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CHEMISTRY, BIOLOGY AND POTENTIAL APPLICATIONS OF HONEY BEE PLANT-DERIVED PRODUCTS

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CHAPTER 4

Chromatography as a Tool for Identification of Bioactive Compounds in Honeybee Products of Botanical Origin

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Abstract: Honey, propolis, and pollen are three important components of the beehive produced by honeybees mixing different plant parts (nectar, resin and pollen) with their own secretions, for further usage with different purposes in the hive. The fact that these natural products have been associated with numerous health benefits has attracted the attention of researchers resulting in a significant raise of scientific studies attesting their biological properties. Among the various constituents of honey, propolis and pollen, the phenolic compounds are the ones most frequently related to the beneficial properties of these products and hence, one of the main investigated groups. Their characterization is important to understand individual contribution(s) and synergistic effects of each compound for the overall biological effects of the bee product. To pursuit this goal, spectrophotometric techniques including HPLC, GC and TLC, alongside with the respective detection methods such as DAD, FLD and MS, have been developed and improved in order to offer better and more accurate separative performances.

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Susana M. Cardoso (Ed)

The aim of this review is to give an approach on the course that the chromatographic techniques have taken until the most recent trends on this field applied to the separation and characterization of the phenolic constituents of honey, propolis and bee pollen as well as an overall perspective of variability in terms of phenolic composition that can be found in the three bee products mentioned.

Keywords: Bee pollen, Benzoic acids, Bioactive compounds, Caffeic acid derivatives, Chromatography, Cinnamic acids, Coumaric acids, DAD, Flavonoids, FLD, GC, Honey, Honeybee-derived products, HPLC, MS, Phenolic compounds, Propolis, TLC.

1. INTRODUCTION

Honey, propolis, and bee pollen are produced from nectar, resin and floral pollen, respectively, mixed with different bee secretions and further used in the beehive for distinct purposes [1, 2]. Notably, these three bee products have also been used for centuries by Men, for food and medicinal purposes [3 - 5].

More recently, Men´s interest for these natural products have significantly raised, since scientific studies have attested their abundance on nutrients and bioactive compounds, together with their association with beneficial properties, including those of cardio-, neuro-, hepato- and chemo-protective, as well as chemo-preventive, antiseptic, antimicrobial, anti-allergic, antioxidant, anticancer, anti-radiation, anti-inflammatory and wound-healing activities, among many others [6 - 9]. Hence, overall, honey, propolis and pollen are now envised as very tempting and useful for a large spectrum of applications in different industries including foods, cosmetics, perfumes and pharmaceutics [7].

Among the numerous compounds from honey, propolis or bee pollen, the phenolic compounds are undoubtedly more frequently associated with the beneficial properties of these products [4, 5, 10, 11]. These compounds are a class of metabolites that are ubiquitously distributed through plant kingdom and plant-derived products [12], where they are important players in growth and reproduction, providing protection against pathogens and predators, besides contributing towards the color and sensory features of fruits, vegetables and their derived products [13].

Chemically, all the phenolic compounds possess at least one phenyl ring in its structure, and most of them arise from a common origin: the amino acids phenylalanine or tyrosine. These amino acids are deaminated to give cinnamic acids, further entering the phenylpropanoid pathway where one or more hydroxyl groups are added to the aromatic ring(s), ranging from simple phenols to complex compounds generally known as polyphenols or phenolic compounds [14]. The most common examples of phenolic compounds that can be found in foods include the phenolic acids (C_6-C_1), cinnamic acids (C_6-C_3) and flavonoids (C_6-C_3-C_6) [13] (Fig. **1**) and hence, in general these are also important groups of phenolic constituents of honey, propolis and bee pollen.

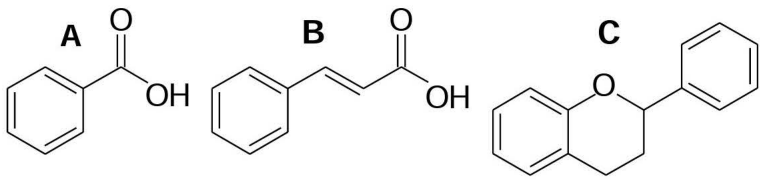


Fig. (1). Basic structure of phenolic acids (**A**), cinnamic acids (**B**) and flavonoids (**C**).

2. CHROMATOGRAPHIC METHODS

The close association between the beneficial properties of honey, propolis and pollen with their phenolic constituents boosted the need for characterizing them so that individual contribution(s) and synergistic effects on their biological activities can be elucidated.

Spectrophotometric assays, including Folin-Ciocalteu and Folin-Denis, for determination of the total phenolic content in plant samples, or reaction with $AlCl_3$ for total flavonoids measurement, are simple and economical, and can be useful for rapid and relatively inexpensive screening of numerous samples. However these techniques only give an estimation of the concentrations of the phenolic compounds over a certain minimum level and do not quantify phenolics individually. Besides, these reagents do not react specifically with phenols, since cross reactions commonly occur in complex samples and hence, unreliable data can be generated [15].

Therefore, it was necessary to replace these traditional methods by more efficient and precise separative equipment. In this field, chromatographic methods are irrefutably the most widely applied techniques due to their excellent separation ability and capability in analyzing multiple compounds in simultaneous [16]. These techniques are characterized by having two distinct phases, *i.e.*, a mobile phase consisting of a sample to be analyzed and a fluid/eluent that carries it out through an immobilized structure, which is known as the stationary phase. Based on differential partitioning between the mobile and stationary phases, the various constituents of the sample travel at different speeds, causing them to separate [17].

Further discussion of chromatographic techniques, namely high-performance liquid chromatography (HPLC), gas chromatography (GC) and thin-layer chromatography (TLC) will be carried out in this section, alongside with the detection systems since they are also important contributors for the characterization of polyphenols.

2.1. High-Performance Liquid Chromatography (HPLC)

Liquid chromatography (LC) is a separation technique that began in the early 1900s´. This technique was initially carried out in a glass cylinder packed with a fine powder and loaded with the sample mixture on top. An eluent was then poured into the column flowing down on it by gravity, dragging the sample compounds through the column at different speeds, causing them to separate [18].

HPLC is an upgraded technique from the traditional low pressure LC, which employ high operational pressures (up to 400 bar) [19]. In this case, smaller sample amounts and solvents are necessary and, therefore, the conventional HPLC typical column dimensions are 250 mm length x 4.6 mm diameter, filled with 5 μm particles, resulting in high plate numbers and consequent faster separations [20, 21]. All these features render this technique a superior resolving power when separating mixtures.

Several different separation modes of HPLC are known. Among them, normal-phase (NP), reverse-phase (RP), ion-exchange (IE) and size-exclusion (SE) are the major four chromatography techniques applied [21]. The NP-HPLC, also known as liquid-solid or adsorption chromatography, is the traditional separation method

based on adsorption/desorption of the sample in a polar stationary phase (typically silica or alumina). On the other hand, the RP-HPLC functioning is quite the opposite of the NP-HPLC, *i.e.*, the separation is based on analytes' partition coefficients between a polar mobile phase and an hydrophobic (nonpolar) stationary phase [22]. Regarding to the IE- and SE-chromatography, as the method names imply, the former is based on the exchange of ionic analytes with the counter-ions of the ionic groups attached to the solid support [23], by while the latter is based in the separation of molecules according to their sizes, *i.e.*, smaller molecules migrate slower through the gel pores than the larger ones which are excluded from the pores migrating faster down the column [24].

Despite the previous four methods are the most common, other chromatographic techniques are known, including the affinity chromatography, chiral chromatography, hydrophilic and hydrophobic interaction chromatography, electrochromatography, supercritical fluid chromatography, among others.

The RP-HPLC is however the most popular and consistent analytical technique for characterization of polyphenolic compounds and hence, this prediction applies to honey, propolis and bee pollen samples (Table **1**). The earliest stationary phases used for this method were solid particles coated with nonpolar liquids, which were later replaced by more permanently bonded hydrophobic groups, made up of hydrophobic alkyl chains such as butyl (C4), octyl (C8) or octadecyl (C18) groups bounded on silica support [21, 25].

Table 1. Selected HPLC conditions for determination of phenolic compounds in honeybee-derived products from the last four years.

Stationary Phase	Mobile phase	Detection method	Reference
Honey			
Betasil RP-C18 column (150 mm × 4.6mm, 3 µm p.s.)	Various compositions of water, acetonitrile, methanol, and mixtures of 1% formic acid aqueous solution with methanol or acetonitrile	PDA	[4]
Luna C-18 RP column (250 × 4.6 mm, 5 µm p.s.)	A: acetonitrile B: 1% formic acid in water	UV-Vis	[30]
Zorbax SB-C18 column (250 mm × 4.6 mm, 5 µm p.s,)	A: 0.5% formic acid in water B: methanol	PDA and ESI-MS/MS	[31]

(Table 1) contd.....

Stationary Phase	Mobile phase	Detection method	Reference
Hypersil gold C18 (100 mm × 2.1 mm, 1.9 µm p.s.)	A: water + 0.1% formic acid B: acetonitrile + 0.1% formic acid	ESI-MS/MS	[32]
C18 column (4.6 × 250 mm, 5 µm p.s.)	A: methanol B: 5% formic acid in water	PDA	[33]
Gemini C18 110A column (150 mm × 4.60 mm, 3 µm p.s.)	A: 0.2 M phosphoric acid B: acetonitrile	PDA	[34]
KinetexTM C-18 column (100 × 2.1 mm, 2.6 µm p.s.)	A: 8 mmol.L^{-1} formic acid in water B: acetonitrile	ESI-MS/MS	[35]
Propolis			
Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 µm p.s.)	A: acetonitrile B: 0.4% acetic acid in water	VWD	[36]
Zorbax Eclipse XDB C18 (50 × 2.1 mm, 1.8µm p.s.)	A: 0.1% formic acid in a water:acetonitrile 98:2 solution B: 0.1% formic acid in an acetonitrile:water 98:2 solution	ESI-MS/MS and NMR	[10]
WondasilTM column C18 (250 × 4.6 mm, 5 µm p.s.)	A: 1% formic acid in water B: methanol	PDA	[16]
Agilent Zorbax SBC18 column (250mm × 4.6mm, 5 µm p.s.)	A: water:methanol:formic acid (93:5:2) B: water:methanol:formic acid (3:95:2)	PDA	[6]
Zorbax Eclipse XDB C18 column (50 mm × 2.1 mm, 1.8 µm, p.s.)	A: 0.1% formic acid in a water:acetonitrile 98:2 solution B: 0.1% formic acid in a acetonitrile:water 98:2 solution	ESI-MS/MS	[37]
Bee Pollen			
RP-18 ODS-A column (250 mm × 4.6 mm, 5 µm p.s.)	A: acetic acid: water (1:20) B: methanol	PDA	[38]
RP Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 µm p.s)	A: 0.1% formic acid in 5% acetonitrile B: 100% acetonitrile	PDA	[39]
RP Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5-µm p.s.)	A: 0.5% acetic acid in acetonitrile:water (1:1) B: 2% acetic acid in water	UV-vis	[40]
RP C18 Zorbax 5B-RP-18 (Hewlett-Packard) column (4.6 × 250 mm, 5 µm p.s.)	A: 0.1% acetic acid in water B: methanol	PDA, and ESI-MS/MS	[41]
Thermo-Hypersil GOLD C18 RP column (250 mm × 4.0 mm, 5 µm p.s.)	A: ortho-phosphoric acid B: methanol	PDA	[42]

ESI-MS – Electrospray ionization mass spectrometer; NMR – Nuclear magnetic resonance; p.s. – Particle size; PDA – Photodiode array; RP – Reversed phase; UV-vis – Ultraviolet visible; VWD – Variable wavelength detector.

Due to the chemical complexity and similarity of polyphenols, the identification and quantification of these compounds is usually performed using a gradient elution of numerous mobile phases instead of an isocratic mode. However, the most common method usually consists of a binary system, where the mobile phase is composed of an acidified aqueous solution (with phosphoric, acetic or formic acids) and a less polar organic solvent (normally acetonitrile or methanol) [26]. The small quantity of acid added to the solvent system aims to suppress the ionization of phenolic and carboxylic groups, improving certain parameters such as retention time and resolution [25].

