accuracy was
478 very good. The r^2 values of fructose and glucose were 0.810 and 0.884, respectively.
479 The values of SEP obtained were 1.6 and 1.3 % (w/w) for fructose and glucose,
480 respectively. The prediction accuracies of fructose and glucose were somewhat lower
481 than those obtained by other authors [15,16]. The prediction accuracy of sucrose (SEP: 0.36
482 % (w/w); r^2: 0.725) was lower than that obtained by Qiu [15] allowing a rough estimation
483 of the sucrose amount. However, the prediction accuracy was poor and unreliable for
484 minor sugars such as maltose, turanose, nigerose, erlose, trehalose, isomaltose,
485 kojibiose, melezitose, raffinose, gentiobiose, melibiose, and maltotriose. The authors
486 considered that the low prediction accuracies of minor sugars obtained are due to the
487 low contents of these compounds in honey, their insufficient separation by the HPLC
488 method used (IHC), and the non-specific absorption bands in NIR. Concerning maltose,
489 it has been determined with good accuracy of prediction by Qiu [15] using NIR
490 spectroscopy although a calibration with fewer samples was used. The determinations
491 of HMF, proline, pH, and free acidity gave also poor prediction accuracy. NIR
492 spectroscopy has also been applied to the determination of low concentrations of
493 perseitol, a sugar that is specific of avocado honey [77].

494 FTIR spectroscopy with micro-attenuated total reflectance (FTIR-mATR) has
495 been applied to the determination of fructose, glucose, sucrose, and maltose in honeys
496 belonging to various floral and geographical origins around the world. Two calibration
497 models were developed; one used 42 standard mixtures (r^2: 0.97-0.99) and another 45
498 samples of honey from different floral and geographical origins around the world (r^2:
499 0.94-0.97). Both models showed a good correlation. However, a calibration model using
500 spectra of honey samples is preferred because it uses samples containing the same

matrix as the real samples improving predictability. On the other hand, this calibration model was developed using a wide variety of samples of different floral and regional sources so that it proves to be more useful to predict amounts of the 4 sugars in a wide variety of honeys [78].

FTIR-ATR spectroscopy was evaluated by Ruoff [19], for the simultaneous determination of a high number of sugars and the main quality physicochemical parameters in honey. Good precision accuracies were obtained for moisture, fructose, glucose, sucrose, melezitose, total monosaccharide content (sum of fructose and glucose), fructose/glucose and glucose/moisture ratios, electric conductivity, pH, and free acidity. The prediction accuracy of the fructose (r^2: 0.84) was similar to that obtained by FT-NIR [18]. Glucose (r^2: 0.94) was predicted with accuracy similar to those obtained by NIR [15,16] and FT-mATR [78]. On the other hand, the prediction accuracy of sucrose (r^2: 0.91) was similar to that obtained by Tewari [78] and Qiu [15], but it was better than that obtained by Ruoff [18]. The prediction accuracy for HMF, proline, and minor sugars (maltose, turanose, erlose, trehalose, isomaltose, and kojibiose) was rather poor [19].

A partial least squares calibration model using second-derivative transformed spectra (PLS-2nd derivative) was developed for the prediction of fructose, glucose, sucrose, and maltose concentrations in honey from different geographical regions around the world by FTIR-ATR. The reference method used to quantify sugars was HPLC-ELSD [79]. Calibration model was developed using a set of 45 standard mixtures. The predicted concentrations had good agreement with those obtained by the reference method (r^2: 0.948-0.988). The correlations found were similar to those obtained in a previous study by Tewari [78] (r^2: 0.971-0.993) where a calibration model with standard mixtures was also developed using FTIR-mATR in combination with PLS. The r^2

526 values for fructose, glucose and maltose were also similar to those found by Qiu [15] using
527 NIR and PLS (r^2: 0.91-0.97). However, r^2 for sucrose was better than that obtained by
528 Qiu [15] (r^2: 0.86).

529 Glucose, fructose, sucrose, trehalose, maltose, melezitose, and turanose were
530 determined in samples of Portuguese honey by FTIR-ATR. HPAEC-PAD was used as
531 the reference method. Good PLS-1st derivative calibration models were obtained for
532 glucose (r^2: 0.879), fructose (r^2: 0.841), melezitose (r^2: 0.890), and turanose (r^2: 0.825)
533 [73]. No calibration model was established for sucrose and trehalose due to the lower
534 amounts of these sugars in samples.

535 Raman spectroscopy is also a valuable technique to evaluate the quality of
536 honey. The main advantages of this over other spectroscopy techniques are that the
537 water present in the samples does not interfere with the Raman measurement. There is
538 only a minimal fluorescence interference of the honey matrix that varies from sample to
539 sample.

540 A method based on Fourier transform Raman (FT-Raman) spectroscopy in
541 combination with PLS was developed to determine fructose and glucose in honey. This
542 method was assessed using a standard HPLC-RID method. Both methods gave
543 statistically similar results being also equivalent with respect to their reproducibility [80].

544 Raman spectroscopy has also used for the determination of maltose and sucrose,
545 as well as fructose and glucose [81]. A method using HPLC-RID was used as the reference
546 method. In this work, commercial honeys were used for the calibration data set. 2
547 multivariate techniques, artificial neural network (ANN) and PLS, were studied. Both
548 techniques provided very good results. The r^2 values between actual and predicted
549 values of sugars ranged between 0.949 and 0.968 for PLS and 0.956 and 0.978 for
550 ANN. As compared to the work of Basoulis [80], a greater number of training and

prediction samples have been used. Therefore, the calibration model obtained seems more reliable in improving prediction efficiency.

Honey adulteration with sweeteners. Due to the their nutritional and healthy properties and unique flavour, the commercial value of honey is much higher than that of other sweeteners such as beet sugar, cane sugar, corn syrup (CS), high fructose corn syrup (HFCS), and invert syrup (IS). Therefore, honey can be adulterated with these sweeteners.

The addition of cane and beet invert syrup is usually difficult to detect. These sweeteners can be added to honey imitating its natural sucrose–glucose–fructose profile. Furthermore, adding only a moderate amount of invert syrup, the levels of glucose and fructose remain within their normal range. However, adulteration with invert syrup may be detected indirectly studying the oligosaccharides profile of honey samples. Genuine and adulterated honeys show different profiles. Oligosaccharides in invert sugar are produced as impurities during the hydrolysis of sugar into glucose and fructose [82].

The presence of high levels of HMF may be due to the adulteration of the honey with inverted syrup since the acid hydrolysis of sucrose produces HMF [83]. However, high levels of HMF are also produced by excessive heating during the honey manufacturing process or by inadequate storage conditions. Old honeys contain the highest concentrations of HMF. Therefore, HMF alone cannot be used for detecting adulteration with invert syrup.

HFCS is produced by hydrolysis and isomerization of corn starch. According to their fructose content, this type of syrup is classified as: HFCS-42 (42 % fructose), HFCS-55 (55 % fructose), and HFCS-90 (90 % fructose). HFCS presence significantly affects the pH and HMF content. HFCS is also used to feed bees, especially in winter

576 months and early spring [84]. Different analytical techniques, such as isotopic analysis,
577 chromatography, and spectroscopy, have been used in order to detect honey adulteration
578 with different sweeteners (Table 5).

579 The stable carbon isotope ratio analysis (SCIRA) is a standard analytical
580 technique for detecting adulteration of honey [85-88]. This technique is based on the
581 determination of the $^{13}C/^{12}C$ isotope ratio. According to their carbon metabolism, the
582 plants that produce substances used to adulterate honeys are classified as C3 or C4
583 plants. The C3 plants fix CO_2 by the Calvin cycle while the C4 plants do using the
584 Hatch-Slack cycle. C3 plants have a lower $^{13}C/^{12}C$ ratio than C4 plants. Most of the
585 honey-contributing plants (wheat, rice, and sugar beets, among others) are C3 plants.
586 The C4 sugars most used to adulterate honeys are corn and cane sugar [84]. SCIRA is an
587 expensive and time-consuming technique that also requires a considerable technical
588 skill.

