Analysis of phenolic acids and flavonoids in honey

Krystyna Pyrzynska, Magdalena Biesaga

Honey is rich in phenolic acids and flavonoids, which exhibit a wide range of biological effects and act as natural antioxidants. The analysis of polyphenols has been regarded as a very promising way of studying floral and geographical origins of honeys. This review surveys recent literature on determination of these active compounds in honey. The analytical procedure to determine individual phenolic compounds involves their extraction from the sample matrix, analytical separation and quantification. We pay particular attention to sample pre-treatment and separation techniques (e.g., high-performance liquid chromatography and electrophoresis).

Keywords: Analysis; Antioxidant; Capillary electrophoresis (CE); Flavonoid; High-performance liquid chromatography (HPLC); Honey; Phenolic acid; Polyphenol; Sample pre-treatment; Separation

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1. Introduction

Phenolic compounds or polyphenols are one of the most important groups of compounds occurring in plants, in which they are widely distributed. Polyphenols are also products of the secondary metabolism of plants. Flavonoids and phenolic acids (both benzoic and cinnamic-acid derivatives) constitute the most important classes of polyphenol, with more than 5000 compounds already described [1].

Flavonoids of dietary significance can be categorized as flavonols, flavanones, flavones, anthocyanidins and isoflavones (Fig. 1). They exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic and antithrombotic activities [2]. Epidemiological studies point to their possible role in preventing cardiovascular diseases and cancer. Flavonoids behave as antioxidants in a variety of ways, including direct trapping of reactive oxygen species, inhibition of enzymes responsible for producing superoxide anions, chelation of transition metals involved in processes forming radicals and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals [3].

Honey is a natural food product well known for its high nutritional and prophylactic-medicinal value. Ancient Egyptians and Greeks used honey as a medicine to treat ailments (e.g., stomach ulcers and skin wounds). Apitheraphy (the medical use of honeybee products) has recently become the focus of attention as a folk and preventive medicine for treating certain conditions and diseases as well as promoting overall health and well-being [4].

Because of its sweetness, color and flavor, honey is often used as a sugar substitute, an ingredient or a natural preservative in many of manufactured foods. It can prevent oxidation reaction in foods (e.g., lipid oxidation in meat [5] and enzymatic browning of fruits and vegetables [6]). From the chemical point of view, honey is a highly concentrated solution of a complex mixture of sugars. Its composition depends strongly on the plant species from which nectar or honeydew was collected and other factors (e.g., environmental conditions and climate [1]). Apart from sugars, honey also has a wide range of minor constituents, many of which, including polyphenols, are known to have antioxidant properties. It has been demonstrated that honey is similar in antioxidant capacity to many fruits and vegetables on a fresh-weight basis, as measured by the assay of absorbance capacity of oxygen radicals [7]. Honeys with dark color have a higher total phenolic content and consequently a higher antioxidant capacity [8].

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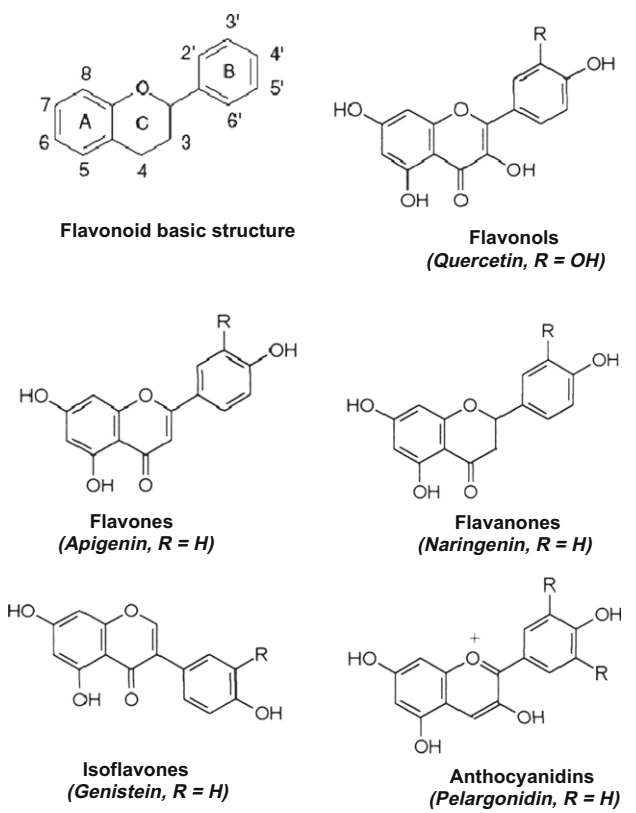

Figure 1. Chemical structures of the common classes of flavonoids. Representative compounds are shown in brackets.