Despite HPLC is a very well established reliable technique, it could still suffer some improvements, especially concerning to the separation times which are usually long in food samples, bee products included. In general, higher efficiencies are obtained from narrower analytical columns diameters (10–150 μm) as well as from smaller particles dimensions (1.5–2 μm) [27]. However, despite the dramatic improvements in the sensitivity, resolution and speed of analysis, there are also some drawback aspects to be considered, being the major one the need for higher pressures in order to turn possible the passage of the mobile phase through the columns. Therefore, the creation of new instruments capable of generating and supporting pressures up to 1000 bar gave rise to what is currently known as ultra-performance LC (UPLC), a term that was firstly used by Waters Corporation [28]. With this new equipment, the same separation that would take over 20 min on RP-HPLC, can now be accomplished under 3 min [29].

Alongside with the separation techniques, the detection systems also play a very important role for the good functioning of these procedures. Particularly, the most common detection system for phenolics employed in HPLC are undoubtedly the ultraviolet–visible (UV–vis), photodiode array (PDA), and UV-fluorescence detectors (FLD) [15] (Table **1**). The existence of conjugated double and aromatic bonds, makes every phenol absorb in the UV or UV–vis region, ranging from 200–290 nm for benzoic acids and 190 to 380 nm for cinnamic acids, while the typical UV–vis spectra for flavonoids comprises two bands: the first with a maximum in the 240–285 nm range, which arises from the A-ring and the second in the 300–550 nm range, that arises from the B-ring. Therefore the most frequent

wavelength used for identification of phenolics is 280 nm, although dual monitoring at 254 and 280 nm, or 280 and 320 nm, can be ideal wavelengths [43, 44].

Although less common, detection by FLD offers a higher selective and sensitive quantification of compounds (λ_{ex} = 280; λ_{em} = 310) [45]. This is very useful to determine phenolic compounds in complex sample matrixes such as honey, allowing the quantification of compounds at trace levels. Indeed, it has been reported that the detection limits for *p*-hydroxybenzoic acid and quercetin in honeys from different botanical origins ranged from 25 $ng.kg^{-1}$ to 0.75 $\mu g.kg^{-1}$, respectively [46].

HPLC coupled to mass spectrometry has tremendously improved the analysis of non-volatile species, including those of phenolic compounds [47]. This analytical technique engages the generation of charged molecules in the apparatus ion source that will be sorted by electromagnetic fields according to their mass-to-charge ratio in the mass analyzer and finally measured, usually by a quantitative method in the detector [48]. Although several MS ionization sources can be applied (*e.g.* electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB) and thermospray (TSP), ESI has been proven to be the most suitable one [49].

The mass spectrometry analysis of phenolic compounds are commonly performed in the negative ionization mode, since this provides the highest sensitivity and results in limited fragmentation of the molecular ion, making it most appropriate to inferring the molecular mass of the separated phenolic compounds, particularly when their concentrations are low [29]. Notably, the use of tandem MS^n in combination with collision-induced dissociation (CID) are crucial for elucidation compounds´ structure or even distinguish between isomers [50], as this technique allows multiple stages precursor ion *m/z* selection followed by product ion detection for successive fragmentation and generation of further product ions.

The coupling of LC to a nuclear magnetic resonance (NMR) spectroscopy is another alternative that has increasingly attracted attention. This technology was initially used in the late 1940s´ to clarify the structure of molecules in organic

chemistry [51] and it consists in the submission of certain atomic nuclei to a magnetic field, allowing to exploit their magnetic properties [52, 53]. Notwithstanding, the low sensitivity, long run times and expensive instrumentation are the major weaknesses of this technique [54, 55].

2.2. Gas Chromatography (GC)

GC is also used in some instances for determining phenolic compounds both qualitatively and quantitatively. As observed in Table **2**, various literature reports describe the employment of this technique in its beginnings as an attempt to enable the determination of polyphenolic compounds either in honey, propolis or bee pollen [56 - 60]. The major drawback of GC in the analysis of phenolic compounds is the need for volatility, limiting the range of compounds that can be analyzed. To surpass this gap, compounds usually must pass through a derivatization process, *i.e.*, a chemical modification of the compounds to produce derivatives with properties that are more suitable for GC analysis [50]. Still, derivatization can be a challenge for analytes of interest in complex food matrixes since the presence of glycosides may interfere with their chemical modification [26]. Another alternative is the high-temperature, high-resolution gas chromatography (HT-HRGC) which is an established technique for separating complex mixtures and identifying high-molecular weight compounds that do not elute when analyzed on ordinary GC columns [15].

Earlier GC work was typically performed with flame ionization detection (FID), which is based on the detection of ions formed during combustion of organic compounds in an hydrogen flame [61 - 63], however the combination of GC with MS became widespread, since MS allows the acquisition of molecular mass data and structural information together with the identification of compounds. Notwithstanding, as reported by Araújo *et al.* [64], some of the compounds contained in propolis are not volatile enough for direct GC–MS analysis even upon derivatization or HT-HRGC–MS. Table **2** resumes the main characteristics applied in diverse studies using GC technique for analysis of phenolic compounds from honey, pollen and propolis.

Table 2. Selected GC conditions for the determination of phenolic compounds on bee-derived products from the last ten years.

Derivatization	Detection method	Column conditions	Reference
Honey			
----	MS	SPB-1 fused silica capillary column (25 m × 0.25 mm, 0.25 μm)	[58]
Methylation	FID-MS	HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm c.t.)	[34]
Pyridine + BSTFA	MS	HP-5MS column (30 m × 0.25 mm, 0.25 μm c.t.)	[65]
BF_3 in methanol	FID	CBP1-Shimadzu non-polar column (20 m × 0.2 mm, 0.25 μm c.t.)	[66]
Propolis			
BSTFA	MS	Borosilicate capillary column (20 mm × 0.3 mm, 0.1 μm c.t.)	[15]
BSTFA	FID	SE-54 capillary column (9 mm × 0.25 mm)	Adapted from [44]
BSTFA	MS	Borosilicate capillary column (20 mm × 0.30 mm, 0.1 μm c.t.)	
BSTFA	FID	SE−54 fused-silica capillary column (9 m × 0.25 mm, 0.25 μm c.t.)	Adapted from [26]
Pyridine + BSTFA including 1% TMCS	MS	OV1 capillary column (25 m × 0.25 mm)	[59]
Pyridine + BSTFA	MS	DB1 column (30 m × 0.32 mm)	Adapted from [67]
Pyridine + BSTFA	MS	HP5-MS capillary column (23 m × 0.25 mm, 0.5 μm c.t.)	
Pyridine + BSTFA	MS	HP5-MS capillary column (23 m × 0.25 mm, 0.5 μm c.t.)	
Methylation	MS	HP1 methyl silicone capillary column (25 m × 0.25 mm)	
BSTFA	MS	Borosilicate capillary column (20 mm × 0.3 mm i.d.)	
Methylation	MS	CBP5 column (30 m × 0.25 mm i.d.)	
BSTFA	FID-MS	Glass column (22 m × 0.2 mm)	
----	FID-MS	Fused silica capillary 55 (10 m × 0.3 mm, 0.1 μc.t)	
Pyridine + BSTFA	MS	HP5-MS capillary column (23 m × 0.25 mm, 0.5 μm c.t.)	[68]
TMSi	MS	Zebron ZB-5HT column (30 m × 0.25 mm, 0.25 μm c.t.)	[69]

(Table 2) contd.....

Derivatization	Detection method	Column conditions	Reference
Bee Pollen			
----	MS	Varian Factor Four column (30 m × 0.25 mm)	[60]
Methylation with ethereal diazomethane	MS	HP-5MS capillary column (30 mm × 0.25 mm, 0.25 μm c.t.)	[38]
Pyridine + BSTFA	EI-MS	HP-5 capillary column (30 m × 0.25 mm, 0.25 μm c.t.)	[11]

AED – atomic emission detection; BF_3 – Boron trifluoride; BSTFA – Bis(trimethylsilyl) trifluoroacetamide; c.t. – coating thickness; EI-MS – Electron ionization mass spectrometer; FID – Flame ionization detector; MS- Mass spectrometer; PA – Polyacrylate fiber; TMCS – trimethylchlorosilane; TMSi – trimethylsilylimidazole.

2.3. Thin-Layer Chromatography (TLC)

TLC is another chromatographic technique which is widely used for qualitative analysis of organic compounds, isolation of the individual compounds from multi-component mixtures, quantitative analysis, and preparative-scale isolation [70]. This technique is fast, inexpensive and several samples can be examined at the same time, side by side, providing a chromatographic fingerprint of the sample which is very useful for identification purposes [15, 44]. In fact, with the adequate fractionated multi-component mixtures, video images of the chromatograms can be obtained, making this technique the only chromatographic method that enables presentation of the obtained results in the picture form [71].

Among the many available TLC pre-coated plates (*i.e.*, those with the inorganic adsorbent layers like silica or silica gel and alumina; organic layers like polyamide and cellulose; organic, polar covalently bonded modifications of the silica gel matrix such as diol, cyanopropyl, and aminopropyl; and organic, non-polar bonded modifications namely RP2, RP8, RP18), it is possible to choose a suitable stationary phase according to the sample characteristics [70]. For example, a classical stationary phase of silica gel is widely used to separate more apolar flavonoids such as flavonols and isoflavonoids from propolis [67].

Likewise, as the samples are eluted with different mobile phases, these may also be adapted to the sample of matter. As an example, for the quantification of flavonoids and phenolic acids in propolis, Medic-Saric *et al.* [72] used two-

dimensional TLC with *n*-hexane/ethyl acetate/glacial acetic acid (31:14:5, nu/nu) (System A) and chloroform/methanol/formic acid (44:3.5:2.5) (System B) as mobile phases.

In this chromatographic method, visualization is usually read using a common wavelength performed in short- and long-wavelength UV light and in some cases spraying with different reagents, including methanolic diphenylboryloxyethylamine or ethanolic polyethyleneglycole 4000 [68, 73 - 75].

However TLC has some limitations including long development times, relative low reproducibility and moderate sensitivity [76, 77]. Moreover, the volatile mobile phase makes contact with the ambient atmosphere around the chamber, so factors such as humidity and temperature can affect the chromatogram [78]. In Table **3** it is possible to observe the main characteristics applied in diverse studies using TLC technique for analysis of phenolic compounds from propolis.

Table 3. Selected TLC conditions for the determination of phenolic compounds in propolis from the last ten years.

Mobile phase	Stationary phase	Detection method	Reference
n-Hexane-Ethyl acetate-Acetic acid (5:3:1, v/v/v)	Silica gel plates 60, 20 cm x 10 cm, 8 mm band	DART-MS	[79]
Ethyl acetate-Methanol-Water (75:15:0), Ethyl acetate-Formic acid-Water (80:10:10), Ethyl acetate-Formic acid-Acetic acid-Water	Silica	Densitometer	Adapted from [55]
Chloroform-Methanol-Formic acid (various v/v) n-Hexane-Ethyl acetate-Acetic acid (31:14:5)	Silica	UV	Adapted from [55]
n-Hexane-Ethyl acetate-Acetic acid, 31 + 14 + 5 (v/v), (mobile phase 1) or Chloroform-Methanol - Formic acid, 44 + 3.5 + 2.5 (v/v), (mobile phase 2)	20 cm x 20 cm silica gel 60 F254 plates	CAMAG Reprostar 3 densitometer	[72]
Petroleum ether/Ethyl acetate 7:3	20 cm x 20 cm silica gel 60 F254 plates	UV	[68]

3. TYPICAL PHENOLIC COMPOUNDS OF HONEYBEE PRODUCTS

The chemical composition of bee products of botanical origin is greatly dependent on the plants found nearby the hive, as well as on the geographic and climatic characteristics of the place. In this way, distinct samples of honey, propolis or bee pollen can greatly differ from each other with respect to their phenolic profile. Besides differences in the type of compounds, samples of honey, propolis or bee pollen also diverge with respect to the quantity of phenolics, even if they are from a close geographic region. *E.g.* Heather (*Calluna vulgaris*) honeys from different regions of Poland have been shown to contain a divergent total content of phenolic compounds (306 mg GAE/kg [80] and 698 mg GAE/kg [81]). Moreover, the three major propolis extracts available on the market are very distinct between them according to the presence of certain phenolic composition. The poplar type of propolis, also known as CAPE-based propolis and commonly found in European, eastern Asia and New Zealand regions, is typically abundant in caffeic acid phenethyl ester. Another very popular type of propolis is the green propolis from Brazil which is commonly found in areas where plants from *Baccharis* species are abundant and is particular rich in artepillin C. The third is the red propolis from Brazil or China which contains neither CAPE nor artepillin C and it is the less studied up to date [82]. Attending to the mentioned differences, as well as to the huge number of phenolic compounds present in bee products of botanical origin (*e.g.* more than 300 in propolis), this chapter will not be devoted to the deep comprehension of phenolic profiles of specific samples of honey, propolis and bee pollen but instead, it will focus on those more commonly found in these three products, as analysed by chromatographic techniques.