589 Profiles of malto-oligosaccharides obtained by HPAEC-PAD were used to detect
590 adulteration with CS or HFCS with different degrees of isomerization: 20 % (20HFCS),
591 40 % (40HFCS), and 80 % (80HFCS). Honey samples were treated with activated
592 charcoal before analysis in order to remove mono and disaccharides [61]. CS and 20HFCS
593 profiles showed oligosaccharides with a degree of polymerization (DP) up to 16 and at
594 relatively high concentrations. Oligosaccharides with DP up to 7 were detected in the
595 40HFCS and 80HFCS syrups and only oligosaccharides with DP up to 6 were
596 quantified presenting much lower concentrations than those found in the other syrups.
597 In honey samples, oligosaccharides up 7 DP were detected and their amounts were very
598 low and variable among samples. A comparison of oligosaccharide profiles of honey
599 samples and the same samples adulterated with one of the different syrups at different
600 amounts (5, 10, and 20 % (w/w)) enabled to detect adulteration with CS (down to 5 %

601 (w/w)), 20HFCS, and 40HFCS. Adulteration with 80HFCS was not detected due to the
602 low amounts of oligosaccharides present in this syrup, comparable to those present in
603 honey samples.

604 A simple chromatographic separation of sugars by HPLC-RID was proposed to
605 detect honey adulteration with starch syrup. A characteristic chromatographic peak of
606 syrup can be found. No such peak at the same retention time is seen in chromatograms
607 of pure honeys. This characteristic peak is an overlapping peak of oligosaccharides with
608 a DP greater than 5. Therefore, this oligosaccharide peak was proposed as a syrup
609 indicator in adulterated honey, with a content of detectable syrup near 2.5 % (w/w). On
610 the other hand, the syrup content in adulterated honeys could be approximately
611 calculated using the height of this peak in the chromatograms. Using this method,
612 preliminary treatment of the samples is not required and no organic solvent is needed.
613 Therefore, the method proposed is simple, of low cost, environmentally
614 unobjectionable, and easy to perform in quality control [27].

615 Spectroscophic techniques (NIR, MIR, Raman) coupled with chemometric
616 methods have proven to be effective to recognize adulterated honey and to determine
617 the amount of adulterant added. For quality-grading purposes, discriminating between
618 different ranges of adulterant concentration might be enough.

619 NIR transflectance spectroscopy was evaluated to discriminate between
620 unadulterated honey and the same honey adulterated by different fructose:glucose
621 mixtures. Honey samples of 6 floral origins were used. Five classification methods were
622 evaluated. The best classification model obtained is known as least square support
623 vector machine (LS-SVM). Wavelet transformation (WT) was more effective than
624 principal component analysis (PCA) for selection of variables [89]. More samples of

625 different places and botanical origins could be included to expand the calibration model
626 and improve the robustness and accuracy of the method.

627 The use of fibre optic diffuse reflectance FT-NIR spectroscopy has been
628 assessed in order to differentiate between adulterated with HFCS and authentic blossom
629 honey samples. Classification of honey samples was realized using discriminant partial
630 least squares (D-PLS) analysis. The main bands responsible for the discrimination of
631 samples were in the range of 6000-10000 cm^{-1}. Honey samples containing HFCS were
632 purchased in the market. The 100 % of genuine honeys and 95 % of adulterated honeys
633 from the test set were correctly classified [90]. This method presented the advantage
634 compared to most methods of using adulterated honey samples not prepared in
635 laboratory. Therefore, adulterated honey samples were truly representative improving
636 the prediction efficiency.

637 FTIR-ATR was evaluated for determine the amount of beet medium invert
638 sugar added to 3 varieties of honey (clover, bluckwheat, and orange blossom honey) [91].
639 Two multivariate techniques, PLS and principal component regression (PCR), were
640 assessed. PLS-1st derivative models for the different varieties of honeys gave slightly
641 better results that those obtained using other calibration models. The r^2 values for
642 validation set ranged between 0.946 and 0.956. SEP values were between 2.1 and 4.5 %
643 (w/w). Adulterated samples were classified into 3 groups according to the levels of beet
644 invert sugar added to honey. For this purpose, linear discriminant analysis (LDA) and
645 canonical variate analysis (CVA) were evaluated. PCA was used as the data
646 compression technique. The best predictive model was achieved using CVA, which
647 successfully classified 88.2-94.1 % of the validation set. On the other hand, a single-
648 calibration model including all varieties of honey gave lower correlations and higher

649 SEC and SEP values than the individual models, and the percentage of correct
650 classification was reduced to 78.4 % for the validation set using CVA.

651 This methodology was used to study adulteration with cane medium invert sugar
652 by the same authors [92]. The same varieties of honey were analyzed. The PLS-1st
653 derivative model gave SEP values between 2.8 to 3.5 % (w/w); the r^2 values ranged
654 between 0.93 and 0.96. The PLS data compression technique was used in discriminant
655 analysis. Optimum classifications of 88.2-96.4 % and 89.3-94.1 % were achieved in the
656 validation set, using LDA and CVA, respectively. However, LDA was slightly better
657 for orange blossom honey and CVA for clover and bluckwheat. A single-calibration
658 model including all varieties of honey was also studied and gave worse results than
659 individual models.

660 An analytical method using FTIR-ATR spectroscopy was developed for
661 determining the amount added of CS to 3 different varieties of honeys (bluckwheat,
662 clover, and orange). Using PLS-1st derivative models, SEP values were in the range
663 1.99-2.22 % (w/w). The r^2 values ranged between 0.899 and 0.940. A combined model
664 for the 3 different varieties of honey was studied and gave worse results than individual
665 models [93]. This result was similar to that obtained by the same authors for other
666 adulterants, such as beet and cane medium invert sugar [91,92].

667 FTIR-ATR spectroscopy was applied to the discrimination of Irish artisanal
668 honeys from those adulterated with 1 of 5 sugar syrups: fully inverted beet syrup,
669 HFCS, partial invert cane syrup, dextrose syrup, and beet sucrose [94]. A soft independent
670 modeling class analogy (SIMCA) model allowed correct identification of 96.2 % of
671 authentic honeys. Furthermore, 97.5 % of beet sucrose-adulterated honeys and 95.8 %
672 of honeys adulterated with dextrose syrup were correctly classified as adulterated
673 honeys. Most of the samples adulterated with the others syrups were incorrectly

674 classified as genuine honeys. Therefore, the model may be used only to detect
675 adulteration with dextrose syrup or beet sucrose in Irish artisanal honeys. Minimum
676 levels of 10 % and 7 % (w/w) for beet sucrose or dextrose syrup, respectively, were
677 detected. On the other hand, a PLS model that allowed differentiate between authentic
678 honeys and honeys adulterated with partial invert cane syrup was obtained. Applying
679 this model to the samples adulterated with HFCS or fully inverted beet syrup, most of
680 them were identified as not cane partial inverted syrup-adulterated honeys, i.e., they
681 were classified as genuine honeys. This model allowed confirmation of the identity of
682 partial invert cane syrup-adulterated honeys with a correct classification rate of 91.7 %.
683 Using the 2 models, SIMCA and PLS, adulteration with beet sucrose, dextrose syrup, or
684 partial invert cane syrup can be detected.

685 FTIR-ATR spectroscopy was used to quantify CS, HFCS, and inverted sugar in
686 honeys of 4 states of México (Estado de México, Oaxaca, Chiapas, and Morelos). PLS
687 models for the 3 adulterants were developed. Good prediction accuracies were obtained.
688 The r^2 values ranged between 0.976 and 0.999. SEP values were between 1.5 and 2.1 %
689 (w/w) for CS, 2.1-3.0 % (w/w) for HFCS, and 1.4-2.5 % (w/w) for inverted sugar.
690 Furthermore, a classification model based on SIMCA was developed in order to
691 discriminate the geographic origin of the Mexican honeys. Using this model a correct
692 classification rate of 100 % was achieved [95].