Analysis of phenolic compounds has also been regarded as a very promising way of studying floral and geographical origins of honeys [9–17]. For example, hesperetin has been used as a marker for citrus honey and kaempferol for rosemary honey as well as quercetin for sunflower honey [10]. Some phenolic acids (e.g., ellagic acid in heather honey [11] and hydroxycinnamates (caffeic, *p*-coumaric and ferulic acids) in chestnut honey [12]) have also been used as floral markers.

The main difference between the Australian and European Eucalyptus honeys is the content of propolis-derived flavonoids (e.g., pinobankin, pinocembrin and chrysin [14]). The botanical origin of honey is one of its main quality parameters and its price is very often related to this floral origin. It has been pointed out that analysis of the volatile compounds in honey [15] as well as its mineral content [18] could be also a useful tool for characterization of botanical and geographical origin.

On the basis of the usefulness and the importance of natural phenolic acids and flavonoids, we review their analysis in honey samples mainly in the period 2000–08. There have been a number of reviews on the analysis of phenolic compounds [19–22], but they mostly related to fruits and vegetables.

In general, an analytical procedure for the determination of individual phenolic compounds involves the basic steps of isolation from a sample matrix, analytical separation, identification and quantification. The recovery step usually involves solid-phase extraction (SPE) or solvent extraction using a range of solvents. Separation is commonly achieved by HPLC or capillary electrophoresis (CE), although gas chromatography (GC) is used in some instances. The most common mode of separation exploits reversed-phase (RP) systems typically with a C_{18} column and various mobile phases. Detection is routinely achieved by ultraviolet (UV) absorption often involving a photodiode detector and various mass-spectral methods. We present the current literature related to analytical procedures that allow the determination of phenolic acids and flavonoids in honey, individually or as a group simultaneously, and discuss their advantages and disadvantages. We pay particular attention to the sample-preparation step.

2. Preparation of honey samples

Typically, the procedure includes sampling a representative sample, homogenization, extraction, removal of matrix and preconcentration (if needed) prior to final analysis. In some cases, depending on the type of compound and the detector, a derivatization step may be necessary. Fig. 2 shows the main steps of the analytical procedure applied to determination of phenolic acids and flavonoids in honey samples.

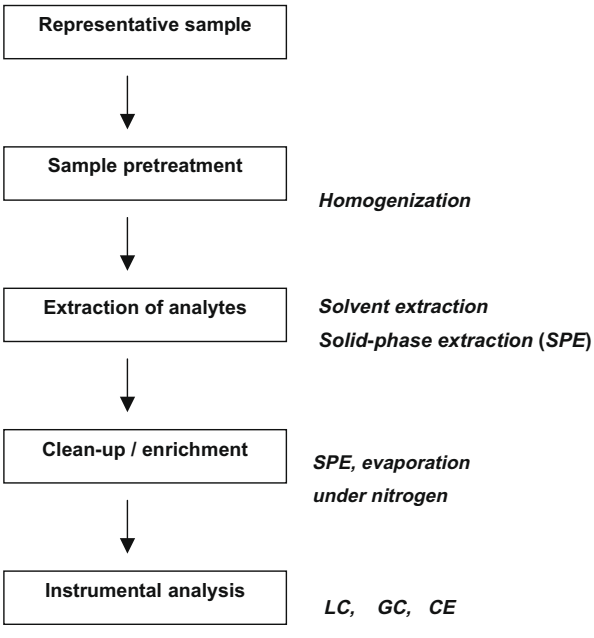

Figure 2. Main steps of the analytical procedure for the determination of phenolic acids and flavonoids in honey.

It is very important for the sample to be representative (i.e. to reflect the average composition of the whole batch of honey). For small portions of sample, the heterogeneity of the raw material is significant and may result in great inconsistency in results, especially when more subsamples of the same material are analyzed. In order to obtain a representative sample, it is advisable to homogenize it by stirring thoroughly [23]. Manual stirring (e.g., 3 min before sonication for 10 min at room temperature) [23] as well as mechanical stirring with a blender have been recommended [24]. If the honey is crystallized, it can be gently heated on a stove or in a thermostatic bath, but at no more than 40–50°C [25].

2.1. Extraction

The ultimate goal is preparation of a sample extract uniformly enriched in all components of interest and free from interfering matrix components [26]. Generally, for analysis of phenolic acids and flavonols in honey, sugars must be removed to start with. Apart from removing matrix components, this can isolate and concentrate analytes. In the case of liquid-liquid extraction (LLE), the solvent is usually ethyl acetate [27,28] or ethanol [29,30]. LLE is usually directed at isolating aglycones, while other methods (e.g., extraction combined with hydrolysis at elevated temperatures) can have as their goal isolation of both aglycones and conjugates.