3.1. Non-Flavonoids

Caffeic acid and its derivatives are among the most frequently described non-flavonoids compounds in chromatographic analysis of honey, propolis and pollen samples. The identification of these compounds has been frequently performed by comparison of the retention time in RP-HPLC, together with spectral data gathered by PDA, which typically present wavelength maxima at approximately 324 nm, with a shoulder around 296 nm [83, 84]. These compounds are also commonly identified in ESI-MS^n analysis in the negative mode. The parent ion of

caffeic acid in full MS spectra appears at *m/z* 179, which in turn fragments with a common base peak ion in MS^2 spectra at *m/z* 135, due to the loss of the carboxylic group, and other at *m/z* 161, which results from the loss of a water molecule [85 - 91]. These characteristic ions are also commonly observed in MS^n spectra of caffeic acid derivatives.

Table 4. Common hydroxycinnamic acids in honey, propolis and bee pollen worldwide.

Compound	Honey (BO)	Propolis (BO)	Pollen (BO)
Caffeic acid **(1)**	*Ziziphus spina-christi* [4, 121]; *Castanea sativa* [90, 95, 102, 122, 123]; *Robinia pseudoacacia* [35, 85, 88, 95, 107, 108, 122, 123,]; *Tilia* spp. [32, 35, 65, 88, 89, 95, 122]; *Eucalyptus* spp. [90, 95, 97, 102, 122]; *Lavandula* spp. [95, 122]; *Brassica* spp. [35, 88, 89, 95, 107, 122] ; *Helianthus annus* [88, 89, 95, 122]; *Rosmarinus officinalis* [92, 95, 122]; *Citrus* spp. [94 - 96, 102, 108, 122, 123]; *Hedysarum* spp. [102, 103, 108, 122, 123]; *Echium plantagineum* [122]; *Erica* spp. [46, 65, 90, 95, 122, ; *Calluna* spp. [35, 46, 65, 81, 90, 122]; *Rubus* spp. [97, 124]; *Leptospermum scoparium* [90, 93, 99, 106]; *Fagopyrum esculentum* [35, 65, 81, 85, 86, 88, 97]; honeydew [85, 89, 123]; *Melaleuca* spp. [93, 104, 125]; *Ananas comosus* spp. [104]; *Acacia* spp. [93, 97, 98]; *Satureja hortensis, Ailanthus altissima* [123]; *Thymus* spp. [65, 123]; *Turbina corymbosa, Ipomoea triloba, Avicennia germinans, Govania polygama, Lysiloma latisiquum* [101]; *Mimosa scabrella* [100]; *Trifollium* spp. 89; *Solidaga virgaurea* [88]; *Ocimum basilicum* 88; pine, hawthorn, nettle, black chokeberry, aloe 65; milk vetch, wild chrysanthemum, jujube, acacia [98]; multifloral/heterofloral [65, 92, 123]; unknown floral origin [105]	*Baccharis dracunculifolia* [109, 112 - 114, 126, 127]; *Populus* spp. [5, 10, 37, 87, 110, 111, 115, 116, 118, 119, 128]; Black propolis [126]; multifloral origin [117]	*Camelia sinensis*[120]; Multifloral [40]; *Cystus incanus* [129]

(Table 4) contd.....

Compound	Honey (BO)	Propolis (BO)	Pollen (BO)
3-Caffeoyl quinic acid **(2)**	*Ziziphus spina-christi*; *Calluna vulgaris* [81]; *F. esculentum* [81, 85, 88, 97]; *Melaleuca* spp. [104, 125]; *Ananas comosus* spp. [104]; *R. pseudoacacia* [85, 88, 107, 123]; *Brassica* spp. [88, 89, 107]; *Hedysarum* spp. [102, 123]; *Eucalyptus* spp. [97, 102]; milk vetch, wild chrysanthemum, jujube, acacia [98]; *Rubus idaeus, Acacia catechu* [97]; *C. sativa* [102, 123]; *S. hortensis, A. altissima, T. vulgare* [123]; *H. annus* [88, 89]; *Tilia* spp. [35,88, 89]; *Trifollium* [89]; *Ocimum basilicum, Solidaga virgaurea* [88]; honeydew [85, 89, 123]; heterofloral/multifloral [123]	*B. dracunculifolia* [109,126,130]; *Populus* spp. [110, 115] Black propolis [126]	*Camelia sinensis* [120]
4-Caffeoyl quinic acid **(3)**	-	*B. dracunculifolia* [130]	-
5-Caffeoyl quinic acid **(4)**	-	*B. dracunculifolia* [130]	-
3,5-Dicaffeoyl quinic acid **(5)**	-	*B. dracunculifolia* [130]	-
3,4-Dicaffeoyl quinic acid **(6)**	-	*B. dracunculifolia* [131]	-
4,5-Dicaffeyol quinic acid **(7)**	-	*B. dracunculifolia* [130]	-
Dicaffeoyl quinic acid	-	*B. dracunculifolia,* Black propolis [126]	-
3,4,5-Tricaffeoyl quinic acid **(8)**	-	*B. dracunculifolia* [130]	-
Tricaffeoyl quinic acid	-	*B. dracunculifolia,* Black propolis [126]	-
Chlorogenic acid derivatives	-	Multifloral origin [132]	-
Rosmarinic acid **(14)**	*C. vulgaris* [81]; *F. esculentum* [81, 97]; *R. idaeus, A. catechu, E. globules* [97]; *R. pseudoacacia, B. napus* [107]	-	-

(Table 4) contd.....

Compound	Honey (BO)	Propolis (BO)	Pollen (BO)
Prenyl caffeate **(15)**	*R. pseudoacacia, H. annus, T. cordata, O. Basilicum, F. esculentum, Solidago virgaurea, B. napus* [88]; *Mimosa scabrella* [100]	*Populus* spp. [87, 118, 133]	-
Phenethyl caffeate **(16)**	Chaste [134]; *R. pseudoacacia, H. annus, T. cordata, O. Basilicum, F. esculentum, Solidago virgaurea* [88]; *Brassica* spp. [88, 134]	*Populus* spp. [5, 87, 111, 117, 119, 133]	-
Cinnamyl caffeate **(17)**	-	*Populus* spp. [5, 37, 87, 118] Multifloral origin [132]	-
Benzyl caffeate **(18)**	-	*Populus* spp. [87, 118, 133]	-
Caffeic acid derivatives	*R. pseudoacacia, C. sativa, C. sinensis, E. camaldulensis, Erica* spp., *Lavandula* spp., *Tilia europea, Brassica* spp., *R. officinalis, H. annus* [95]	Multifloral origin [132], *B. dracunculifolia* [112]	-
Caffeic acid esters	*Erica* spp., *Brassica* spp., *Aesculus* spp., *Calluna* spp., *Helianthus* spp., *Rosmarinus* spp., *Abies* spp., *Frangula* spp., *Lavandula* spp., *Citrus* spp., *Rhododendron* spp., *Tilia* spp. [135]	-	-

(Table 4) contd.....

Compound	**Honey (BO)**	**Propolis (BO)**	**Pollen (BO)**
Ferulic Acid **(9)**	*Ziziphus spina-christi* [4, 121]*; R. pseudoacacia* [35, 85, 95, 107, 136]*; F. esculentum* [35, 85, 86, 97]*; C. vulgaris* [35]*; Tilia* spp. [35, 95]; *Brassica* spp. [35, 95, 107, 134; *Melaleuca* spp. [104, 125]; *Ananas comosus* spp. [104]; *Citrus* spp. [94, 95, 102, 136]; *R. idaeus* [97]*; Acacia* spp. [97, 121]; *Eucalyptus* spp. [95, 97, 102, 136]; *Hedysarum* spp. [102, 103]; *C. sativa* [95, 102, 136]*; Erica* spp., *R. officinalis, H. annus* [95]; chaste [134]; *Mimosa scabrella* [100]; *Turbinia corymbosa, Ipomoea triloba, Avicennia germinans, Govania polygama, Lysiloma latisiquum* [101]; *Abies alba, Quercus* spp. [136]; *Lavandula* spp. [95, 136]; *Prosopis julifora, L. scoparium* [121]; *Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]; *Gossypium hirsutum* [138]*; Quillaja saponaria* [139]; milk vetch, wild chrysanthemum, jujube, acacia [98]; multifloral/heterofloral [35, 121]; honeydew [85]	*B. dracunculifolia* [109], [112 - 114]*; Populus* spp. [5, 6, 10, 37, 110, 111, 115, 116, 118, 119, 128]; Multifloral origin [132]	*Camelia sinensis* [120]; multifloral [40, 140]

(Table 4) contd.....

Compound	Honey (BO)	Propolis (BO)	Pollen (BO)
p-Coumaric acid **(10)**	*Ziziphus spina-christi* [4, 121]; milk vetch, wild chrysanthemum, jujube, acacia [98]; *Prosopis julifora,* [121]; *Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]; *Gossypium hirsutum* [138]*; C. sativa* [95, 102, 122, 123]*; R. pseudoacacia* [35, 85, 88, 95, 107, 108, 122, 123, 141]*; Tilia* spp. [32, 88, 89, 95, 122, 142]; *Eucalyptus* spp. [95, 97, 122]; *Lavandula* spp. [95, 122]; *Brassica* spp. [35, 88, 89, 95, 107, 122 , 142]; *H. annus* [88, 898,122]; *R. officinalis* [92, 95, 122]*; Citrus* spp. [31, 92, 94, 95, 102, 108, 122, 123, 143]; *Hedysarium* spp. [102, 108, 122]; *Echium plantagineum* [122]*;* Heather (*Erica* spp./*Calluna* spp.) [35, 65, 81, 95, 122]; *F. esculentum* [35, 65, 81, 85, 86, 88, 97, 141]; *Melaleuca* spp. [93, 104, 125]; *Ananas comosus* spp. [104]; *L. scoparium* [31, 93, 99, 121]*; Acacia* spp. [93, 97, 121]; *Rubus* spp. [97, 124]; *S. hortensis, A. altissima, T. vulgaris* [123] *; Mimosa scabrella* [100]*; Trifollium* spp. [89, 142, 144]; *Turbinia corymbosa, Ipomoea triloba, Avicennia germinans, Govania polygama, Lysiloma latisiquum* [101] *; O. basilicum, S. virgaurea* [88]*; Rhododendron* spp. [33]; *Salix* spp., fruit tree [142]; *Epilobium angustifolium, Nyssa aquatica, Schinus terebinthinfolius, Melilotus* spp., *Glycine max* [141]*;* gallberry, palmetto, tupelo [31]; Blueberry [144]; honeydew [85, 89, 145]; heterofloral/multifloral [31, 35, 65, 92, 112, 121, 123, 143]	*B. dracunculifolia* [109, 113, 114, 130] *Populus* spp. [5, 6, 10, 37, 110, 111, 115, 116, 118, 119]	*Camelia sinensis*[120]; *Schisandra chinensis* [146]; multifloral [40, 140]; *Cistus ladaniferus* [147]

(Table 4) contd.....