693 Raman spectroscopy was used to detect honey adulteration with cane and beet
694 medium invert sugar in 3 varieties of honey (bluckwheat, clover, and orange blossom
695 honey) [82]. PLS analysis was used to predict amounts added of the adulterants. Models
696 were made for each adulterant and type of honey. The prediction models had an
697 acceptable accuracy. Values of r^2 between 0.909 and 0.952 were obtained. SEP values
698 were between 1.56-2.20 % (w/w). As compared with results obtained by Sivakesava [91,92]

699 using FTIR-ATR, the r^2 values were somewhat lower. However, SEP values were
700 better. Discriminant analysis by CVA with PCA compressed data was used in order to
701 classify adulterated honey based on the different concentration ranges for the
702 adulterants. About 96 % was the correct minimum classification rate obtained. Results
703 were better than those obtained by Sivakesava [91,92], using FTIR-ATR. CVA was also
704 used to differentiate between the 2 different types of adulterants regardless of the
705 honeys floral origin. The percentages of correct classification obtained were 90 %
706 (approximately) and 91 % for the calibration and validation sets, respectively. Better
707 predictions were obtained when discriminant models were developed for each type of
708 honey. In this case, the classification accuracy from all floral varieties was
709 approximately 96 % for both calibration and validation data sets.
710
711 **ANALYTICAL METHODS FOR METHYLGLYOXAL AND OTHER 1, 2-**
712 **DICARBONYL COMPOUNDS**
713 The 1,2-dicarbonyl compounds in honey, such as glyoxal (GO), 2-oxopropanal
714 or MGO, and 3-deoxyglucosone (3-DG), are formed from reducing carbohydrates by
715 the Maillard reaction or caramelization reactions**.** GO and MGO are considered markers
716 for an advanced state of glucose degradation. GO is the product of autoxidation for
717 glucose and MGO is formed by retroaldolization of the intermediate 3-DG [96]. Analytical
718 methods used for determining MGO and other 1,2-dicarbonyl compounds in honey are
719 shown in Table 6.
720 1,2-dicarbonyl compounds were determined in honey by RP-HPLC with UV-
721 detection at 312 nm, preceded by derivatization to their corresponding quinoxalines
722 with orthophenylenediamine (OPD) [96]. The derivatization procedure was done at room
723 temperature in the absence of light for 12 h. The chromatographic separation was

724 carried out using gradient elution: solvent A: 0.075 % (v/v) acetic acid and solvent B:
725 80 % (v/v) aqueous methanol containing 0.075 % (v/v) acetic acid. The LODs obtained
726 were: 0.16, 0.03, and 0.04 mg/L for 3-DG, GO, and MGO, respectively. The ranges of
727 concentration used for the calibration showed good linearity: 0.32-81 mg/L for 3-DG,
728 0.06-1.7 mg/L for GO, and 0.07-2.2 mg/L for MGO. HMF, formed preferably under
729 acidic conditions from 3-DG, was separately quantified using RP-HPLC. Elution was
730 performed in isocratic mode with a buffer containing 15 % (v/v) methanol and 85 %
731 (v/v) 0.05 M phosphate-buffer (pH 5.5). The concentration of HMF was much lower
732 than that of 3-DG and no correlation between the 2 compounds was found.

733 Another 1,2-dicarbonyl compound, glucosone, unknown to occur in foods before
734 2004, was isolated and identified by HPLC-MS and NMR spectroscopy. The estimated
735 amount of glucosone in all samples analyzed was between 18 and 262 mg/kg, with an
736 average value of 90 mg/kg. On the other hand, an increase of 3-DG and HMF was
737 observed when the samples of honey were stored at 35 ºC and 45 ºC. However, the
738 concentrations of GO and MGO did not show changes and no significant increase in the
739 content of glucosone was observed. 3-DG proved to be a more sensitive indicator for
740 heat treatment than HMF [96].

741 GO, MGO and 3-DG were also determined in honey using the Mavric method,
742 which is a slight modification of the Weigel method [97]. The LODs obtained were 0.3
743 mg/kg for 3-DG and 0.2 mg/kg for GO and MGO. Higher concentration ranges for
744 calibration curves were used showing good linearity: 10-500 mg/L for 3-DG, 0.1-20
745 mg/L for GO, and 0.1-300 mg/L for MGO. HMF was determined separately. The
746 amounts of 3-DG were also much higher than those of HMF, and no correlation
747 between the 2 compounds was found. Furthermore, GO and MGO were not affected by
748 storage conditions.

HPLC-DAD coupled to mass spectrometry with an electrospray ionization interface (HPLC-DAD-ESI-MS) has also been used for the determination of GO, MGO, and 3-DG in honeys [98]. 1,2-dicarbonyl compounds were derivatized with OPD before analysis. Electrospray ionization in positive mode was performed. Gradient elution was used: solvent A: methanol/acetic acid (99.97:0.03 v/v) and solvent B methanol/acetic acid (99.85:0.15 v/v). The detection wavelength used was 312 nm. The estimated LODs for 3-DG, GO, and MGO were 0.130, 0.083, and 0.063 μg/mL, respectively. LODs for GO and MGO were higher than those reported by Weigel [96]. However, the LOD of 3-DG was similar. The concentration ranges used for the calibration curves were 3−520, 0.1-60, and 0.1-250 mg/L, for 3-DG, GO, and MGO, respectively.

Honey is valued for its healing properties and can be used as a topical antibacterial agent for surface wound infections. Acidity, high osmolality, and hydrogen peroxide contribute to the antimicrobial activity of honey. Non-peroxide factors include lysozyme, phenolic acids, and flavonoids. MGO was identified as the component mainly responsible for the non-peroxide antimicrobial activity in New Zealand manuka honey (*Leptsopermum scoparium*) [97,99]. *Leptospermum* honeys are well known for their healing properties and include New Zealand manuka honey (*Leptsopermum scoparium*) and Australian jelly bush honey (*Leptospermum polygalifolium*).

Amounts of MGO in New Zealand manuka honey (*Leptsopermum scoparium*) between 38.4 and 761 mg/kg have been found[97]. These values were much higher than those found in conventional honey [96-98] when MGO values below 13 mg/kg were found. Concentrations of MGO in honeys from Australian native plants have also been determined [100]. The values obtained ranged from 8 to 1755 mg/kg, with an average concentration of 357 mg/kg. The samples from the *L. polygalifolium* (jelly bush) floral

773 source had the highest MGO concentrations (279-1755 mg/kg) with an average value of
774 604 mg/kg.

775 It has been reported that the high levels of MGO arise from the nonenzymatic
776 conversion of dihydroxyacetone (DHA) to MGO in maturating honey[99]. High levels of
777 DHA and low levels of MGO have been found in nectar and honey freshly produced by
778 bees.

779 A strong positive correlation between the amount of MGO in manuka honey and
780 antibacterial activity on *Staphylococcus aureus* was reported [101]. The MGO content has
781 been used commercially as an indicator of the bioactivity of *Leptospermum* honeys.
782 MGO amount in honeys is expressed as a MGO number. For instance, '500 + MGO'
783 indicates that the honey contains a minimum amount of methylglyoxal of 500 mg/kg [100].

784 In order to classify manuka honey by antimicrobial strength, unique manuka
785 factor (UMF) is used. For its determination, the size of the zone of inhibition of
786 *Staphylococcus aureus* induced by the honey is compared with that induced by phenol
787 solutions. Honey having an antibacterial activity equivalent to that of a 5 % phenol
788 solution has a UMF 5+ and so on up to a 30 % phenol solution rated as UMF 30+ [102].
789 Manuka honey with a high UMF value has a good antibacterial activity. Therefore,
790 UMF 12+ manuka honey is commercialized for therapeutic purposes and that with
791 UMF 16+ would be more potent for specific medical applications.

792 MGO content is usually measured according to the above methods [96,97]. These
793 methods are time-consuming and involve a number of steps and numerous reagents and
794 solvents. A direct method has been developed involving a mixed mode size-
795 exclusion/ligand exchange separation with 2 columns and with refractive index
796 detection [103]. This method was compared to the Weigel method [96]. The direct method
797 presented disadvantages. It proved to be much less sensitive with a detection limit ca.

50 mg/kg. Therefore, it is not suitable to determine MGO in conventional honeys (non-manuka honeys) with relatively low amounts of MGO. Another disadvantage is that the standard additions method was necessary for quantification, known amounts of MGO being added to a honey solution (50-900 mg/kg). Instead, the indirect method using derivatization with OPD required an external calibration. On the other hand, better resolution of the MGO peak was obtained using the indirect method, less prone to interference with co-eluting peaks.