Pinelo et al. [31] tested the possibility of quercetin degradation under different solvent and temperature conditions. An initial increase and then a decrease in its anti-radical activity were observed in ethanol and methanol solutions when storage time was prolonged. By contrast, a progressive decrease in antioxidant activity was determined in 10% (v/v) ethanol-water solution due to oxidative cleavage, which is favored under these conditions.

In recent years, some novel extraction methods of flavonoids have been developed [e.g., microwave-assisted extraction (MAE) and ultrasonic extraction (UE)]. The experimental results demonstrated that extraction time is dramatically reduced and the yields of flavonoids are effectively improved [30,32]. However, MAE selectivity was low, with significant amounts of non-phenolic material. Longer irradiation times in UE resulted in a decrease in the percentage of extracted components, presumably due to degradation processes [30]. The alternative extraction methods {e.g., supercritical fluid extraction (SFE) [33–35] and pressurized liquid extraction (PLE) [36]}, due to shorter extraction time and reduced solvent consumption, have gained in popularity for isolating polyphenolic acids and flavonoids. Due to the apolar property of CO_2 in SFE, a significant amount of polar organic modifiers has to be added to obtain a high extraction yield, but this reduces selectivity [35].

Several authors have carried out SPE procedures to remove matrix components from honey [14,37–53]. According to Tomás-Barberán et al. [37] and repeated in other procedures [14,38–47], Amberlite XAD-2 adsorbs honey phenolic compounds with a recovery rate of 80–90%. Generally, the honey samples were mixed with five parts of water acidified to pH 2 with HCl and filtered through cotton to remove solid particles. The filtrate was then passed through the column containing Amberlite XAD-2. The phenolic compounds remained on the column, while sugars and other more polar compounds were eluted with acidified water. The whole phenolic fraction was desorbed using methanol and dried under reduced pressure at 40°C. In some cases, the filtrate was mixed with Amberlite particles and stirred in a magnetic stirrer for 10 min before filling the column [14,42–45]. For the clean-up step, the residue obtained after evaporation of methanol was redissolved in distilled water and extracted with diethyl ether, then the solvent was removed by flushing with nitrogen. The dried residue was then redissolved with methanol and filtered through a 0.45-μm membrane filter, ready for HPLC analysis [14,42,44].

C18 SPE cartridges have also been used for the recovery of phenolic compounds from honey [50,51]. Honey samples were subjected to basic hydrolysis and extracted with ethyl acetate [50]. The dry extract was redissolved in acidified deionized water (pH 3.5) and the phenolic compounds were adsorbed onto preconditioned (with methanol and acidified water) Isolute C18 columns. The analytes were eluted by passing 25% (v/v) methanol-water solution at a drop-wise flow rate. The recovered fraction was then dried under nitrogen and subjected to further analysis.

Dimitrova et al. [51] proposed SPE isolation and enrichment of phenolic acids on BondElut C18 cartridges using an acetonitrile-tetrahydrofuran (1:1, v/v) elution system. The behavior of the compounds studied was predicted from preliminary calculations involving the pK_a constants of the carboxylic groups, the *n*-octanol-water partition coefficients and the distribution coefficients at different pH values of the conditioning and washing solvents.

To meet the objective of isolating polyphenolic compounds from honey, the performance of several cartridges packed with different sorbents (C18 BondElut and polymeric Strata-X, Oasis HLB as well as Amberlite XAD-2) has been compared [52]. As can be seen in Fig. 3, C_{18} silica was found to be less appropriate for recovery of compounds tested. However, some polyphenols (e.g., quercetin) displayed recovery >90%. Better performance of polymeric sorbents in comparison with C_{18} can be attributed to their aromatic structure, which can sorb aromatic phenolic compounds via π-π interactions. Kaempferol, *p*-coumaric acid and syringic acid were completely sorbed onto Amberlite XAD-2, but recovery of quercetin by methanol was only 54%. It was found that increasing the amount of Oasis HLB sorbent up to 2.5 g gave a mean recovery above 80% for rutin [52].