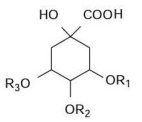
Compound	**Honey (BO)**	**Propolis (BO)**	**Pollen (BO)**
o-Coumaric acid (**11**)	*C. sativa, R. pseudoacacia, Tilia* spp., *Eucalyptus* spp., *Lavandula* spp., *Brassica napus, H. annus, R. officinalis, C. aurantium, C limon, Hedysarum* spp., *E. plantagineum,* Heather (*Erica* spp./*Calluna* spp.) [122]	*Populus* spp. [115, 116]	Multifloral/heterofloral [40, 140]; *Cistus ladaniferus* [147]
m-Coumaric acid **(12)**	*Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]; *C. sativa, R. pseudoacacia, Tilia* spp., *Eucalyptus* spp., *Lavandula* spp., *Brassica napus, H. annus, R. officinalis, C. aurantium, C limon, Hedysarum* spp., *E. plantagineum,* Heather (*Erica* spp./*Calluna* spp.) [122]	-	-
Methyl *p*-coumarate	-	*Populus* spp. [87]	-
Prenyl *p*-coumarate	-	*Populus* spp. [87, 118]	-
Cinnamyl *p*-coumarate	-	*Populus* spp. [118]	-
Benzyl *p*-coumarate	-	*Populus* spp. [118]	-
p-Coumaric acid derivatives	-	Multifloral origin [132]; *B. dracunculifolia* [112]	-

(Table 4) contd.....

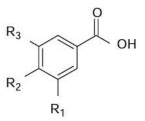
Compound	Honey (BO)	Propolis (BO)	Pollen (BO)
trans-Cinnamic acid **(13)**	*Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]; *Ziziphus spina-christi* [4, 121]; *Gossypium hirsutum* [138]; *Epilobium angustifolium, Nyssa aquatica, Schinus terebinthinfolius, Melilotus* spp., *Glycine max* [141]; Blueberry [144]; *F. esculentum* [88, 87, 141]; *Eucalyptus* spp. [97, 122]; *Tilia* spp., *H. annus, Brassica* spp., *R. pseudoacacia* [88, 122]; *Citrus* spp. [122, 143]; Heather (*Erica* spp./*Calluna* spp.) [122]; *C. sativa, R. officinalis, Lavandula* spp., *Hedysarum* spp., *E. plantagineum* [122]; *O. basilicum, S. virgaurea* [88], *Clidemia* spp., *Serjania* spp., *Myrcia* spp. [148]; Heterofloral/multifloral [121, 143, 148]; unknown floral origin [105]	*B. dracunculifolia* [109, 112 - 114]; *Populus* spp. [10, 87, 115]	Multifloral/heterofloral [40, 140]; *Cistus ladaniferus* [147]
3,4-Dimethoxy cinnamic acid	*R.pseudoacacia, C.sativa, Citrus* spp., *Eucalyptus camaldulensis, Lavandula* spp. [95, 136]; *R. officinalis, H. annus, Brassica* spp.,*Tilia europea, Erica* spp. [95]; *Abies alba, Quercus* spp. [136]	*Populus* spp. [5, 87, 118]	-
p-Methoxy cinnamic acid	*Citrus* spp., multifloral [143], *Gossypium hirsutum* [138]	-	-
m-Methoxy cinnamic acid	*Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]	-	-
3,5-Diprenyl-4-hydroxy cinnamic acid	-	*B. dracunculifolia* [112, 149]	-
3-Prenyl-4-hydroxy cinnamic acid	-	*B. dracunculifolia* [112, 149]	-
Cinnamylideneacetic acid	-	*Populus* spp. [5, 111, 119]	-
Cinnamyl methoxycinnamate	-	*Populus* spp. [118]	*Populus* spp. [118]
Cinnamic acid derivatives	-	*B. dracunculifolia* [150] Multifloral origin [132]	-
Methoxycinnamic acid derivative	-	Multifloral origin [132]	-

(Table 4) contd.....

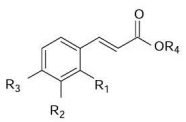
Compound	Honey (BO)	Propolis (BO)	Pollen (BO)
Ellagic acid	*Citrus*spp. [92, 95, 135]; Heather (*Erica* spp./*Calluna* spp. [81, 95, 135]; *F. esculentum* [81] *; R. pseudoacacia* [95, 107]*; Brassica* spp. [89, 95, 107, 135]*; Tilia* spp., *H. annus* [89, 95, 135]*; Eucalyptus camaldulensis, R. officinalis, C. sativa* [95]*; Lavandula* spp. [95, 135]; *Melaleuca* spp. [104, 125]; *Ananas comosus* spp. [104]; *Aesculus* spp, *Abies* spp., *Frangula* spp., *Rhododendron* spp. [135]; *Trifollium* spp., honeydew [89]; multifloral/heterofloral [151]	*Populus* spp. [87]	-



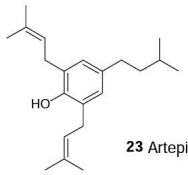
2 R_1=Caf; R_2=R_3=H
3 R_1=R_3=H; R_2=Caf
4 R_1=R_2=H; R_3=Caf
5 R_1=R_2=Caf; R_3=H
6 R_1=H; R_2=R_3=Caf
7 R_1=R_3=Caf; R_2=H
8 R_1=R_2=R_3=Caf



19 R_1=R_2=R_3=H
20 R_1=R_2=R_3=OH
21 R_1=R_2=OH; R_3=H
22 R_1=R_3=OCH_3; R_2=OH



1 R_1=R_4=H; R_2=R_3=OH
9 R_1=R_4=H; R_2=OH; R_3=OCH_3
10 R_1=OH; R_2=R_3=R_4=H
11 R_1=R_3=R_4=H; R_2=OH
12 R_1=R_2=R_4=H; R_3=OH
13 R_1=R_2=R_3=R_4=H
14 R_1=H; R_2=R_3=OH; R_4= 3,4-dihydroxyphenil lactic acid
15 R_1=H; R_2=R_3=OH; R_4= 3-methyl-2butenyl
16 R_1=H; R_2=R_3=OH; R_4= phenetyl alcohol
17 R_1=H; R_2=R_3=OH; R_4= cinnamyl
18 R_1=H; R_2=R_3=OH; R_4= Benzyl



23 Artepilin C

Fig. (2). Structure of the main non-flavonoid components found in honey, propolis and/or pollen. Caf – Caffeic acid.

Caffeic acid (**1** in Fig. **2**) has been described as a phenolic constituent of honey, propolis and bee pollen from many countries (see Table **4**), with concentration levels ranging from 0.001 to 33 μg/g of honey, 0.02 to 32.20 mg/g of propolis extract and 0.446 to 410 μg/g of bee pollen [4, 5, 10, 32, 37, 46, 72, 81, 85 - 90, 92 - 119]. These values show that the composition is highly variable and dependent on the geographic and floral origin of samples. Among the literature, it is clear that the most enriched bee products in caffeic acid are the Brazilian honeys from *Mimosa scrabella* (33 μg/g honey), Chinese *Populus* spp. propolis (32.20 mg/g extract) and Taiwanese *Camellia sinensis* bee pollen (410 μg/g pollen) [5, 100, 120].

Ester derivatives of caffeic acid are quite abundant in honey, propolis and bee pollen. Among these derivatives, 3-*O*-caffeyoilquinic acid (**2** in Fig. **2**), commonly known as chlorogenic acid, is clearly the most abundant in these three bee products, being detected in samples of diverse geographical regions including Brazil, China, Germany, Italy, Lithuania, Malaysia, Poland, Taiwan and Yemen (Table **4**).

In addition to this, several other ester derivatives can be found in honeybee products of botanical origin. Propolis is undoubtedly the most enriched in these compounds typically containing distinct mono-caffeoylquinic acids like 3-*O*-caffeoylquinic acid (3-CQA), 4-*O*-caffeoylquinic acid (4-CQA), 5-*O*-caffeoylquinic acid (5-CQA), together with di-caffeoylquinic acids 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) and 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA) and also 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA) (**2–8** in Fig. **2** and Table **4**). All these compounds show an identical UV_{max} which is close to that of caffeic acid, but caffeic acid and mono-, di- and tri-CQA acids can be easily distinguishable through full MS detection [126]. Moreover all these isomers can be accurately identified in RP-HPLC coupled to tandem MS^n analysis [130, 152]. Alternatively, identification of individual CQA derivative compounds can be achieved through GC-FID or GC-MS analysis, though a derivatization step is needed first. However this technique have fallen out of favor since the RP-HPLC is more appropriate for the analysis of complex samples [153].

The content of chlorogenic acid in honey has been described to reach 79 µg/g (in Lithuanian multifloral samples), while substantially higher concentrations have been described on *Camelia sinensis* bee pollen samples from Taiwan [81, 97, 120]. However, as mentioned, it is in propolis samples where higher concentrations of chlorogenic acid and of its isomers and/or derivatives have been detected. The values vary from 0.7 to 7.2 mg/g of crude propolis for monoCQAs (3-, 4- and 5-), 5 to 31 mg/g for diCQAs (3,4-, 3,5- and 4,5-) and 1 to 4 mg/g for 3,4,5-triCQA, with 4,5-diCQA clearly predominating [115, 130, 131].

Besides the mono-, di- and triCQAs, other ester derivatives have been frequently detected in honey, propolis and bee pollen samples. These comprise rosmarinic acid, prenyl caffeate, phenethyl caffeate, cinnamyl caffeate and benzyl caffeate (**14-18** in Fig. **2**, Table **4**), which also show an UV_{max} close to that of caffeic acid [118, 154] but are easily identified when HPLC is coupled to MS^n analysis. Honey samples from different botanical and geographical origins are particularly enriched in rosmarinic acid, prenyl caffeate and phenethyl caffeate, with the former being the most predominant, where it varies from 0.012 to 15.85 µg/g [81, 97, 107].

In opposition, caffeates including caffeic acid phenethyl, cinnamyl and/or benzyl esters, prevail in propolis samples, and particularly in CAPE-based propolis. In this regard, CAPE has been found particularly abundant in samples collected from Spain, Argentina and China in concentrations of 19.2, 11.9 and 8.7 mg/g of ethanolic extracts, respectively. Caffeic acid cinnamyl ester is another compound that can also be found abundantly in propolis, being recorded to reach 25.1 mg/g of ethanolic extract in Chinese propolis [5, 111, 119, 155]. On the other hand, prenyl caffeate has been described to be abundant in polar extracts of propolis from Italy (0.01-4.1 mg/g extract) [118] and Macedonia (0.95 and 1.6 mg/g extract, respectively) [133] while the Italian (0.02-1.1 mg/g extract) [118] and Chinese samples are a good source of benzyl caffeate (4.1 and 1.5 mg/g extract, respectively) [133].

Besides caffeic acid and its derivatives, honey products of botanical origin contain considerable amounts of other hydroxycinnamic acids, including ferulic, coumaric and cinnamic acid (**9-13** in Fig. **2**), along with their derivatives (Table **4**). Ferulic

acid (typically characterized by UV_{max} at 215, 287 and 312sh nm and $[M - H]^-$ at *m/z* 193, with products ions at *m/z* 149, 178 and 134) is commonly detected in honey and propolis from different geographical and botanical origins, while occurring at a lesser extent in bee pollen. Naturally, the content of this compound in each of the three products is very variable, with reported amounts ranging from 0.004 to 174 µg/100 g honey [4, 85, 97, 121, 156], 0.005 to 12.5 mg/g of propolis extract [111, 112, 115, 132] and 0.37 to 450 µg/g of bee pollen [40, 120, 140].

p-Coumaric acid (**10** in Fig. **2**), which is characterized by having an absorption maxima peak around 310 nm and a $[M - H]^-$ at *m/z* 193 with a corresponding base product ion at *m/z* 119 in ESI-MS analysis in negative mode, has been described in honey samples in concentrations that range from 0.004 to 77.9 µg/g [84, 86, 125, 143, 148, 157] (highest values belonging to the multifloral and *Fagopyrum esculentum* honeys from Brazil) [35, 81, 143]. Typically, *p*-coumaric acid is also found in propolis. Samples from Chinese, Brazilian and Italian beehives, have been described as important sources of this phenolic acid (52.2, 16.0 and 13.5 mg/g extract, respectively) [5, 10, 112]. Instead, its isomer *o*-coumaric acid (**11** in Fig. **2**) has been described to occur in lower concentrations in several other propolis samples, namely in those of Turkish origin in which concentration may vary between 2.1 to 23.3 µg/g raw propolis [115]. Moreover, contrasting to the two other honeybee-derived products, propolis is a source of coumaric acid derivatives, mainly in an ester form such as methyl *p*-coumarate, prenyl *p*-coumarate and cinnamyl *p*-coumarate (Table **4**) [10, 85 - 87]. Quantification of prenyl and cinnamyl *p*-coumarates demonstrates that despite the variations observed between samples from different origins (ranging from 0.02 to 0.65 mg/g extract and 0.01 to 1.12 mg/g extract, respectively) *p*-coumaric acid cinnamyl ester may be found in more abundance than its prenyl counterpart [87, 118].