A method for the simultaneous determination of MGO and DHA, its precursor, in Australian honeys has been proposed [104]. The method involves a derivatization with O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine·HCl (PFBHA). Subsequent analysis of PFBHA derivatives by RP-HPLC with UV-detection at 263 nm was carried out. Hydroxyacetone (HA) was used as an internal standard. Gradient elution was used for separation: solvent A: 30 % (v/v) acetonitrile and solvent B: acetonitrile. The usable linear range of the method for MGO was from 20 to 1800 mg/kg. This range was wider than that used (50-900 mg/kg) by Adams [103]. The method using PFBHA was less sensitive than the methods developed by Weigel, Mavric, and Marshall [96-98]. However, it was sensitive enough to detect MGO and DHA in honeys from 6 *Leptospermum* species (*L. polygalifolium*, *L. trinervia, L. leavigatum*, *L. liversidgei*, *L. semibaccatum*, and *L. speciosum*) and blends with other species. High levels of MGO and DHA were found ranging from 43 to 1723 mg/kg and from 412 to 2403 mg/kg for MGO and DHA, respectively. High levels of DHA and MGO were present at the genus level, not being restricted to manuka honey [97,103]. This method presents some advantage over other methods using HPLC-UV and previous derivatization with OPD [96,97] which does not detect monocarbonyl compounds such as DHA. Furthermore, the time for sample preparation was reduced from 12-16 h to 2 h.

823 The Windsor method was applied to the simultaneous determination of MGO,
824 DHA, and HMF in Austalian *Leptospermun* honeys [105]. The PFBHA derivate of HMF
825 resulted in 2 chromatographic peaks corresponding to their cis and trans-isomers.
826 GC has also been employed to determine MGO in some New Zealand manuka
827 and kanuka honeys. A method using headspace solid-phase microextraction coupled to
828 gas chromatography with nitrogen/phosphorus detector (HS-SPME-GC–NPD) was
829 described [106]. Before SPME, derivatization of MGO with OPD was carried out in vials
830 that were capped and left for 1 h to allow complete derivatization before analysis. 2,3-
831 Butanedione was used as an internal standard. The LOD of the method was 5 mg/kg.
832 This method was less sensitive than those developed by Weigel [96] and Mavric [97] using
833 HPLC-UV and derivatization with OPD. However, it was more sensitive than the direct
834 method developed by Adams [103] and the Windsor method [104] which uses HPLC-UV and
835 derivatization with PFBHA.
836 Besides chromatography, other analytical techniques have been used. A NMR
837 method was proposed [102]. MGO was present in sample solutions as the mono- and
838 dihydrate which were independently measured. The values obtained were added to
839 determine the methylglyoxal concentration. The method was applied to samples of
840 manuka honey. The amounts of methylglyoxal ranged from 338 to 817 mg/kg. This
841 method was compared with the Mavric method using HPLC-UV detection and another
842 using HPLC coupled with time of flight mass spectrometry detection (HPLC/TOF-MS),
843 being MGO previously derivatized with OPD. The amounts of MGO obtained with
844 these 2 methods were in most of the cases somewhat higher than those obtained with the
845 NMR method. This could be explained by the Maillard reaction between sugars and
846 OPD which would generate MGO or by the conversion of DHA to MGO due to the
847 elevated temperature used during the process of derivatization [102]. The NMR method

848 presented advantages because it is applied directly on the diluted honey with neither
849 chromatographic separation nor sample derivatization.

850 Recently, MIR spectroscopy with ATR in combination with a PLS1 regression
851 model has been proposed to predict MGO content in Australian honeys from several
852 native plants [100]. The reference method used derivatization of MGO with OPD and
853 posterior analysis by HPLC-UV detection. A r^2 value of 0.75 between the predicted and
854 reference values was obtained. Robustness of the method could be improved for
855 commercial applications.

856 Sugars and several physicochemical parameters used in quality control can be
857 determined simultaneously with FTIR-ATR spectroscopy. Moreover, this technique
858 allows discrimination of adulterated honey samples containing several added
859 sweeteners, and determination of the amount added of each one. It is nondestructive,
860 rapid, requires only limited sample preparation, and is easy to perform. Therefore,
861 FTIR-ATR has been proposed for routine quality control by several researchers.
862 However, IR spectroscopy did not allow a good quantitative determination of HMF and
863 enzyme activities, two criteria of great importance for the honey trade to assess storage
864 and heat damage.

865 As conclusion, several analytical techniques are needed due to the complex
866 chemical composition of honey. Chromatographic techniques are widely used. More
867 recently, methods based on other techniques, such as Raman, IR, and NMR
868 spectroscopy, in combination with chemometric methods, have been proposed for
869 honey analysis. These methods rely on the inherent advantages of spectroscopy. They
870 are rapid, inexpensive, nondestructive, and not needing sample preparation or expensive
871 reagents. Official methods based on spectroscopic techniques should be developed and
872 validated for routine quality control of honeys.

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875 References

876 1. Iglesias, M.; De Lorenzo, C.; Polo, M.; Martin-Alverez, P.; Pueyo, E. Usefulness of
877 amino acid composition to discriminate between honeydew and floral honeys.
878 Application to honeys from a small geographic area. *J. Agric. Food Chem.* **2004,** *52,*
879 84-89.

880 2. Anonymous EU Directive 110/2001. *http://eur-lex. europa.*
881 *eu/LexUriServ/LexUriServ. do?uri=OJ:L:2002:010:0047:0052:EN:PDF* **2001,**

882 3. Cajka, T.; Hajslova, J.; Pudil, F.; Riddellova, K. Traceability of honey origin based
883 on volatiles pattern processing by artificial neural networks. *J. Chromatogr. a* **2009,**
884 *1216,* 1458-1462.

885 4. Ouchemoukh, S.; Louaileche, H.; Schweitzer, P. Physicochemical characteristics and
886 pollen spectrum of some Algerian honeys. *Food Control* **2007,** *18,* 52-58.

887 5. de la Fuente, E.; Sanz, M.L.; Martinez-Castro, I.; Sanz, J. Development of a robust
888 method for the quantitative determination of disaccharides in honey by gas
889 chromatography. *J. Chromatogr. a* **2006,** *1135,* 212-218.

890 6. Low, N.H.; Sporns, P. Analysis and Quantitation of Minor Di- and Trisaccharides in
891 Honey, Using Capillary Gas Chromatography. *J. Food Sci.* **1988,** *53,* 558-561.

892 7. Ruiz-Matute, A.L.; Brokl, M.; Soria, A.C.; Sanz, M.L.; Martinez-Castro, I. Gas
893 chromatographic-mass spectrometric characterisation of tri- and tetrasaccharides in
894 honey. *Food Chem.* **2010,** *120,* 637-642.

895 8. Sanz, M.; Polemis, N.; Morales, V.; Corzo, N.; Drakoularakou, A.; Gibson, G.;
896 Rastall, R. In vitro investigation into the potential prebiotic activity of honey
897 oligosaccharides. *J. Agric. Food Chem.* **2005,** *53,* 2914-2921.

898 9. Cavia, M.; Fernandez-Muino, M.; Gomez-Alonso, E.; Montes-Perez, M.; Huidobro,
899 J.; Sancho, M. Evolution of fructose and glucose in honey over one year: influence of
900 induced granulation. *Food Chem.* **2002,** *78,* 157-161.

901 10. Gheldof, N.; Engeseth, N. Antioxidant capacity of honeys from various floral
902 sources based on the determination of oxygen radical absorbance capacity and
903 inhibition of in vitro lipoprotein oxidation in human serum samples. *J. Agric. Food*
904 *Chem.* **2002,** *50,* 3050-3055.

905 11. Conti, M.E. Lazio region (central Italy) honeys: a survey of mineral content and
906 typical quality parameters. *Food Control* **2000,** *11,* 459-463.

907 12. Khalil, M.I.; Sulaiman, S.A.; Gan, S.H. High 5-hydroxymethylfurfural
908 concentrations are found in Malaysian honey samples stored for more than one year.
909 *Food Chem. Toxicol.* **2010,** *48,* 2388-2392.