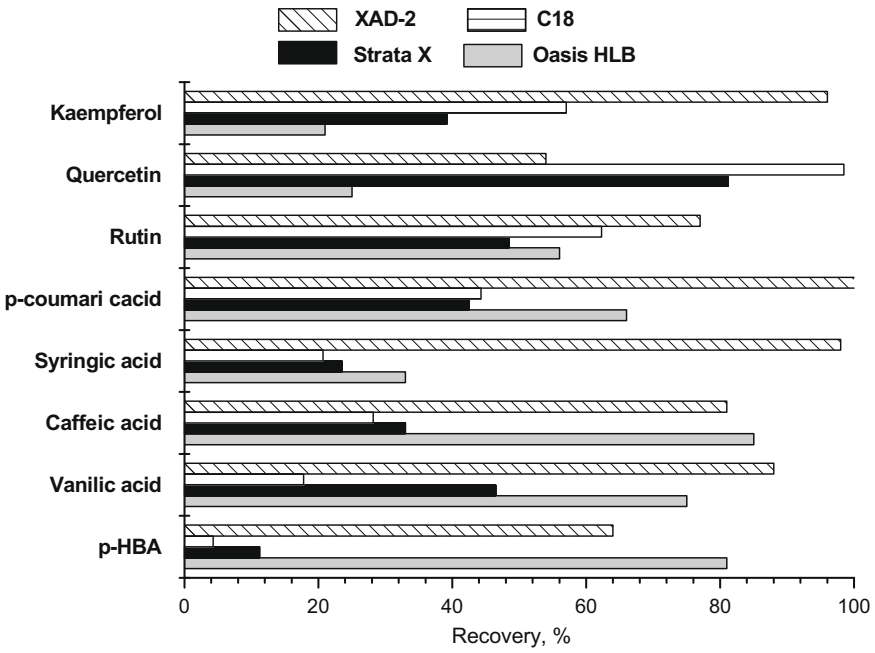

Figure 3. Recoveries of phenolic acids and flavonoids extracted from deionized water (pH 2) spiked at the 10 μg/mL level using different SPE cartridges (based on [52]).

2.2. Hydrolysis

If aglycones are the target analytes, chemical hydrolysis is usually performed with hydrochloric acid or formic acid at elevated temperature (80–100°C) [19,26,54]. The efficiency of recovery depends on acid concentration, hydrolysis time and temperature. In most publications, the hydrolysis of flavonoid glycosides from vegetables and fruits is carried out in 1.2 mol/L HCl at 90°C for 2 h, following a procedure presented by Hertog et al. [55]. However, the extended exposure time to HCl could cause degradation of some flavonoids (e.g., quercetin [56]).

Generally for the hydrolysis process, the optimum compromise is to achieve complete release of aglycones and to minimize degradation reactions of compounds involved. For this purpose, one experimental design was described [57]. Applying multiple-regression analysis on the data set, it was possible to obtain a mathematical model that took into account linear, quadratic and cross-product terms (e.g., optimum conditions for rutin hydrolysis corresponded to HCl concentration of 1.5 mol/L and a hydrolysis time of 1 h).

3. Chromatographic and electrophoretic analysis of polyphenols in honey

In general, separations of phenolic acids and flavonoids have been carried out by HPLC equipped with RP columns, generally packed with spherical particles of silica bonded with octadecyl (C_{18}) chains [21,22].

HPLC columns packed with monolithic supports, comprising a single piece of porous material, provide an alternative means of performing fast separations. The main advantage of this type of support is its excellent hydrodynamic property, which allows back-pressure to be reduced and the flow rate increased. Monolithic columns are increasingly being applied in phytochemical analysis [58]. However, in the field of food analysis, they have been used for determination of only phenolic compounds in wine [59] and phenolic acids in fruits [60].

Gradient elution is usually used in recognition of the complexity of the phenolic profile of honey samples (Table 1). Numerous mobile phases have been employed but binary systems comprising an aqueous component and a less polar organic solvent (e.g., acetonitrile or methanol) remain common. Acids (e.g., formic, acetic or phosphoric) have usually been added to maintain appropriate pH during gradient runs. Isocratic elution has been employed for analysis of phenolic acids in strawberry-tree honey [27]. The elution pattern is usually benzoic acids, cinnaminic acids, flavonone glycoside followed by flavonol and flavone glycosides and then the free aglycones in the same order. Table 1 presents selected examples of application of HPLC to the determination of phenolic acids and flavonoids in honeys. Fig. 4 shows the HPLC profiles of rosemary honeys from

Table 1. Selected examples for separation of phenolic acids and flavonoids of honey using HPLC methods