Cinnamic acid (**13** in Fig. **2**, UV_{max} at approximately 277 nm, typical $[M - H]^-$ at *m/z* 147 with a fragment at *m/z* 103) has been detected in honeys, propolis and bee pollens from several geographic areas and origins (Table **4**), but its major abundance is actually in propolis samples, with concentrations reaching up to 0.1 mg/g raw propolis [87, 115]. Propolis is also the bee product with further diversity in cinnamic acid derivatives, like 3,4-dimethoxycinnamic acid, *p*- and *m*-methoxycinnamic acid, artepillin C, 3-prenyl-4-hydroxycinnamic acid,

cinnamylideneacetic acid and cinnamyl methoxycinnamate. Indeed, depending on the geographical and botanical origin, different amounts of these compounds can be found in propolis samples worldwide. As referred before, artepillin C (**23** in Fig. **2**) is a very important compound characteristic of the Brazilian green propolis. In fact, the quantification of this compound has become an important indicative of the Brazilian green propolis quality, and therefore it is generally used as a chemical marker for the quality control of this product [158]. Other cinnamic acids have also been reported as relevant constituents of propolis. *E.g.* 3,4-dimethoxycinnamic acid in propolis from China [5], has been described to amount up to concentrations of 5.8 and 57.4 mg/g. Likewise, concentrations of cinnamylideneacetic acid in the range of 0.8 to 45.4 mg/g extract were detected in propolis of Chinese origin [5, 111, 119].

In addition to hydroxycinnamic acids, honeybee-derived products are also enriched in phenolic acids such as benzoic vanillic , gallic, protocatechuic , syringic acid (**19–22** in Fig. (**2**), respectively), as well as several derivatives of these compounds (Table **5**). Similarly to what was previously mentioned for hydroxycinnamic acids, the identification of these compounds has been mainly carried out by HPLC coupled to DAD and/or MS^n analysis.

Table 5. Common phenolic acids in honey, propolis and bee pollen worldwide.

Compound	Honey (BO)	Pollen (BO)	Propolis (BO)
Benzoic acid (**19**)	*Ziziphus spina-christi* [4]; *C. sativa, Citrus* spp., *Eucalyptus* spp [102, 122, 136]; *R. pseudoacacia* [122, 136]; *Tilia* spp., *B. napus, H. annus, R. officinalis, E. plantagineum* [122]; Heather (*Erica* spp./*Calluna* spp.) [65, 122]; *Lavandula* spp. [122, 136]; *Hedysarum* spp. [102, 103, 122]; *L. scoparium,* Tualang tree, *Melaleuca* spp., *Acacia mangium* [93]; *F. esculentum* [65, 86]; *Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]; *Mimosa scabrella* [100]; *Gossypium hirsutum* [138]; *Centaurea cyanus* [161]; Pine, hawthorn, nettle, thyme, black chokeberry, aloe [65]; Multifloral/Heterofloral [65, 112]	Multifloral/Heterofloral [40]	*B. dracunculifolia* [112]; *Populus* spp. [87, 115]; Multifloral origin [132]

(Table 5) contd.....

Compound	Honey (BO)	Pollen (BO)	Propolis (BO)
Vanillic acid	*Ziziphus spina-christi* [4, 121]; *C. sativa* [122]; *R. pseudoacacia* [35, 85, 108, 122, 141] ; *Tilia* spp. [35, 46, 89, 122]; *Hedysarium* spp. [108, 122]; *F. esculentum* [35, 81, 85, 141]; *Citrus* spp. [108,122,143]; Heather (*Erica* spp./*Calluna* spp.) [35, 46, 81, 122]; *Eucalyptus* spp. [122]; *Brassica* spp. [35, 89, 122]; *L. scoparium* [93, 121]; *H. annus* [89, 122]; *Hedysarum* spp. [108, 122]; *Acacia* spp. [93, 121]; *Lavandula* spp., *R. officinalis, E. plantagineum* [122]; Tualang tree, *Melaleuca* spp. [93]; *Turbina corymbosa, Ipomoea triloba, Avicennia germinans, Govania polygama, Lysiloma latisiquum* [101]; *Trifolium* spp. [89, 144]; *Gossypium hirsutum* [138]; *Pinus* spp., *Thymus* spp., *Abies cephallonica* [162]; *E. angustifolium, N. aquatic, S. terebinthifolius, Melilotus* spp., *G. max* [141] ; *Clidemia* spp., *Serjania* spp., *Myrcia* spp. [148]; *Prosopis julifora* [121]; Honeydew [85, 89]	*Schisandra chinensis* [146]; Multifloral [40]; *Cistus ladaniferus* [147]	*Populus* spp. [115]; Multifloral origin [132]
Gallic acid	*Clidemia* spp., *Serjania* spp., *Myrcia* spp. [148]; *Pinus* spp., *Thymus* spp., *Abies cephallonica* [162]; *E. angustifolium, N. aquatic, S. terebinthifolius, Melilotus* spp., *G. max* [141] ; *Ziziphus spina-christi* [4, 121]; *C. sativa* [90,102,122]; *Kunzea ericoides, Knightia excelsa* [163]; *Quillaja saponaria* [139]; milk vetch, wild chrysanthemum, jujube, acacia [98]; *R. pseudoacacia* [85, 88, 107, 122, 141]; *F. esculentum* [85, 88, 97, 141]; *Tilia* spp. [32, 46, 88, 89, 122]; *Eucalyptus* spp. [90, 97, 102, 122]; *Trifollium* spp. [89, 163]; *Hedysarum* spp. [102, 103, 122]; *Citrus* spp. [102, 122, 143]; *Brassica* spp. [88, 89, 107, 122]; *H. annus* [88, 89, 122]; Heather (*Erica* spp./*Calluna* spp.) [46, 90, 122]; *E. plantagineum, R. officinalis* [122]; *L. scoparium* [90, 121, 163]; *Melaleuca* spp. [104, 125]; *Ananas comosus* spp. [104]; *Acacia* spp. [97, 121]; *R. idaeus* [97]; *O. basilicum, S. virgaurea* [88]; Honeydew [85, 89]; Multifloral/Heterofloral [121, 143, 148]	*Camelia sinensis* [120]; *Schisandra chinensis* [146]	*Populus* spp. [110, 115]

(Table 5) contd.....

Compound	Honey (BO)	Pollen (BO)	Propolis (BO)
Protocatechuic acid	*Citrus* spp. [122, 143]; *F. esculentum* [81, 86, 88]; *R. pseudoacacia* [88, 122]; *Tilia* spp., *Brassica* spp., *H. annus* [88, 89, 122]; Heather (*Erica* spp./*Calluna* spp.) [81, 122]; *Eucalyptus* spp., *Lavandula* spp., *R. officinalis*, *Hedysarum* spp., *E. plantagineum* [122]; *Trifolium* spp., honeydew [89]; *O. basilicum, S. virgaurea* [88]; multifloral/heterofloral [143]; unknown floral origin [105]	*Schisandra chinensis* [146]; Multifloral [40]; *Cistus ladaniferus* [147]	*Populus* spp. [115]
p-Salicylic acid	*Ziziphus spina-christi* [4]; *Pinus* spp., *Thymus* spp., *Abies cephallonica* [162]; *Gossypium hirsutum* [138]; milk vetch, wild chrysanthemum, jujube, acacia [98]; *C. sativa* [90, 122]; *R. pseudoacacia* [85, 107, 122, 141]; *Tilia* spp. [46, 89, 122]; *Eucalyptus* spp. [90, 122]; *F. esculentum* [65, 81, 85, 86, 122, 141]; *Brassica* spp. [89, 122]; *Citrus* spp. [89, 122, 143]; Heather (*Erica* spp./*Calluna* spp.) [46,65,81,90,122]; *H. annus* [89, 122]; *Lavandula* spp., *R. officinalis, Hedysarum* spp., *E. plantagineum* [122]; *L. scoparium* [90]; Pine, hawthorn, nettle, thyme, black chokeberry, aloe [65]; honeydew [85, 89]; *Trifolium* spp. [89, 144]; multifloral/heterofloral [65, 122, 143]; *E. angustifolium, N. aquatica, S. terebinthifolius, Melilotus* spp., *G. max* [141]	Multifloral/Heterofloral [40]	*Populus* spp. [115]
Syringic acid	*E. angustifolium, N. aquatic, S. terebinthifolius, Melilotus* spp., *G. max* [141]; *Clidemia* spp., *Serjania* spp., *Myrcia* spp. [148]; *Pinus* spp., *Abies cephallonica* [164]; *Thymus capitatus* [164, 165]; *Kunzea ericoides, Knightia excelsa* [163]; milk vetch, wild chrysanthemum, jujube, acacia [98]; *Ziziphus spina-christi* [4, 121]; *L. scoparium* [93, 121, 163]; *F. esculentum* [81, 85, 86, 141]; *Citrus* spp. ; *Turbina corymbosa, Ipomoea triloba, Avicennia germinans, Govania polygama, Lysiloma latisiquum Trifolium* spp. [89, 163]; *Tilia* spp. [46, 89, 122]; *R. pseudoacacia* [85, 107, 122, 141]; Heather (*Erica* spp./*Calluna* spp.) [46, 81, 122]; *Brassica* spp. [89, 107, 122]; *Eucalyptus* spp., *Lavandula* spp., *R. officinalis, Hedysarum* spp., *E. plantagineum* [122]; Tualang tree, *Melaleuca* spp.[93]; honeydew [85, 89]; *Acacia* spp. [93, 121]; multifloral/heterofloral [121, 143, 148]; *Prosopis julifora* [121]	Multifloral [40]; *Cistus ladaniferus* [147]	*Populus* spp. [115]

(Table 5) contd.....

Compound	Honey (BO)	Pollen (BO)	Propolis (BO)
o-Anisic acid	*L. scoparium* [90, 163]; *Kunzea ericoides, Trifollium* spp., *Knightia excelsa* [163]	-	-
o-Salicylic acid	*Gossypium hirsutum* [138]; *C. sativa, R. pseudoacacia, Eucalyptus* spp., *Citrus* spp., *Lavandula* spp. [122, 136]; *Abies alba, Quercus* spp. [136]; *Tilia* spp., *Brassica napus, H. annus, R. officinalis, Hedysarum* spp., *E. plantagineum,* Heather (*Erica* spp./*Calluna* spp.) [122]	-	-
m-Salicylic acid	*C. sativa, R. pseudoacacia, Tilia* spp., *Eucalyptus* spp., *Lavandula* spp., *B. napus, H. annus, R. officinalis, Citrus aurantium, Citrus limon, Hedysarum* spp., *E. plantagenium,* Heather (*Erica* spp./*Calluna* spp.) [122]	-	-
Methyl anisate	-	Heterofloral [38]	-
p-Anisic acid	*L. scoparium, Kunzea ericoids, Knigthia excelsa, Trifollium* spp. [163], *Citrus* spp., Multifloral/heterofloral [143]; *Gochnatia* spp., *Croton* spp., *Vernonia* spp. [137]	-	
Benzoic acid derivatives	-	-	Multifloral origin [132]
Methyl benzoate	-	Heterofloral [38]	-
Methyl gallate	-	*Camelia sinensis* [120]	-
Methyl syringate	*L. scoparium* [90]; Heather (*Erica* spp./*Calluna* spp.) [80, 90]; *C. sativa* [90, 136]; *Eucalyptus* spp. [90, 136]; *Mimosa scabrella* [100]; *F. esculentum* [80, 136]; *R. pseudoacacia* [80, 136]; *A. alba, C. sinensis, Lavandula* spp., *Quercus* spp. [136]; *Tilia* spp., *Solidago* spp., *B. napus* [80]; *C. cyanus* [161]	-	-

BO – Botanical origin

From those, benzoic acid (typical UV_{max} at 229 and 274 nm [86, 87, 90] and deprotonated molecular ion at *m/z* 121 in ESI-MS analysis in negative mode and a typical fragment peak base at *m/z* 77, caused by the loss of the carboxylic acid moiety [159]) is perhaps the mostly widely distributed amongst bee products. This has been described in honeys from different geographical and botanical origins with variable abundance, being particular prevalent in honeys obtained from Brazilian *Gochnatia* spp. and in Yemeni *Ziziphus spina-christi* (141 and 56 µg/g, respectively) [4, 112, 137], as well as in propolis (amounts varying from 5.3 µg to

48.6 mg/g extract) [112, 115, 132] and in pollen (very scarce quantification).