910 13. Martelo, M.S. Chemical characterization in honey harvested in Madrid's northern
911 mountains. *Ars Pharm.* **2000,** *41,* 387-396.

912 14. Osborne, B.G.; Fearn, T.; Hindle, P.H. *Practical NIR spectroscopy with*
913 *applications in food and beverage analysis.* Longman scientific and technical: 1993;

914 15. Qiu, P.Y.; Ding, H.B.; Tang, Y.K.; Xu, R.J. Determination of chemical composition
915 of commercial honey by near-infrared spectroscopy. *J. Agric. Food Chem.* **1999,** *47,*
916 2760-2765.

917 16. García-Alvarez, M.; Huidobro, J.F.; Hermida, M.; Rodríguez-Otero, J.L. Major
918 components of honey analysis by near-infrared transflectance spectroscopy. *J. Agric.*
919 *Food Chem.* **2000,** *48,* 5154-5158.

920 17. Cozzolino, D.; Corbella, E. Determination of honey quality components by near
921 infrared reflectance spectroscopy. *J. Apic. Res.* **2003,** *42,* 16-20.

922 18. Ruoff, K.; Luginbühl, W.; Bogdanov, S.; Bosset, J.-.; Estermann, B.; Ziolko, T.;
923 Kheradmandan, S.; Amadò, R. Quantitative determination of physical and chemical
924 measurands in honey by near-infrared spectrometry. *Eur. Food Res. Technol.* **2007,** *225,*
925 415-423.

926 19. Ruoff, K.; Iglesias, M.T.; Luginbühl, W.; Bosset, J.-.; Bogdanov, S.; Amadò, R.
927 Quantitative analysis of physical and chemical measurands in honey by mid-infrared
928 spectrometry. *Eur. Food Res. Technol.* **2006,** *223,* 22-29.

929 20. de Rodriguez, G.; de Ferrer, B.; Ferrer, A.; Rodriguez, B. Characterization of honey
930 produced in Venezuela. *Food Chem.* **2004,** *84,* 499-502.

931 21. Bogdanov, S.; Ruoff, K.; Persano Oddo, L. Physico-chemical methods for the
932 characterisation of unifloral honeys: a review. *Apidologie* **2004,** *35,* 4-17.

933 22. Meda, A.; Lamien, C.; Romito, M.; Millogo, J.; Nacoulma, O. Determination of the
934 total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their
935 radical scavenging activity. *Food Chem.* **2005,** *91,* 571-577.

936 23. Can, Z.; Yildiz, O.; Sahin, H.; Turumtay, E.A.; Silici, S.; Kolayli, S. An
937 investigation of Turkish honeys: Their physico-chemical properties, antioxidant
938 capacities and phenolic profiles. *Food Chem.* **2015,** *180,* 133-141.

939 24. Bogdanov, S.; Lullmann, C.; Martin, P.; von der Ohe, W.; Russmann, H.; Vorwohl,
940 G.; Odo, L.; Sabatini, A.; Marcazzan, G.; Piro, R.; Flamini, C.; Morlot, M.; Lheritier, J.;
941 Borneck, R.; Marioleas, P.; Tsigouri, A.; Kerkvliet, J.; Ortiz, A.; Ivanov, T.; D'Arcy, B.;
942 Mossel, B.; Vit, P.; Int Honey Commission Honey quality and international regulatory
943 standards: review by the International Honey Commission. *Bee World* **1999,** *80,* 61-69.

944 25. Ough, C. Rapid Determination of Proline in Grapes and Wines. *J. Food Sci.* **1969,**
945 *34,* 228-&.

946 26. Truzzi, C.; Annibaldi, A.; Illuminati, S.; Finale, C.; Scarponi, G. Determination of
947 proline in honey: Comparison between official methods, optimization and validation of
948 the analytical methodology. *Food Chem.* **2014,** *150,* 477-481.

949 27. Wang, S.; Guo, Q.; Wang, L.; Lin, L.; Shi, H.; Cao, H.; Cao, B. Detection of honey
950 adulteration with starch syrup by high performance liquid chromatography. *Food Chem.*
951 **2015,** *172,* 669-674.

952 28. Azeredo, L.; Azeredo, M.; de Souza, S.; Dutra, V. Protein contents and
953 physicochemical properties in honey samples of Apis mellifera of different floral
954 origins. *Food Chem.* **2003,** *80,* 249-254.

955 29. Escuredo, O.; Míguez, M.; Fernández-González, M.; Carmen Seijo, M. Nutritional
956 value and antioxidant activity of honeys produced in a European Atlantic area. *Food*
957 *Chem.* **2013,** *138,* 851-856.

30. Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.* **1998,** *63,* 549-562.

31. Nozal, M.; Bernal, J.; Toribio, L.; Jimenez, J.; Martin, M. High-performance liquid chromatographic determination of methyl anthranilate, hydroxymethylfurfural and related compounds in honey. *J. Chromatogr. a* **2001,** *917,* 95-103.

32. LoCoco, F.; Valentini, C.; Novelli, V.; Ceccon, L. High-performance liquid chromatographic determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in honey. *J. Chromatogr. a* **1996,** *749,* 95-102.

33. Makawi, S.Z.A.; Taha, M.I.; Zakaria, B.A.; Siddig, B.; Mahmod, H.; Elhussein, A.R.M.; Kariem, E.A.G. Identification and quantification of 5-hydroxymethyl furfural HMF in some sugar-containing food products by HPLC. *Pak. J. Nutr.* **2009,** *8,* 1391-1396.

34. Nassberger, L. Influence of 5-Hydroxymethylfurfural (5-Hmf) on the overall Metabolism of Human Blood-Cells. *Hum. Exp. Toxicol.* **1990,** *9,* 211-214.

35. Bruce, W.R.; Archer, M.C.; Corpet, D.E.; Medline, A.; Minkin, S.; Stamp, D.; Yin, Y.; Zhang, X. Diet, aberrant crypt foci and colorectal cancer. *Mutat. Res. -Fund. Mol. M.* **1993,** *290,* 111-118.

36. Severin, I.; Dumont, C.; Jondeau-Cabaton, A.; Graillot, V.; Chagnon, M. Genotoxic activities of the food contaminant 5-hydroxymethylfurfural using different in vitro bioassays. *Toxicol. Lett.* **2010,** *192,* 189-194.

978 37. Zhang, X.-.; Chan, C.C.; Stamp, D.; Minkin, S.; Archer, M.C.; Bruce, W.R.
979 Initiation and promotion of colonic aberrant crypt foci in rats by 5-hydroxymethyl-2-
980 furaldehyde in thermolyzed sucrose. *Carcinogenesis* **1993,** *14,* 773-775.

981 38. Surh, Y.J.; Liem, A.; Miller, J.A.; Tannenbaum, S.R. 5-Sulfooxymethylfurfural as a
982 possible ultimate mutagenic and carcinogenic metabolite of the Maillard reaction
983 product, 5-hydroxymethylfurfural. *Carcinogenesis* **1994,** *15,* 2375-2377.

984 39. Lee, Y.; Shlyankevich, M.; Jeong, H.; Douglas, J.; Surh, Y. Bioactivation of 5-
985 Hydroxymethyl-2-Furaldehyde to an Electrophilic and Mutagenic Allylic Sulfuric-Acid
986 Ester. *Biochem. Biophys. Res. Commun.* **1995,** *209,* 996-1002.

987 40. Janzowski, C.; Glaab, V.; Samimi, E.; Schlatter, J.; Eisenbrand, G. 5-
988 hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and
989 reactivity towards cellular glutathione. *Food Chem. Toxicol.* **2000,** *38,* 801-809.

990 41. Durling, L.J.; Busk, L.; Hellman, B.E. Evaluation of the DNA damaging effect of
991 the heat-induced food toxicant 5-hydroxymethylfurfural (HMF) in various cell lines
992 with different activities of sulfotransferases. *Food Chem. Toxicol.* **2009,** *47,* 880-884.