Sample	Column	Mobile phases	Detection	Identified compounds	Ref.
Eucalyptus honey	Lichrocart RP-18 (125 × 40 mm, 5 μm)	A: water-formic acid (19:1, v/v) B: methanol	DAD λ = 290 nm and 340 nm	Myricetin, tricetin, quercetin, luteolin, quercetin-3-methyl ether, kaempferol, pinocembrin, chrysin, pinobankins, genkwanin, isorhamnetin	[14]
Sunflower honey	Lichrosorb RP-18 (200 × 3 mm, 7 μm)	A: water-phosphate acid (pH 2.6) B: acetonitrile	DAD λ = 280 nm and 310 nm	Benzoic acid, ferulic acid, pinocembrin, chrysin, galangin	[29]
Australian *Eucalyptus* honeys	Lichrocart RP-18 (125 × 40 mm, 5 μm)	A: water-formic acid (19:1, v/v) B: methanol	DAD λ = 290 nm and 340 nm	Gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid	[39]
New Zealand and Australian *Leptospermum* honeys	Lichrocart RP-18 (125 × 40 mm, 5 μm)	A: water-formic acid (19:1, v/v) B: methanol	DAD λ = 290 nm and 340 nm	Myricetin, tricetin, quercetin, luteolin, kaempferol, kaempferol 8-methyl ether, pinocembrin, chrysin, gallic acid, ellagic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, syringic acid	[42]
Tunisian honeys (eucalyptus, thyme, rosemary, orange, sunflower, rape, multi-floral)	Lichrocart RP-18 (125 × 40 mm, 5 μm)	A: water-formic acid (19:1, v/v) B: methanol	DAD λ = 290 nm and 340 nm, NMR, EIMS	Ellagic acid, pinobankisin, hesperetin, quercetin, luteolin, 3-methylquercetin, 8-methoxykaempferol, quercetin 3,7-dimethyl ether, galangin, apigenin, isorhamnetin, pinocembrin, chrysin	[43]
Australian honeys from botanical species (*Melaleuca, Banksia, Lophostemon, Guioa, Helilianthus*)	Lichrocart RP-18 (125 × 40 mm, 5 μm)	A: water-formic acid (19:1, v/v) B: methanol	DAD λ = 290 nm and 340 nm	Myricetin, tricetin, quercetin, luteolin, quercetin-3-methyl ether, quercetin-3,3′-dimethyl ether, kaempferol, pinocembrin, chrysin, pinobankins, genkwanin	[44]
Honeybee-collected pollen	Nucleosil C18 (250 × 46 mm, 10 μm)	A: water-phosphate acid (pH 2.6) B: methanol	DAD λ = 280 nm and 350 nm	Vanillic acid, syringic acid, *p*-coumaric acid, rutin, quercetin, protocatechuic acid, myricetin, kaempferol, isorhamnetin	[45]
Acacia, eucalyptus, lime, chestnut, heather, lavender, rosemary, orange, sunflower, rapeseed honeys	Spherisorb ODS-2 (250 × 4.6 mm, 5 μm)	A; phosphate buffer (20 mM, pH 2.92) B: methanol	DAD λ = 220 nm and 280 nm	Benzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, gallic acid, syringic acid. vanillic acid, *p*-coumaric acid, caffeic acid, ferulic acid, phenylacetic acid	[51]
Linden, heather honeys	Ascentis (C18, 150 × 4.6 mm, 5 μm)	A: formic acid (2 mM, pH 2.7) B: methanol	DAD λ = 254 nm MS	Gallic acid, *p*-HBA, vanillic acid, caffeic acid, syringic acid, rutin, quercetin, kaempferol	[52]
Acacia, Chinese milk vetch, buckwheat, manuka honeys	Discovery RP Amide C16 (150 × 4.6 mm, 5 μm)	A: 0.5% acetic acid (95:5, v/v) B: methanol - 0.5% acetic acid (95:5, v/v)	Electrochemical detection and MS	Methyl siringate	[53]
Strawberry-tree honey	Spherisorb ODS-2 (250 × 4.6 mm, 5 μm)	Methanol- sulphuric acid (10:90, v/v, 10^{-2} N)	DAD λ = 292 nm MS, NMR	Homogentisic acid	[27]