Vanillic acid is also very commonly found in floral honeybee derived products. Typically, the UV analysis of this acid reveals wavelength maxima peaks at 260 and 294 nm [101] and its full MS spectrum in the negative mode is characterized by a deprotonated molecular ion at *m/z* 167 [85, 89] and a typical ion fragment at *m/z* 152, originated by the loss of a CH_3 group [85, 160], along with ions at *m/z* 122 and 108 [160]. As can be observed in Table **5**, this phenolic acid has been described in honeys, propolis and pollen collected worldwide and according to literature, its amounts can be up to 211 µg/g in honey (as described for honey from Brazilian *Citrus* spp. [81, 143, 148]) or to 334 µg/g extract in propolis, as described for Finnish Coniferous Forest propolis samples. Quantification in pollen is scarce, although values of 0.23 to 15.0 µg/g have been reported for Chinese *Schisandra chinensis* and Turkish pollen [115, 146].

Besides benzoic and vanillic acids, gallic acid (MW 170 g/mol and UV_{max} at approximately 272 nm), protocatechuic acid (MW 154 g/mol and UV_{max} at nearly 260 and 290 nm), *p*-salicylic (MW 138 g/mol and UV_{max} around 254 nm), and syringic acid (MW 198 g/mol and UV_{max} close to 276), among others, are often detected in bee-floral products (Table **5**). Considerable amounts of these compounds have been reported in specific samples, *e.g.* the levels of gallic acid in honeys of Italian *Hedysarium* spp. and in a multifloral honey from Brazil accounted for 89.5 and 77.3 µg/g, respectively [103, 143], while the highest levels of protocatechuic acid in honeys were described to occur in those from Brazilian *Citrus* spp. and from Polish *Fagopyrum esculentum* (66.7 and 37.0 µg/g, respectively) [81, 143]. Notably, *p*-salicylic acid levels in Brazilian *Baccharis dracunculifolia* have been reported to account for up to 267 µg/g. Despite lower, the concentrations of this phenolic in multifloral Brazilian honey and Polish *Fagopyrum esculentum* honey were yet significant (98.1 and 62.1 µg/100 g, respectively) [81, 112, 143]; On the other hand, syringic acid was generally found in lower concentrations in honeys, although heterofloral *Clidemia* spp./*Mimosa pudica* and monofloral *Citrus* spp. were described as a potential source of this acid (15.7 and 13.8, 6.4 µg/100 g, respectively) [4, 143, 148].

Amongst propolis worldwide samples, gallic, syringic and *p*-salicylic acids seem to appear in considerable amounts in Turkish temperate propolis (8.7-70.1, 4.4-11.9 and 41.5-139 μg/g raw propolis, respectively). In turn the concentrations of these acids in pollen samples are in general much lower than those found in honey and propolis. From the reported literature, one can ascribe Brazilian, Turkish and Chinese bee pollen as sources of gallic acid (0.08 to 5.6 μg/g) and protocatechuic acid (0.05 and 4.6 μg/g) [40, 146].

3.2. Flavonoids

Flavonoids, whose structures are based on a C_6-C_3-C_6 skeleton (Fig. **1**) are sub-divided into different subclasses differing in the oxidation state of the central heterocyclic ring, including flavonols, flavones, flavanones, dihydroflavonols, anthocyanidins and flavanols [166]. Depending on the matrix, these compounds may be prevalent in the form of aglycones or as glycosides, both varying according to their pattern of hydroxylation and/or methoxylation. Glycosylation can occur as *O*-glycosylation of their hydroxyl groups as well as *C*-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. Such derivatives are thermo-labile and their isolation and further purification without partial degradation is difficult [167].

The structural elucidation of the different subclasses of flavonoids is commonly achieved by comparison of their chromatographic behavior, UV spectra and MS information, with those of reference compounds. In fact, the chromatographic behavior and UV spectroscopy provide particularly wealthy information when applied to flavonoids. Interestingly, a reasonable idea of the structure from many of the most common flavonoid glycosides can be obtained only looking at these two parameters [168].

Notably, one of the most important features of flavonoids is the existence of two main wavelength maxima. In a general perspective, the longer wavelength absortion corresponds to the Band I absortion that ranges between 300 – 380 nm and is considered to be associated to the B-ring cinnamoyl function, while the shorter corresponds to Band II absortion which ranges between 240 – 280 nm and

is correlated with the absorption involving the A-ring benzoyl function (Fig. **3**) [169, 170]. However, the exact wavelength and magnitudes of the absorption maximas depend on several factors including the nature of the C-ring, the point of attachment of the B-ring, and the nature of the substituents at A- and B-rings [168].

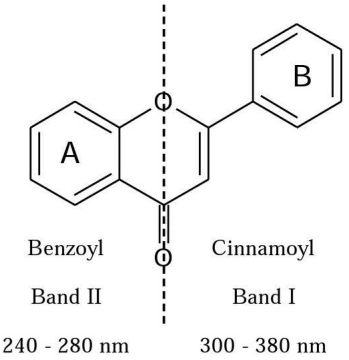


Fig. (3). Representation of the two distint chromophore functions of flavonoids (benzoyl function that comprises the A-ring and C-4 carbonyl group, and cinammoyl function comprising the B-ring and the three carbons of the C-ring) and respective association with the Band I and Band II absortion maxima regions.

Just as important as UV–vis detectors, mass spectrometers are crucial when it comes to the identification of flavonoids. Notably, the fragmentation profile of compounds from distinct subclasses is clearly influenced by their substitution pattern, although several common features can be found. The MS^2 spectrum of many flavonoids reveal the fragments at *m/z* 151 or at *m/z* 165, which are resultant from the retro Diels-Alder mechanism [171]. Besides, neutral losses commonly described to occur in these compounds, such as the small molecules CO (-28 Da), CO_2 (-44 Da), C_2H_2O (-42 Da), as well as the successive losses of these molecules, are also observed [171].

The flavonoid content in honeybee-products is very variable, however it has been reported that their concentration can reach up to 6 $mg.kg^{-1}$ in honey, and about 10% and 0.5% of propolis and bee pollen extracts respectively [172].

Among them, flavonols and flavones are particularly important constituents from

bee plant-derived products, with special relevance in honey and bee pollen [173]. In general, flavonols exhibit a Band I and II between 350 – 385 and 250 – 280 nm, respectively, while the absence of the 3-hydroxyl group in flavones causes an hypsochromic shift of the Band I to 310 – 350 nm. Also, the *O*-substitution 3-hydroxyl group in flavonols (*O*-alkyl or *O*-glycosyl), modify the general shape of the Band I absortion wavelength that tends to enlarge to 330 – 360 nm (bathochromic shift), approaching to those of flavones [168].

From flavonols, quercetin (**23** in Fig. (**4**)), kaempferol (**24** in Fig. (**4**)), isorhamnetin (**25** in Fig. (**4**)) and myricetin (**26** in Fig. (**4**)) are the most commonly found, along with several of their methyl and/or glycosidic derivatives (Table **6**).

All these compounds exhibit an UV maxima at 370 nm with exception of kaempferol which reveals a hypsochromic shift of its UV maxima to 366 nm [174]. Despite their similarity in the UV spectra, these compounds can be easily distinguishable through MS spectrometry, because of their distinct MS ($[M-H]^-$ at *m/z* 301, 285, 315 and 317, repectively) and characteristic MS^n data.

Table 6. Relevant flavonols in honey, propolis and bee pollen worldwide.

Flavonol	Honey (BO)	Propolis(BO)	Pollen (BO)
Quer **(23)**	*Eucalyptus* spp. [95,102,176,177]; *R. pseudoacacia, H. annus, Lavandula* spp., *Erica* spp., *Brassica* spp. [95]; *F. esculentum* [35, 86]; *C. sativa* [95, 102, 177]; *Citrus* spp. [31,95,102,164,177]; *Tilia* spp. [32, 95]; *Q. saponaria* [139]; *Mimosa caesaepiniifolia* [178]; *R. officinalis* [95, 177]; *Thymus* spp. [164,165,177]; palmetto berry, *L.scoparium*, tupelo [31]; *Pinus* spp., *Abies* spp. [164]; Heather [35, 177]; *Ziziphus spina-christi* [4]; *Hedysarum* spp. [102]; multifloral [31,102,177]	*Populus* spp. [37,72,87,115,116,133, 177]; *B. dracunculifolia* [73, 126]; *Dalbergia* spp. [179]; *Clusia* spp. [126]; Commercial [16, 118]	Anzer* [40]; *Cystus incanus* [129]; *Cistus ladaniferus* [147]; *Eupatorium* spp., *Ricinus* spp., *Mimosa arenosa, Eucalyptus* spp., *Cecropia* spp., *Mimosa pudica, Elaeis* spp. [180]; *Typha angustifolia* [175]
3-*O*-CH_3Quer	*Eucalyptus* spp. [176], La Alcarria* [181]	*Populus* spp. [182]; Commercial [118]	*Eucalyptus globulus* [183]

(Table 6) contd.....

Flavonol	Honey (BO)	Propolis(BO)	Pollen (BO)
7-*O*-CH_3Quer	-	Commercial [118]	-
3,7-diCH_3Quer	*Citrus* spp., *H. annus*, *Erica* spp. [95]	-	-
3,3'-diCH_3Quer	*H. annus*, *Erica* spp. [95]; *R. officinalis* [2]	-	-
7-3'-diCH_3Quer	*R. officinalis* [2]	-	-
diCH_3Quer	-	*Populus* spp. [87]; Commercial [118]; *B. dracunculifolia*, *Clusia* spp. [126]	-
tetraCH_3Quer	-	*Populus* spp. [87]	-
Quer-3-*O*-Glc	Multifloral (6)	*Populus* spp. [87]; *B. dracunculifolia*, *Clusia* spp. [126]	*Prosopis juliflora* [184]; *Eupatorium* spp., *Ricinus* spp., *Mimosa arenosa, Cecropia* spp., *Eucalyptus* spp., *Mimosa pudica, Elaeis* spp. [180]
Quer-3-*O*-Rha	Multifloral [185]	*Populus* spp. [87]; *B. dracunculifolia*, *Clusia* spp. [126]	*E. australia* [183]; Multifloral [11]; Multifloral [41]
Quer-3-*O*-Gal			Multifloral [11]
Quer-3-*O*-Rut	Orange blossom [186], *Q. saponaria* [139]; *C. sativa, Eucalyptus* spp., *Citrus* spp., *Hedysarum* spp. [102]; *R. pseudoacacia, F. esculentum,* Heather, *Brassica* spp., *Tilia* spp. [35]; palmetto berry, *L. scoparium* [31]; *Brassica napus* [186]	*Populus* spp. [87, 115]; Commercial [16]	*Ranunculus petiolaris* [187]; Anzer* [40]; *Cistus ladaniferus* [147]; Multifloral [41]
Quer-3-*O*-Hex(1→2)Hex	*Citrus* spp., *Brassica* spp. [186]	-	-
Quer-3-*O*-Rut-7-*O*-Rha	*Brassica napus* [186]	-	-
Quer-3-*O*-Gluc	-	*Populus* spp. [87]	Multifloral [11]
Quer-3-*O*-Soph	-	-	*E. globulus* [183]; *R. petiolaris* [187]
Quer-3-*O*-Ara	-	*B. dracunculifolia*, *Clusia* spp. [126]	Multifloral [41]
diCH_3Quer-*O*-Rut	-	*Populus* spp. [87]	-
diCH_3Quer-*O*-Gluc	-	*Populus* spp. [87]	-

(Table 6) contd.....