993 42. Jeuring, H.; Kuppers, F. High-Performance Liquid-Chromatography of Furfural and
994 Hydroxymethylfurfural in Spirits and Honey. *J. Assoc. Off. Anal. Chem.* **1980,** *63,*
995 1215-1218.

996 43. White, J. Spectrophotometric Method for Hydroxymethylfurfural in Honey. *J Assoc*
997 *Off Agric Chem.* **1979,** *62,* 509-514.

998 44. Zappala, A.; Fallico, B.; Arena, E.; Verzera, A. Methods for the determination of
999 HMF in honey: a comparison. *Food Control* **2005,** *16,* 273-277.

45. Truzzi, C.; Annibaldi, A.; Illuminati, S.; Finale, C.; Rossetti, M.; Scarponi, G. Determination of Very Low Levels of 5-(Hydroxymethyl)-2-furaldehyde (HMF) in Natural Honey: Comparison Between the HPLC Technique and the Spectrophotometric White Method. *J. Food Sci.* **2012,** *77,* C784-C790.

46. Cabras, P.; Angioni, A.; Tuberoso, C.; Floris, I.; Reniero, F.; Guillou, C.; Ghelli, S. Homogentisic acid: A phenolic acid as a marker of strawberry-tree (Arbutus unedo) honey. *J. Agric. Food Chem.* **1999,** *47,* 4064-4067.

47. Spano, N.; Casula, L.; Panzanelli, A.; Pilo, M.; Piu, P.; Scanu, R.; Tapparo, A.; Sanna, G. An RP-HPLC determination of 5-hydroxymethylfurfural in honey - The case of strawberry tree honey. *Talanta* **2006,** *68,* 1390-1395.

48. Spano, N.; Ciulu, M.; Floris, I.; Panzanelli, A.; Pilo, M.I.; Piu, P.C.; Salis, S.; Sanna, G. A direct RP-HPLC method for the determination of furanic aldehydes and acids in honey. *Talanta* **2009,** *78,* 310-314.

49. Schade, J.; Marsh, G.; Eckert, J. Diastase activity and hydroxy-methyl-furfural in honey and their usefulness in detecting heat alteration. *J. Food Sci.* **1958,** *23,* 446-463.

50. White Jr, J.W.; Pairent, F. Report on the analysis of honey. *J Assoc Off Agric Chem* **1959,** *42,* 341-348.

51. Zurcher, K.; Hadorn, H. Eine einfache kinetische Methode zur Bestimmung der Diastasezahl in Honig. *Deut Lebensmittel rundsch* **1972,** *68,* 209-216.

52. Oddo, L.P.; Pulcini, P. A scientific note on the Phadebas method for honeys with low enzyme content. *Apidologie* **1999,** *30,* 347-348.

1021 53. McCleary, B.V.; Sturgeon, R. Measurement of alpha-amylase in cereal, food, and
1022 fermentation products. *Cereal Foods World* **2002,** *47,* 299-+.

1023 54. Bentabol Manzanares, A.; Hernández García, Z.; Rodríguez Galdón, B.; Rodríguez
1024 Rodríguez, E.; Díaz Romero, C. Physicochemical characteristics of minor monofloral
1025 honeys from Tenerife, Spain. *LWT - Food Sci. Technol.* **2014,** *55,* 572-578.

1026 55. Sakac, N.; Sak-Bosnar, M. A rapid method for the determination of honey diastase
1027 activity. *Talanta* **2012,** *93,* 135-138.

1028 56. Baltrusaityte, V.; Venskutonis, P.R.; Ceksteryte, V. Radical scavenging activity of
1029 different floral origin honey and beebread phenolic extracts. *Food Chem.* **2007,** *101,*
1030 502-514.

1031 57. Fell, R. Color Grading of Honey. *Am. Bee J.* **1978,** *118,* 782-&.

1032 58. González-Miret, M.L.; Terrab, A.; Hernanz, D.; Fernández-Recamales, M.A.;
1033 Heredia, F.J. Multivariate correlation between color and mineral composition of honeys
1034 and by their botanical origin. *J. Agric. Food Chem.* **2005,** *53,* 2574-2580.

1035 59. Junk, W.; Pancoast, H. Boiling point elevations of sugar solutions. *Handbook of*
1036 *Sugars: For Processors, Chemists & Technologists, 1st Ed. (HM Pancoast, ed.)* **1973,**
1037 42-66.

1038 60. García-Alvarez, M.; Ceresuela, S.; Huidobro, J.F.; Hermida, M.; Rodríguez-Otero,
1039 J.L. Determination of polarimetric parameters of honey by near-infrared transflectance
1040 spectroscopy. *J. Agric. Food Chem.* **2002,** *50,* 419-425.

1041 61. Morales, V.; Corzo, N.; Sanz, M.L. HPAEC-PAD oligosaccharide analysis to detect
1042 adulterations of honey with sugar syrups. *Food Chem.* **2008,** *107,* 922-928.

1043 62. Arias, V.C.; Castells, R.C.; Malacalza, N.; Lupano, C.E.; Castells, C.B.
1044 Determination of oligosaccharide patterns a in honey by solid-phase extraction and
1045 high-performance liquid chromatography. *Chromatographia* **2003,** *58,* 797-801.

1046 63. Ni, C.; Zhu, B.; Wang, N.; Wang, M.; Chen, S.; Zhang, J.; Zhu, Y. Simple column-
1047 switching ion chromatography method for determining eight monosaccharides and
1048 oligosaccharides in honeydew and nectar. *Food Chem.* **2016,** *194,* 555-560.

1049 64. Horvath, K.; MolnarPerl, I. Simultaneous quantitation of mono-, di- and
1050 trisaccharides by GC-MS of their TMS ether oxime derivatives .2. In honey.
1051 *Chromatographia* **1997,** *45,* 328-335.

1052 65. Barez, J.; Garcia-Villanova, R.; Garcia, S.; Pala, T.; Paramas, A.; Sanchez, J.
1053 Geographical discrimination of honeys through the employment of sugar patterns and
1054 common chemical quality parameters. *Eur Food Res Technol* **2000,** *210,* 437-444.

1055 66. Sanz, M.; Sanz, J.; Martinez-Castro, I. Characterization of O-trimethylsilyl oximes
1056 of disaccharides by gas chromatography-mass spectrometry. *Chromatographia* **2002,**
1057 *56,* 617-622.

1058 67. Bogdanov, S.; Vit, P.; Kilchenmann, V. Sugar profiles and conductivity of stingless
1059 bee honeys from Venezuela. *Apidologie* **1996,** *27,* 445-450.

1060 68. Pourtallier, J. Ueber die Benutzung der GC für die Bestimmung der Zucker in
1061 Honig. *Z. für Bienenforschung* **1967,** *9,* 217-221.

1062 69. Sabatini, G.; Nanetti, A.; Maurizi, M.; Lercker, G. Studio del'origine dei mieli
1063 attraverso il profilo gaschromatografico dei componenti neutri. *Rivista di merceologia*
1064 **1984,** *23,* 71-81.

1065 70. Leite, J.; Trugo, L.; Costa, L.; Quinteiro, L.; Barth, O.; Dutra, V.; De Maria, C.
1066 Determination of oligosaccharides in Brazilian honeys of different botanical origin.
1067 *Food Chem.* **2000,** *70,* 93-98.

1068 71. White, J.; Meloy, R.; Probst, J.; Huser, W. Sugars Containing Galactose Occur in
1069 Honey. *J. Apic. Res.* **1986,** *25,* 182-185.

1070 72. Ouchemoukh, S.; Schweitzer, P.; Bey, M.B.; Djoudad-Kadji, H.; Louaileche, H.
1071 HPLC sugar profiles of Algerian honeys. *Food Chem.* **2010,** *121,* 561-568.

1072 73. Anjos, O.; Campos, M.G.; Ruiz, P.C.; Antunes, P. Application of FTIR-ATR
1073 spectroscopy to the quantification of sugar in honey. *Food Chem.* **2015,** *169,* 218-223.

1074 74. de la Fuente, E.; Ruiz-Matute, A.I.; Valencia-Barrera, R.M.; Sanz, J.; Martínez
1075 Castro, I. Carbohydrate composition of Spanish unifloral honeys. *Food Chem.* **2011,**
1076 *129,* 1483-1489.