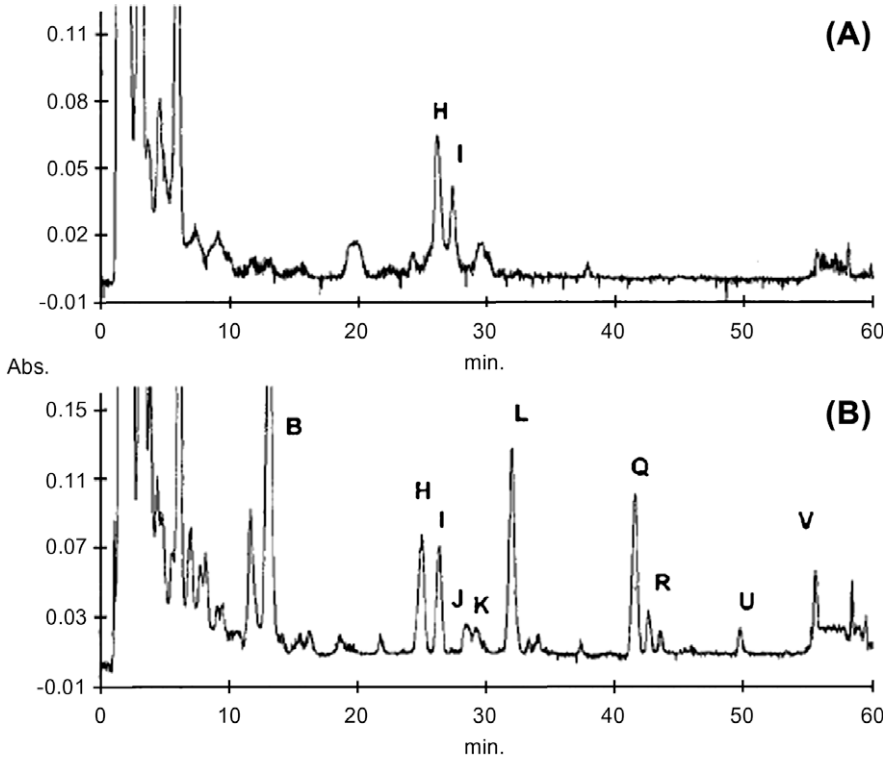

Figure 4. HPLC phenolic profiles of Tunisian (A) and Spanish (B) rosemary honeys detected at 290 nm [43]. LiChroCART RP-18 column (12.5 × 0.4 cm, 5 μm particle); gradient eluent water/formic acid (19:1, v/v); flow rate 1 mL/min. Peak identification: B, Pinobanksin; H, 8-metoxykaempferol; I, Kaempferol; J, Apigenin; K, Isorhamnetin; L, Pinocembrin; Q, Chrysin; R, Galagin; U< Pinocembrin 7-Me; V, Tetochrysin.

Tunisia and Spain [43]. In the Tunisian honey, only the floral-derived metabolites, kaempferol and 8-metoxykaempferol, were presented, and these compounds appeared in amounts and proportions similar to those found in the Spanish sample.

Ultra-performance LC (UPLC) takes advantage of technological strides made in particle-chemistry performance [61]. Using 1.5–2 μm particles, narrower analytical columns and instrumentation that operates at higher pressures than those used in HPLC, dramatic increases in resolution, sensitivity and speed of analysis can be obtained. The same separation on RP-HPLC that takes over 20 min, can be accomplished in under 3 min by UPLC. This new chromatographic methodology has been applied so far for separation and quantification of the major chocolate polyphenols [62].

Several attempts have been made to correlate flavonoid structures to their chromatographic retention using different parameters for structure characterization [63,64]. Stefanova et al. [64] proposed a method based on the assumption that the effects of substituents would be additive. The influence of two different substituents (OH and OCH_3 groups) in eight possible positions in the flavone ring on RP-HPLC retention was studied in a group of 21 flavones. The results obtained were used for evaluating the structure of an unknown compound in the methanolic extract and the predicted pattern of substituents was then experimentally confirmed by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy of the isolated flavone.

GC has been also employed for the analysis of polyphenols, mostly phenolic acids in honey [65–67]. However, this technique does not seem to be the most suitable, because the vast majority of these compounds are not volatile, so a derivatization step is necessary; and mainly methylated or trimethylsilyl derivatives are used.

Although HPLC remains the most dominant separation technique for polyphenolic compounds, CE is gaining popularity and represents an alternative method for the analysis of plant materials (Table 2). CE modes primarily used for these purposes are capillary-zone electrophoresis (CZE) [45–47,68] and micellar electrokinetic chromatography (MEKC) [69].

To achieve ionization of hydroxy compounds (as relatively weak acids) enabling their separation by CZE, background electrolytes based on borate or acetate at pH 9–10 is used. CZE are mostly applied to charged analytes and the charge-to-size ratios determine the electrophoretic migration times. In MEKC in the presence of surfactant [e.g., sodium dodecylsulfate (SDS)], separation is based on hydrophobicity, which affects the analyte partitioning between the aqueous phase (moving with

Table 2. Capillary electrophoretic methods for analysis of phenolic compounds in honey

Sample	Buffer	Detection	Identified compounds	Ref.
Rosemary honey	Ammonium acetate (100 mmol/L, pH 10) + 2-propanol (90:10, v/v)	MS	Kaempferid, kaempferol, quercetin 3′,3′-dimethyl ether, quercetin 7,3′-dimethyl ether, monogalloyl, pinobanksin, pinocembrin, chrysin, myricetin	[45]
Citrus, thyme, rosemary, lavender honeys	Sodium borate (100 mmol/L, pH 9.5) with 20% (v/v) methanol	DAD $\lambda = 280$ nm	Syringic acid, *p*-coumaric acid, caffeic acid, cinnamic acid, chlorogenic acid, ferulic acid, gallic acid	[50]
Rosemary honey	Ammonium acetate (100 mmol/L, pH 9.5)	DAD $\lambda = 280$ and 340 nm	Kaempferol, ferulic acid, chrysin, pinocembrin, *p*-coumaric acid	[51]
Propolis extract	Sodium borate (100 mmol/L, pH 9)	DAD $\lambda = 254$ nm	Pinocembrin, acacetin, chrysin, rutin, catechin, naringenin, luteolin, cinnamic acid, galangin, quercetin, kaempferol, apigenin, myricetin, caffeic acid	[68]
Propolis extract	Phosphate buffer (50 mmol/L, pH 7) + 25 mmol/L SDS + 25 mmol/L sodium cholate	DAD $\lambda = 380$ nm	Quercetin, kaempferol, galangin, chrysin	[69]

the electro-osmotic flow) and the micellar phases (charged and migrating with a different velocity). Compounds, such as flavonoids, strongly interact with micelles and consequently selectivity may be varied by modifying the micellar phase. The used of sodium cholate in combination with SDS is interesting due to the type and the properties of the micelles that each forms [68].