Flavonol	Honey (BO)	Propolis(BO)	Pollen (BO)
Quer-3-O-diGly	-	-	*R. sardous* [183]
Kaemp **(24)**	*Eucalyptus* spp. [176]; *Tilia* spp. [35, 95]; *Citrus* spp., *Brassica* spp., *R. officinalis*, *Erica* spp. [95], *R. pseudoacacia* [35, 95]; *F. esculentum* [86]; *Q. saponaria* [139]; Heather [35]; *Pinus* spp., *Thymus* spp., *Abies* spp., *Citrus* spp. [164]; *Ziziphus spina-christi* [4]; palmetto berry, *L. scoparium* [31]	*Populus* spp. [6, 37, 72, 74, 87, 116, 119, 177, 188]; *B. dracunculifolia* [73,74,109]; Commercial [118, 189]	*Cystus incanus* [129]; *Cistus ladaniferus* [147]; *Eucalyptus* spp., *Mimosa pudica, Elaeis* spp., *Cecropia* spp., *Eupatorium* spp., [180]; *Typha angustifolia* [175]
CH_3Kaemp	*Tilia* spp., *Brassica* spp., *R. officinalis*, *Erica* spp., *Citrus* spp., *Lavandula* spp., *R. pseudoacacia* [95], La Alcarria* [181]	*Populus* spp. [87]	-
OCH_3-CH_3Kaemp	-	*Populus* spp. [87]	-
diCH_3Kaemp	-	*Populus* spp. [87]; Finnish* [132]	-
Kaemp-*O*-*p*-CouRha	-	*Populus* spp. [87]	-
Kaemp-3,4'-di-*O*-Hex	*Tilia* spp., *Brassica* spp. [186]	-	-
CH_3Kaemp-*O*-Glc	-	*Populus* spp. [87]	-
CH_3Kaemp-*O*-Rut		*B. dracunculifolia*, *Clusia* spp. [126]	
8-OCH_3kaemp-3-*O*-Hex(1→2)Hex	*Brassica napus, Prunus avium, Eucalyptus* spp., *Mendicago sativa* [186]	-	-
8- OCH_3kaemp -3-*O*-Neohesp	*Brassica napus, Brassica* spp. [186]	-	-
Kaemp-3-*O*-Hex(1→2)Hex	*Tilia* spp., *Citrus* spp., *Brassica* spp., *R. officinalis* [186]	-	-
Kaemp-3-*O*-Neohesp	*Prunus avium, Eucalyptus* spp., *Rhododendron* spp., *R. officinalis, Taraxacum* spp. [186]	-	*Salix atrocinera* [183]
Kaemp-3-*O*-Rut	*Brassica napus* [186]	*Populus* spp. [87]	
Kaemp-3-*O*-Glc	-	-	Multifloral [41]
Kaemp-3-*O*-Rha	-	-	Multifloral [11]
Kaemp-3-*O*-Rha-Glc	-	-	Multifloral [41]

(Table 6) contd.....

Flavonol	**Honey (BO)**	**Propolis(BO)**	**Pollen (BO)**
Kaemp-3-*O*-Hex(1→2)Hex-7-*O*-Rha	*Brassica napus, Prunus avium, Brassica* spp., *Taraxacum* spp. [186]	-	-
Kaemp-3-*O*-Rut-7-*O*-Rha	*Brassica napus, Brassica* spp. [186]	-	-
Kaemp-3-*O*-(Hex)Rob-7-*O*-Rha	*R. pseudoacacia* [190]	-	-
Kaemp-3-*O*-(Hex)Rob	*R. pseudoacacia* [190]	-	-
Kaemp-3-*O*-Hex-7-*O*-Rha	*R. pseudoacacia* [190]	-	-
Kaemp-3-*O*-Rob-7-*O*-Rha	*R. pseudoacacia* [190]	-	-
Kaemp-7-*O*-Rob	*R. pseudoacacia* [190]	-	-
Kaemp -7-*O*-Rha	*R. pseudoacacia* [190]	-	-
Kaemp-3-*O*-Soph	-	-	*Raphanus raphanistrum* [183]
Isorhm **(25)**	La Alcarria* [181], *Mimosa caesaepiniifolia* [178], *F. esculentum* [86]	*Populus* spp. [87]; *B. dracunculifolia* [73, 126], *Clusia* spp. [126]; Commercial [118, 189]	*Cystus incanus* [129]; *Cistus ladaniferus* [147]; *Eupatorium* spp., *Eucalyptus* spp., *Cecropia* spp., *Mimosa pudica, Elaeis* spp., *Elephantopus* spp. [180]
Isorhm-*O*-Pen	-	*Populus* spp. [87]	-
Isorhm-Glc		*B. dracunculifolia*, *Clusia* spp. [126]	
Isorhm-3-*O*-(He--Hex)-7-*O*-Hex	*Taraxacum* ssp. [186]	-	-
Isorhm-3-*O*-(2'', 3''-diRha)Glc	-	-	Multifloral [41]
Isorhm-3-*O*-Rha-Glc	-	-	Multifloral [41]
Isorhm-3-*O*-Hex(1→2)Hex	*Tilia* spp., *Brassica* spp., *Rhododendron* spp. [186]	-	-
Isorhm-3-*O*-Neohesp	*Prunus avium, Brassica* spp., *Taraxacum* spp., *Tilia* spp. [186]	-	-
Isorhm-3-*O*-Xyl(1→6)Glc	*Brassica napus, Brassica* spp., *H. annus* [186]	-	Multifloral [11]
Isorhm-3-*O*-Rut-7-*O*-Hex	*Mendicago sativa* [186]		

(Table 6) contd.....

Flavonol	Honey (BO)	Propolis(BO)	Pollen (BO)
Isorhm-3-O-Rut		*Populus* spp. [87]	
Isorhm-3-O-diGlc			Multifloral [41]
Isorhm-O-Gluc		*Populus* spp. [87]	
Isorhm*O*-AcRut		*Populus* spp. [87]	
Isorhm-3-*O*-Sop--diGly			*Taraxacum* ssp. [183]
Myr **(26)**	*Eucalyptus* spp.; La Alcarria*; *F. esculentum* [35], heather [135]; *Q. saponaria* [139]*; C.sativa, Citrus* spp., *Hedysarum* spp. [102]; *Pinus* spp., *Thymus* spp., *Abies* spp., *Citrus* spp. [164]; *Ziziphus spina-christi* [4]*;R. officinalis,* Heather [177]	*Populus* spp. [110]	*E. globulus* [183]; *Cistus ladaniferus* [147]*; Eucalyptus globulus, Elaeis* spp., *Cecropia* spp., *Mimosa pudica, Scoparia* spp. [180]
3-CH_3Myr	Heather [191, 192]	-	-
3,7,4',5'-tetraCH_3Myr	*Eucalyptus* spp., *Thymus algeriensis*, *R. officinalis, C. sinensis, B. campestris, H. annus,* multifloral [193]	*Dalbergia* spp. [182]	-
Myr-3,7-di-*O*-Glc	*Eucalyptus* spp. [186]	-	-
Myr-3-*O*-Gal	-	-	*R. raphanistrum* [183]
Myr-3-*O*-Rha-Glc	-	-	Multifloral [41]
Rhm	Heather, *R. pseudocacia*, *F. esculentum*, *Tilia* spp., *Brassica* spp. [35]	*Populus* spp. [87]; Black propolis [126]; *B. dracunculifolia*, *Clusia* spp. [126]	-
Fis	*Citrus* spp., *R. officinalis*, Heather, *Eucalyptus* spp., *C. sativa, Thymus* spp [177]	-	-
Kaempf	*R. officinalis* [2]	*Populus* spp. [74, 87,133]; *B. dracunculifolia* [74, 109]	-
Gala	La Alcarria* [181]; *F. esculentum*, heather [86, 7]; *Tilia* spp. [32, 194]; *Citrus* spp. [194]; *Ziziphus spina-christi* [4]; palmetto berry, *L. scoparium* [31]	*Populus* spp. [37, 68, 72, 87, 111, 133, 188, 195, 196]; Commercial [118, 189]	*Cystus incanus* [129]
5-CH_3Gala	-	*Populus* spp. [87]; Commercial [118]	-

(Table 6) contd.....

Flavonol	Honey (BO)	Propolis(BO)	Pollen (BO)
Herb-7-*O*-CH_3-3-*O*-Glc-8-*O*-Gal	-	-	-
Herb-7-*O*-CH_3-3-*O*-diGly	-	-	*R. sardous* [183]; *R. petiolaris* [187]
Herb-8-*O*-CH_3-3-*O*-diGly	-	-	*R. sardous* [183]
Herb-7-*O*-CH_3-3-*O*-Soph	-	-	*R. sardous* [183]; Multifloral [11]
Herb-8-*O*-CH_3-3-*O*-Soph	-	-	*Ulex europeaus, Lotus corniculatus* [183]

BO – Botanical origin; "*" after the name refer to geographic origin since no botanical origin is referred. (Hex)Rob – (Hexosyl)robinoside; AcRut – Acetylrutinoside; Ara – Arabinoside; CH_3 – Methyl; CouRha – Coumaroylrhamnoside; Fis – Fisetin; Gal – Galactoside; Gala – Galangin; Glc – Glucoside; Gluc – Glucoronide; Gly – Glycoside; Herb – Herbacetin; Hex – Hexoside; Hex(1→2)Hex – Hexosyl(1→2)Hexoside; Hex-Hex – hexosyl-hexoside; IsoRhm – Isorhamnetin; Kaemp – Kaempferol; Kaempf – Kaempferide; Myr – Myricetin; Neohesp – Neohesperidoside; OCH3 – Methoxy; OH – Hydroxyl; Pen – Pentoside; Quer – Quercetin; Rhm – Rhamnetin, Rha – Rhamnoside; Rut – Rutinoside: Soph – Sophoroside; Xyl(1→6)Glc – Xylosyl(1→6)glucoside

Naturally, the concentrations of each of these compounds is rather variable according to the botanic and geographical origin of each of the three honey-bee derived products.

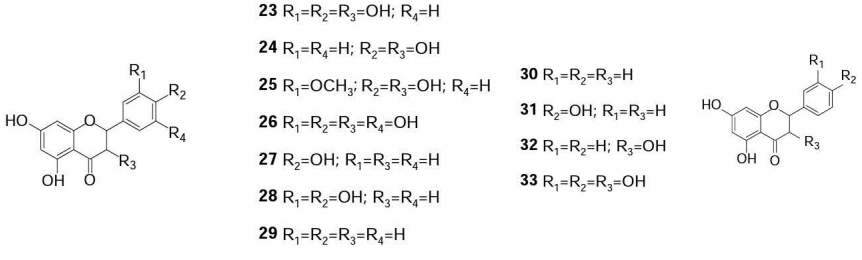


Fig. (4). Structures of relevant flavonoid compounds found on honeybee-derived products.

Bee pollen is specially rich in quercetin when compared to the remaining flavonols (high values registered in *Typha angustifolia* pollen from China (920 µg/100g) [175]). Propolis, on the other hand, is particularly enriched in kaempferol (*e.g.* concentration of 19.5 mg/g of extract from *Populus* spp. origin from Argentina [119]) while myricetin is the most abundant compound in honeys (244.7 µg/g in *Thymus capitatus* honeys from Greece) [165].

As flavones lack hydroxylation in the position C-3, the molecular ion of these compounds exhibit 18 Da less than their respective flavonols [197]. Significant concentrations of these compounds have also been reported in honeybee products. In particular, apigenin (**27** in Fig. (**4**)), luteolin (**28** in (Fig.(**4**)) and chrysin (**29** in Fig. (**4**), together with their methylated derivatives can be found in those products, although with distinct frequency and concentrations [198 - 200] (Table **7**).

Table 7. Relevant flavones in honey, propolis and bee pollen worldwide.