1077 75. Sanz, M.; Sanz, J.; Martinez-Castro, I. Gas chromatographic-mass spectrometric
1078 method for the qualitative and quantitative determination of disaccharides and
1079 trisaccharides in honey. *J. Chromatogr. a* **2004,** *1059,* 143-148.

1080 76. Kelly, J.D.; Downey, G.; Fouratier, V. Initial study of honey adulteration by sugar
1081 solutions using midinfrared (MIR) spectroscopy and chemometrics. *J. Agric. Food*
1082 *Chem.* **2004,** *52,* 33-39.

77. Dvash, L.; Afik, O.; Shafir, S.; Schaffer, A.; Yeselson, Y.; Dag, A.; Landau, S. Determination by near-infrared spectroscopy of perseitol used as a marker for the botanical origin of avocado (Persea americana Mill.) honey. *J. Agric. Food Chem.* **2002,** *50,* 5283-5287.

78. Tewari, J.; Irudayaraj, J. Quantification of saccharides in multiple floral honeys using fourier transform infrared microattenuated total reflectance spectroscopy. *J. Agric. Food Chem.* **2004,** *52,* 3237-3243.

79. Wang, J.; Kliks, M.M.; Jun, S.; Jackson, M.; Li, Q.X. Rapid analysis of glucose, fructose, sucrose, and maltose in honeys from different geographic regions using Fourier transform infrared spectroscopy and multivariate analysis. *J. Food Sci.* **2010,** *75,* C208-C214.

80. Batsoulis, A.; Siatis, N.; Kimbaris, A.; Alissandrakis, E.; Pappas, C.; Tarantilis, P.; Harizanis, P.; Polissiou, M. FT-Raman spectroscopic simultaneous determination of fructose and glucose in honey. *J. Agric. Food Chem.* **2005,** *53,* 207-210.

81. Ozbalci, B.; Boyaci, I.H.; Topcu, A.; Kadilar, C.; Tamer, U. Rapid analysis of sugars in honey by processing Raman spectrum using chemometric methods and artificial neural networks. *Food Chem.* **2013,** *136,* 1444-1452.

82. Paradkar, M.; Irudayaraj, J. Discrimination and classification of beet and cane inverts in honey by FT-Raman spectroscopy. *Food Chem.* **2002,** *76,* 231-239.

83. Swallow, K.W.; Low, N.H. Determination of honey authenticity by anion-exchange liquid chromatography. *J. AOAC Int.* **1994,** *77,* 695-702.

84. Zábrodská, B.; Vorlová, L. Adulteration of honey and available methods for detection–a review. *Acta Veterinaria Brno* **2015,** *83,* 85-102.

85. White, J.; Winters, K.; Martin, P.; Rossmann, A. Stable carbon isotope ratio analysis of honey: Validation of internal standard procedure for worldwide application. *J. AOAC Int.* **1998,** *81,* 610-619.

86. Martín, I.G.; Macías, E.M.; Sánchez, J.S.; Rivera, B.G. Detection of honey adulteration with beet sugar using stable isotope methodology. *Food Chem.* **1998,** *61,* 281-286.

87. Padovan, G.; De Jong, D.; Rodrigues, L.; Marchini, J. Detection of adulteration of commercial honey samples by the 13 C/12 C isotopic ratio. *Food Chem.* **2003,** *82,* 633-636.

88. Cabañero, A.I.; Recio, J.L.; Ruperez, M. Liquid chromatography coupled to isotope ratio mass spectrometry: a new perspective on honey adulteration detection. *J. Agric. Food Chem.* **2006,** *54,* 9719-9727.

89. Zhu, X.; Li, S.; Shan, Y.; Zhang, Z.; Li, G.; Su, D.; Liu, F. Detection of adulterants such as sweeteners materials in honey using near-infrared spectroscopy and chemometrics. *J. Food Eng.* **2010,** *101,* 92-97.

90. Chen, L.; Xue, X.; Ye, Z.; Zhou, J.; Chen, F.; Zhao, J. Determination of Chinese honey adulterated with high fructose corn syrup by near infrared spectroscopy. *Food Chem.* **2011,** *128,* 1110-1114.

91. Sivakesava, S.; Irudayaraj, J. Detection of inverted beet sugar adulteration of honey by FTIR spectroscopy. *J. Sci. Food Agric.* **2001,** *81,* 683-690.

1126 92. Sivakesava, S.; Irudayaraj, J. Prediction of inverted cane sugar adulteration of honey
1127 by Fourier transform infrared spectroscopy. *J. Food Sci. -Chicago* **2001,** *66,* 972-978.

1128 93. Sivakesava, S.; Irudayaraj, J. A rapid spectroscopic technique for determining honey
1129 adulteration with corn syrup. *J. Food Sci.* **2001,** *66,* 787-792.

1130 94. Kelly, J.D.; Petisco, C.; Downey, G. Application of Fourier transform midinfrared
1131 spectroscopy to the discrimination between Irish artisanal honey and such honey
1132 adulterated with various sugar syrups. *J. Agric. Food Chem.* **2006,** *54,* 6166-6171.

1133 95. Gallardo-Velazquez, T.; Osorio-Revilla, G.; Zuniga-de Loa, M.; Rivera-Espinoza,
1134 Y. Application of FTIR-HATR spectroscopy and multivariate analysis to the
1135 quantification of adulterants in Mexican honeys. *Food Res. Int.* **2009,** *42,* 313-318.

1136 96. Weigel, K.; Opitz, T.; Henle, T. Studies on the occurrence and formation of 1,2-
1137 dicarbonyls in honey. *Eur. Food Res. Technol.* **2004,** *218,* 147-151.

1138 97. Mavric, E.; Wittmann, S.; Barth, G.; Henle, T. Identification and quantification of
1139 methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum
1140 scoparium) honeys from New Zealand. *Mol. Nutr. Food Res.* **2008,** *52,* 483-489.

1141 98. Marshall, S.M.; Schneider, K.R.; Cisneros, K.V.; Gu, L. Determination of
1142 Antioxidant Capacities, alpha-Dicarbonyls, and Phenolic Phytochemicals in Florida
1143 Varietal Honeys Using HPLC-DAD-ESI-MSn. *J. Agric. Food Chem.* **2014,** *62,* 8623-
1144 8631.

1145 99. Adams, C.J.; Manley-Harris, M.; Molan, P.C. The origin of methylglyoxal in New
1146 Zealand manuka (Leptospermum scoparium) honey. *Carbohydr. Res.* **2009,** *344,* 1050-
1147 1053.

1148 100. Sultanbawa, Y.; Cozzolino, D.; Fuller, S.; Cusack, A.; Currie, M.; Smyth, H.
1149 Infrared spectroscopy as a rapid tool to detect methylglyoxal and antibacterial activity in
1150 Australian honeys. *Food Chem.* **2015,** *172,* 207-212.

1151 101. Atrott, J.; Henle, T. Methylglyoxal in Manuka Honey - Correlation with
1152 Antibacterial Properties. *Czech J. Food Sci.* **2009,** *27,* S163-S165.

1153 102. Donarski, J.A.; Roberts, D.P.T.; Charlton, A.J. Quantitative NMR spectroscopy for
1154 the rapid measurement of methylglyoxal in manuka honey. *Anal. Method.* **2010,** *2,*
1155 1479-1483.

1156 103. Adams, C.J.; Boult, C.H.; Deadman, B.J.; Farr, J.M.; Grainger, M.N.C.; Manley-
1157 Harris, M.; Snow, M.J. Isolation by HPLC and characterisation of the bioactive fraction
1158 of New Zealand manuka (Leptospermum scoparium) honey. *Carbohydr. Res.* **2008,**
1159 *343,* 651-659.

1160 104. Windsor, S.; Pappalardo, M.; Brooks, P.; Williams, S.; Manley-Harris, M. A
1161 convenient new analysis of dihydroxyacetone and Methylglyoxal applied to Australian
1162 Leptospermum honeys. *J. Pharmacogn. Phytother.* **2012,** *4,* 6-11.