Wang et al. [70] compared the electrophoretic behavior of 13 flavonoids using these two modes of CE. The separation selectivity of MEKC was shown to be better than that of CZE, because electrophoretic behavior in the latter is affected by more factors (e.g., degree of saturation and the stereochemistry of the C-ring, alkyl substitution and the number and position of phenolic hydroxy groups, methylation and glycosylation of the hydroxy groups as well as the complexation of flavonoids with borate buffer). Non-aqueous CE separation of a group of flavonoids was investigated in methanol at high pH to alter the selectivity of the separation [71].

As a separation technique, CE is still evolving and a new mode of separation, called capillary electrochromatography (CEC), has been developed. This hybrid method combined CZE and μ-HPLC [72]. It combines the advantages of both these techniques, offering a separation mode exploiting chromatographic retention and electrophoretic mobility. The application of CEC was explored for analysis of quercetin [73] and biologically-relevant flavonols [74]. However, in spite of such promise, CEC still faces serious problems (e.g., column cost and conditioning). The excessive time and the labor required to obtain a stable baseline and reproducible retention times not only increase considerably the overall price of analysis but also contribute to bubble formation.

4. Detection and identification

Routine detection in HPLC and CE is typically based on measurement of UV absorption, often using diode-array detection (DAD). A match of both UV-Vis spectrum and retention time can lead to strongly positive identification of the separated analytes. DAD can simultaneously detect chromatograms at different wavelengths. This feature significantly enhances the performance of the separation system, particularly when different groups of polyphenols are mixed in one sample. When suitable wavelengths are chosen (e.g., at maximum absorption), all groups can be detected with the highest sensitivity. An appropriate selection of the detection wavelength can also make possible quantification of an unresolved or poorly resolved peak [20]. However, use of conventional approaches based on spectra is often limited when samples contain very similar compounds.

Detection based on fluorescence is generally more sensitive than UV absorption. Quercetin and its glycosides do not show intense native fluorescence, although Rodriguez-Delago et al. [75] reported optimum excitation (260 nm and 264 nm) and emission wavelengths (426 nm and 420 nm) for quercitrin and quercetin, respectively. Many flavonoids can form fluorescent

chelates with several cations [e.g., Mg(II), Be(II), Zn(II), Sc(III), Ga(III), In(III) and Al(III)], which could be used as post-column derivatization reagents for HPLC with fluorescence detection [75–77]. The limits of detection (LODs) for determination of quercetin, based on formation of its fluorescent complex with Al(III) [76] and Ga(III) [77], was found to be 0.15 μg/L and 16.2 μg/L, respectively. The study shows that the 3-hydroxyl-4-keto oxygen site is essential for fluorescence as rutin – containing a sugar bound to the 3-hydroxyl group – does not form a fluorescent chelate [76].

HPLC or CE with electrochemical detection can be a useful complete technique because most flavonoids contain phenolic hydroxyl groups that are electro-active at modest oxidation potentials [53,78–80]. Romani et al. [80] compared HPLC procedures with DAD and electrochemical detection (differential pulse voltammetry and amperometric biosensor with bare graphite screen-electrodes) for analysis of phenolic compounds in natural extracts. The most accurate data were obtained from HPLC-DAD analysis, while differential pulse voltammetry was considered a good, quick method for screening. In the electrochemical methods reported for flavonoid compounds, carbon was most used as electrode material (e.g., carbon-disc electrode [81], graphite-carbon-paste electrode [82], porous-graphite electrode [57] and glassy-carbon electrode [83]). Recently, multi-walled carbon nanotubes have been found to be excellent electrode material for the determination of flavonoids at trace levels due to their strong surface adsorption [84,85]. Electroanalytical methods are very useful for determination of flavonoids due to their sensitivity, selectivity and accuracy, and the vital information on electrochemical mechanisms, which are sometimes comparable to those occurring in metabolic processes in living organisms.

Modern MS detections are very suitable for the analysis of flavonoids and phenolic acids in foodstuffs, since they can achieve very high sensitivity and provide information on the molecular mass and on structural features [21,22,45,49,50,86]. More detailed structural information can be obtained subsequently by resorting to tandem MS (MS^2) in combination with collision-induced dissociation (CID). With regard to structural characterization, information can be obtained on the flavonoid aglycone part, the types of carbohydrates or other substituents present, the stereochemical structure of terminal monosaccharide units, the sequence of the glycan part, interglycosidic linkages and attachment points of the substituents to the aglycone. The different MS techniques that can be applied to analyze flavonoids [e.g., electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB) and thermospray (TSP)] have been reviewed [87–89]. It was experimentally confirmed that quercetin sensitivity with the ESI mode is better than that with the APCI mode [90]. The negative mode provides the highest sensitivity and results in limited fragmentation, making it most suited to inferring the molecular mass of the separated flavonoids, particularly when their concentrations are low [88,90,91].

The analytical performance of APCI and ESI techniques, in both positive and negative modes, using two different mass spectrometers (a triple-quadruple and ion-trap instrument) were compared by de Rijke et al. [92]. In general, the use of APCI in the negative mode gave the best response for the 15 flavonoids used as test compounds, with the signal intensities and the mass-spectral characteristics not differing significantly between the two instruments. Under optimum conditions for LC eluent (methanol-ammonium formate, pH 4), full-scan LODs of 0.1–30 mg/L were achieved. It is interesting to add that the results obtained with APCI and ESI, both in negative mode, were closely similar for all aglycones. This has some practical importance since, in many cases, glycosides are hydrolyzed prior to quantification.

Several papers discussed in more details the possible fragmentation pathways of flavonoid aglycones, *O*-glycosides, *C*-glycosides and acylated glycosides [93–96]. An alternative approach for distinguishing isomers that differ by only their glycosylation site is to form flavonoid glucoside/metal complexes of the type $[M(II)\ (L)\ (L–H)]^+$, where M is the metal ion and L is the flavonoid glycoside [95,97,98]. Davis and Brodbelt [96] found that CID and Mg(II) complexes resulted in distinctive fragmentation patterns that are indicative of five commonly-observed flavonoid-glycosylation sites. Additional information for identification and structural characterization of target analytes could be derived from LC-retention behavior, UV-absorbance spectra and sometimes fluorescence or electrochemical characteristics, due to comparison being made with standard injection. On-line coupling of LC and NMR has increasingly attracted attention. The main advantages (e.g., high information content, differentiation of isomers and substitution patterns) and disadvantages (low sensitivity, expensive instrumentation and long run times) have been discussed in reviews [22,99]. For a more comprehensive structural elucidation of a novel natural product, preparative isolation is often necessary because, in LC-NMR, part of the ^1H spectra region is usually lost and, in most cases, LC-NMR does not provide the ^{13}C NMR data that are indispensable [100].

5. Conclusions

Honey possesses valuable nourishing, healing and prophylactic properties, which result from its chemical composition. The content of polyphenolic compounds (e.g., flavonoids and phenolic acids) in honey is strongly

affected by floral and geographical origin as well as by climate characteristic of the site. These antioxidants reportedly have a beneficial effect on human health. In addition, honey can be considered a biomarker for environmental pollution and can accumulatively indicate the level of air, water, plant and soil contamination over the forage area of the bees [18]. Because of the importance of natural polyphenols, interest in their identification and quantification in honey samples has significantly increased in recent years.

Many analytical procedures have been directed towards determination of the complete phenolic profile of honey. The separation techniques employed have been HPLC and CE, mostly combined with DAD and MS. Rarely does MS, even multiple MS (MS^n), provide an unambiguous structure, and it becomes necessary to combine MS with other spectroscopic techniques (e.g., UV and NMR) for elucidation of flavonoids. The application of LC, where the eluent is split between MS and NMR, constitutes a powerful combination for the determination of molecular structure, but this technique has not yet been applied significantly to flavonoids. Electrochemical detection and fluorescence detection have also been used in some cases in the analysis of flavonoids due to their sensitivity and selectivity.

Ávila et al. [101] proposed a novel electrochemical route to estimate the antioxidant capacity in honey samples. The analytical strategy involved the selective oxidation of polyphenolic compounds using two different target potentials, +0.8 and +0.5 V, at two different pHs. Using this fast procedure, food samples could be screened quickly according to their antioxidant activity prior to using separation techniques that allow recognition of the individual polyphenol responsible for the antioxidant activity.

Since honey has a complex matrix and low concentration levels of some polyphenolic compounds, it is necessary to apply several steps (e.g., extraction, preconcentration or purification) in order to obtain a sample extract uniformly enriched in all components of interest and free from interfering matrix components. This procedure is usually the most time-consuming and most error prone. It is necessary to validate the method to ensure suitable levels of recovery and repeatability. Environmental and economical concerns have led analysts towards smaller sample sizes and reduction of the required solvents. However, more realizable results are obtained with larger sample sizes.

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