1163 105. Windsor, S.; Kavazos, K.; Brooks, P. The quantitation of hydroxymethylfurfural in
1164 Australian Leptospermum honeys. *J. Pharmacogn. Phytother.* **2013,** *5,* 21-25.

1165 106. Stephens, J.M.; Schlothauer, R.C.; Morris, B.D.; Yang, D.; Fearnley, L.;
1166 Greenwood, D.R.; Loomes, K.M. Phenolic compounds and methylglyoxal in some New
1167 Zealand manuka and kanuka honeys. *Food Chem.* **2010,** *120,* 78-86.

1168 107. Ajlouni, S.; Sujirapinyokul, P. Hydroxymethylfurfuraldehyde and amylase
1169 contents in Australian honey. *Food Chem.* **2010,** *119,* 1000-1005.

1170 108. Bentabol-Manzanares, A.; Hernandez Garcia, Z.; Rodriguez Galdon, B.; Rodriguez
1171 Rodriguez, E.; Diaz Romero, C. Differentiation of blossom and honeydew honeys using
1172 multivariate analysis on the physicochemical parameters and sugar composition. *Food*
1173 *Chem.* **2011,** *126,* 664-672.

1174 109. León-Ruiz, V.; Vera, S.; González-Porto, A.V.; Andrés, M.P.S. Vitamin C and
1175 sugar levels as simple markers for discriminating spanish honey sources. *J. Food Sci.*
1176 **2011,** *76,* C356-C361.

1177 110. Santos, A.; Moreira, R.F.A.; De Maria, C.A.B. Study of the principal constituents
1178 of tropical angico (Anadenanthera sp.) honey from the atlantic forest. *Food Chem.* **2015,**
1179 *171,* 421-425.

1180 111. Nayik, G.A.; Dar, B.N.; Nanda, V. Physico-chemical, rheological and sugar profile
1181 of different unifloral honeys from Kashmir valley of India. *Arab. J. Chem.* **2015,**

1182 112. Goodall, I.; Dennis, M.; Parker, I.; Sharman, M. Contribution of High-Performance
1183 Liquid-Chromatographic Analysis of Carbohydrates to Authenticity Testing of Honey.
1184 *J. Chromatogr. a* **1995,** *706,* 353-359.

1185 113. Cotte, J.; Casabianca, H.; Chardon, S.; Lheritier, J.; Grenier-Loustalot, M.
1186 Application of carbohydrate analysis to verify honey authenticity. *J. Chromatogr. a*
1187 **2003,** *1021,* 145-155.

1188 114. Cordella, C.B.Y.; Militao, J.S.L.T.; Clement, M.C.; Cabrol-Bass, D. Honey
1189 characterization and adulteration detection by pattern recognition applied on HPAEC-
1190 PAD profiles. 1. Honey floral species characterization. *J. Agric. Food Chem.* **2003,** *51,*
1191 3234-3242.

115. Sanz, M.; Gonzalez, M.; de Lorenzo, C.; Sanz, J.; Martinez-Castro, I. A contribution to the differentiation between nectar honey and honeydew honey. *Food Chem.* **2005,** *91,* 313-317.

116. Kaskoniene, V.; Venskutonis, P.R.; Ceksteryte, V. Carbohydrate composition and electrical conductivity of different origin honeys from Lithuania. *Lwt-Food Science and Technology* **2010,** *43,* 801-807.

117. Cho, H.; Hong, S. Acacia honey quality measurement by near-infrared spectroscopy. *J. Near Infrared Spectrosc.* **1998,** *6,* A329-A331.

118. Ha, J.; Koo, M.; Ok, H. Determination of the constituents of honey by near infrared spectroscopy. *J. Near Infrared Spectrosc.* **1998,** *6,* A367-A369.

Table 1

Classification of quality parameters of honey.

Parameters related to preservation	Moisture
	pH
	Free acidity and total acidity
Parameters related to freshness	Diastase activity
	Invertase activity
	Hydroxymethylfurfural
Parameters related to ripeness	Proline
	Moisture
	Sucrose
Parameters related to the botanical origin	Ash content
	Moisture
	Color
	Electric Conductivity
	Specific rotation
	Fructose/glucose
	Glucose/moisture
	pH
	Free acidity and total acidity
	Proline
	Sugar composition
Parameters related to adulteration	Sucrose
	Glucose-fructose-sucrose profile
	Proline
	Hydroxymethylfurfural
Parameters related to crystallization	Fructose/glucose
	Glucose/moisture

Table 2

Composition criteria of honey (EU Directive 110/2001).

Parameter	Concentration
Fructose and glucose content (sum of both):	
- Blossom honey	≥ 60 % (w/w)
- Honeydew honey and blends of honeydew honey with blossom honeys	≥ 45 % (w/w)
Sucrose content, in general (with exceptions)	≤ 5 % (w/w)
Moisture content, in general (with exceptions)	≤ 20 % (w/w)
Water-insoluble content:	
- in general	≤ 0.1 % (w/w)
- pressed honey	≤ 0.5 % (w/w)
Electric conductivity:	
- Blossom honey (with exceptions)	≤ 0.8 mS/cm
- Honeydew and chestnut honey	≥ 0.8 mS/cm
Free acid, in general	≤ 50 milli-equivalents/kg
Diastase activity:	
- In general	≥ 8 (Schale units)
- Honeys with low natural enzyme content	≥ 3 (Schale units)
- (e.g. citrus honey) and an HMF content ≤ 15 mg/kg	
Hydroxymethylfurfural (HMF):	
- In general except baker´s honey	≤ 40 mg/kg
- Honeys of declared origin from regions with tropical climate and blends of these honeys	≤ 80 mg/kg
Honeys with low natural enzymatic level (e.g. citrus honey)	≤ 15 mg/kg

Table 3

Analytical techniques for the determination of hydroxymethylfurfural and other furanic alhehydes and acids in honey.

Compound	Analytical techniques	References
Hydroxymethylfurfural	RP-HPLC (detection UV)	[12,31,33,42,44,47,48,96,105,107]
Hydroxymethylfurfural	UV/VIS spectrophotometry	[43,44]
Hydroxymethylfurfural	RP-HPLC (UV-detection), previous derivatization with PFBHA	[105]
Others furanic aldehydes and acids (2-F, 3-F, 2- FA, 3- FA)	RP- HPLC (detection UV)	[31,48]

2-F: 2-furfuraldehyde or furfural, 3-F: 3-furfuraldehyde, 2-FA: 2-furfuroic acid, 3-FA: 3-furfuroic acid

PFBHA: O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine·HCl

Table 4

Analytical techniques for the determination of carbohydrates.

Analytical techniques	References
HPLC-RID	27,54,67,70,80,108-111
HPLC-ELSD	79
HPLC-DAD	62
HPAEC-PAD	29,61,72,73,112-114
GC-FID	68,69,113,115,116
GC-MS	74,75
NIR spectroscopy	15,16,18,77,117,118
FTIR-ATR spectroscopy	19,73,78,79
Raman spectroscopy	80,81

Table 5

Analytical techniques for the detection of honey adulteration with sweeteners.

Technique	References
Stable carbon isotope ratio analysis (SCIRA)	85-88
Chromatography	27,61
NIR spectroscopy	89,90
FTIR-ATR spectroscopy	91,92,94
Raman spectroscopy	82

Table 6.

Analytical methods for the determination of methylglyoxal

Methods	References
RP-HPLC with UV-detection, previous derivatization with OPD	[96,97]*
RP-HPLC with UV-detection, previous derivatization with PFBHA	[104,105]**
RP-HPLC with RI detection (direct method)	[103]
HPLC-TOF-MS, previous derivatization with OPD	[102]
HPLC-DAD-ESI-MS, previous derivatization with OPD	[98]*
Headspace SPME/GC-NPD, previous derivatization with OPD	[106]
NMR spectroscopy	[102]
MIR spectroscopy-ATR	[100]

OPD: ortho-phenylenediamine, PFBHA: O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine·HCl,

* 3-deoxyglucosone (3-DG), methylglyoxal (MGO), and glyoxal (GO) were determined.

** hydroxymethylfurfural (HMF), methylglyoxal (MGO), and dihydroxyacetone (DHA) were determined.