Flavone	Honey (BO)	Propolis(BO)	Pollen (BO)
Aca	*R. pseudoacacia* [204]	*Populus* spp. [72, 133]	-
Aca-Gly	-	-	*R. petiolaris* [187]
Api **(27)**	*Erica* spp., *Citrus* spp., *Lavandula* spp. [95]; La Alcarria* [181]; *F. esculentum* [86]; *R. pseudoacacia, Brassica* spp. [35]; *Tilia* spp. [32, 35]; *Ziziphus spina-christi* [4]	*Populus* spp. [6, 37, 72, 74, 87, 116, 119, 133, 188, 195]; Commercial [118, 189]; *B. dracunculifolia* [73]; Finnish* [132]	-
Api-7-*O*-Gly	-	-	*R. petiolaris* [187]
Api-6,8-di-*C*-Gly	-	-	*R. petiolaris* [187]
Baic	*Citrus* spp., *R. officinalis,* Heather, *Eucalyptus* spp., *C. sativa, Thymus* spp. [177]	-	-
Chr **(29)**	*Eucalyptus* spp. [95,176,177,194]; *Tilia* spp. [32,95,194]; *C. sativa, Citrus* spp. [95,177,194]; Heather, *R. officinalis* [95, 177]; *H. annus, Lavandula* spp., *R. pseudoacacia* [95]; *F. esculentum* [86], [7]; *Pinus* spp., *Thymus* spp., *Abies* spp., *Citrus* spp. [164]; *Ziziphus spina-christi* [4]; palmetto berry, *L. scoparium* [31]	*Populus* spp. [6, 37, 68, 72, 74, 87, 111, 119, 133, 177, 188, 195, 196, 205]; African Propolis [206]; Commercial [118, 189]; *B. dracunculifolia* [73]	*Cystus incanus* [129]
5-CH_3Chr	-	*Populus* spp. [87]	-
6-OCH_3Chr	-	*Populus* spp. [87]	-
5,7-diCH_3Chr	-	*Populus* spp. [87]	-
6-CnnChr	-	*Populus* spp.[195]	-
CH_3Chr	-	*Populus* spp. [87]	-
Genk	La Alcarria*[181]	-	-

(Table 7) contd.....

Flavone	Honey (BO)	Propolis(BO)	Pollen (BO)
Lut **(28)**	*Eucalyptus* [176]*; R. officinalis, Citrus* spp., *H. annus, Lavandula* spp. [95]; *R. pseudoacacia, F. esculentum,* Heather, *Tilia* spp., *Brassica* spp. [35]; palmetto berry [31]*; L. scoparium* [31]	*Populus* spp. [87, 110]; *Dalbergia* spp. [179]; Commercial [16]	*E. globulus* [183]; *Eucalyptus globulus, Cecropia* spp., *Mimosa pudica, Elaeis* spp. [180]; *Cistus incanus* [129]
7-CH_3Lut	La Alcarria*[181]	*Populus* spp. [87]	
Sel	-	-	*Eucalyptus* spp., *Cecropia* spp., [180]
Tect	*R. officinalis* [95]	*Populus* spp. [74, 87, 119, 133, 195, 196, 205], *B. dracunculifolia* [74]	-
Tri	*Eucalyptus* spp. [176]; *R. pseudoacacia, F. esculentum,* Heather, *Tilia* spp., *Brassica* spp. [35]	-	*E. globulus* [183]; *Mimosa pudica, Cecropia* spp., *Elaeis* spp., *Eupatorium* spp., *Eucalyptus globulus*

BO – Botanical origin; "*" after the name refer to geographic origin since no botanical origin is referred. Aca – Acacetin; Api – Apigenin; Baic – Baicalein; CH_3 – Methyl; Chr – Chrysin; Cnn – Cinnamyl; Genk – Genkwanin; Gly – Glycoside; OCH_3 – Methoxy; Sel – Selagin; Tect – Tectochrysin; Tri – Tricetin.

With the exception of some Brazilian propolis, chrysin may account up to 4% of the flavonoid content in propolis [200]. The concentration of this flavone in Argentinian *Populus* spp. propolis reach values as high as 68.7 mg/g extract [119]. High concentrations of chrysin have also been reported in Tunisian *H. annuus* honey (1.3 mg/100g) [193].

Apigenin is also widespread in honey and propolis, although with less extent and abundancy than chrysin [119], while luteolin is most of the times absent from the flavonoid profile of the majority of propolis [6, 201 - 203]. Commonly these flavones are detectable in bee pollen samples though in concentrations below the apparatus limit of quantification thus no relative abundance is found [129, 180, 183].

Flavanones and dihydroflavonols (stucturally equivalents of flavones and flavonols respectively, except for the saturated bond between C2-C3 of the C-ring) have also been identified in honeybee-derived products, though with less representativeness (*i.e.*, only few compounds of these groups have been detected).

Both of these groups are characterized by an intense Band II absorption in the range 277 – 295 nm with only a shoulder or low intensity peak representing Band I in the 300 – 330 nm. This happens because the reduction of the C2=C3 double bond eliminates the cinnamoyl chromophore, leaving only the A-ring benzoyl function intact [168, 169]. Interestingly, the UV spectra of compounds belonging to flavanones are almost identical to those obtained for the equivalent dihydroflavanols, indicating that, contrarily to what happens between flavones and flavonols, the presence or absence of the C-3 hydroxyl group in flavonoids lacking a C2-C3 double bond makes little difference in the UV spectra [169].

Despite less represented, flavanones and dihydroflavanols ocupy a place no less important than flavones and flavonols in the composition of bee products. Notably, pinocembrin (**30** in Fig. (**4**)), pinobanksin (**31** in Fig.(**4**)) and their respective derivatives are commonly present in several honeys and propolis samples of different origins, although they are absent in bee pollen (Tables **8** and **9**). In propolis, these two compounds represent, right after chrysin, the main flavonoid constituents of this bee product, reaching up to 4 and 3% of its total flavonoid content. However, concentrations of pinocembrin and pinobanksin may vary from 1 to 85 mg/g extract and 2 to 77 mg/g extract respectively [72, 111, 118, 196], being these associated with their concentrations in honey, *i.e.*, the higher content of these flavonoids in propolis, the greater their concentration in the honey of the hive [192, 200, 207].

Table 8. Relevant flavanones in honey, propolis and bee pollen worldwide.

Flavanone	**Honey (BO)**	**Propolis(BO)**	**Pollen (BO)**
Pinoc **(30)**	*Tilia* spp. [32,95,194]; *Citrus* spp., *Eucalyptus* spp., *C. sativa* [95, 194]; *Erica* spp., *R. officinalis, H. annus, R. pseudoacacia, Lavandula* spp. [95]; *F. esculentum* [86]; palmetto berry, *L. scoparium* [31]	*Populus* spp. [6, 37, 68, 72, 74, 87, 111, 119, 133, 196, 205]; African propolis [206]; Commercial [118]; *Dalbergia* spp. [179]; *B. dracunculifolia* [73]	*Cystus incanus* [129]
5-CH_3Pinoc	-	*Populus* spp. [87]	-
7-CH_3Pinoc	*Eucalyptus* spp., *Thymus* spp., *R. officinalis*, *Citrus* spp., *B. campestris, H. annuus*, multifloral [193]	*Populus* spp. [133]	-

(Table 8) contd.....

Flavanone	**Honey (BO)**	**Propolis(BO)**	**Pollen (BO)**
Pinoc-5-*O*-3-O--4-OCH_3PhProp	-	*Populus* spp. [87]	-
Hesp **(32)**	*Citrus* spp., [95,177,194]; *C. sativa, Eucalyptus* spp. [95, 102, 177, 194]; *Tilia* spp. [35,95,177,194]; *R. pseudoacacia, Brassica* spp.,*F. esculentum* [35]*; Hedysarum* spp. [102]; *R. officinalis, Thymus* spp. [177]; Heather [35, 177]	-	-
5,7-diCH_3Hesp	-	*Populus* spp. [210]	-
Hesp-7-*O*-Rut	Multifloral [185]; *Q. saponaria* [139]	-	-
Nar **(33)**	*H. annus* [193]; *Q. saponaria* [139]; *Mimosa caesaepiniifolia* [178]; *Tilia* spp. [32]; *Citrus* spp., *R. officinalis,* Heather, *Eucalyptus* spp., *C. sativa, Thymus* spp. [177]	*Dalbergia* spp. [182]; *Populus* spp. [37,72,116,177]	*Cystus incanus* [129]
Nar-7-*O*-Hesp	Multifloral [185]; *C. sativa, Citrus* spp. [194]; *Ziziphus spina-christi* [4]*; Tilia* spp. [32, 194]	-	-
Liqu	-	*Dalbergia* spp. [182]	-
Sak	-	*Populus* spp. [133]	-
3-OH-5-CH_3 flavanone	-	*Populus* spp. [87]	-
SophoB	-	African propolis [206]	-

BO – Botanical origin; "*" after the name refer to geographic origin since no botanical origin is referred. CH_3 – Methyl; Hesp – Hesperitin; Liqui – Liquiritigenin; Nar – Naringenin; OCH_3 – Methoxy; OH – Hydroxyl; Pinoc – Pinocembrin; Rut – Rutinoside; Sak – Sakuranetin; SophoB – Sophoraflavanone B.

Table 9. Relevant dihydroflavonols in honey, propolis and bee pollen worldwide.

Di-hydroflavonols	**Honey (BO)**	**Propolis(BO)**	**Pollen (BO)**
Pinob **(31)**	*Eucalyptus* spp. [95, 176]; *Tilia* spp., *Citrus* spp., *H. annus, C. sativa, Erica* spp., *R. officinalis, Lavandula* spp., *R. pseudoacacia* [95]*; F. esculentum* [86]	*Populus* spp. [6, 37, 68, 72, 74, 87, 111, 119, 133, 196]; *Dalbergia* spp. [179]; African propolis [206]; Commercial [118]	-
5-CH_3Pinob	-	*Populus* spp. [87, 196]; Commercial [118]; African propolis [206]	-

(Table 9) contd.....

Di-hydroflavonols	Honey (BO)	Propolis(BO)	Pollen (BO)
5-CH_3Pinob-3-*O*-Ac	-	*Populus* spp. [87]; Commercial [118]	-
5,7-diCH_3Pinob	-	*Populus* spp. [200]	-
Pinob-3-*O*-Ac	-	*Populus* spp. [74,87,188]; *Dalbergia* spp. [179]; African propolis [206]	-
Pinob-3-*O*-Ac-5-*O*-*p*-OHPhProp	-	*Populus* spp. [87]	-
Pinob-3-*O*-Prop	-	*Populus* spp. [87]; Commercial [118]	-
5-CH_3Pinob-3-*O*-Pent	-	*Populus* spp. [87]; Commercial [118]	-
7-CH_3Pinob-5-*O*-*p*-OHPhProp	-	*Populus* spp. [87]	-
Pinob-3-*O*-But or IBut	-	*Populus* spp. [87]	-
Pinob-3-*O*-Pente	-	*Populus* spp. [87]	-
Pinob-3-*O*-Pent or 2-CH_3But	-	*Populus* spp. [87]	-
Pinob-*O*-Hexe	-	*Populus* spp. [87]; Commercial [118]	-
Pinob-3-*O*-PhProp	-	*Populus* spp. [87]	-
Pinob-3-*O*-Hex	-	*Populus* spp. [87]	-
Taxifolin	*Clidemia* spp., *Serjania* spp., *Myrcia* spp., *Mimosa pudica, Mora* spp., *Tapirira* spp., *Schefflera* spp. [148]	-	*Cystus incanus* [129]

BO – Botanical origin; "*" after the name refer to geographic origin since no botanical origin is referred. Ac – Acetate; But – Butyrate; CH_3 – Methyl; Hex – Hexoside; Hexe – Hexenoate; IBut – Isobutyrate; OHPhProp – Hydroxyphenylpropionate; Pent – Pentanoate; Pente – Pentenoate; PhProp – Phenylpropionate; Pinob – Pinobanksin; Prop – Propionate.

Although unconstant, the flavanones naringenin (**33** in Fig. (**4**)) and hesperitin (**32** in Fig. (**4**)) also participate in the composition of certain honeys. Notwithstanding, when these compounds are detected, they normally give valuable information about the origin of this bee product. Hesperitin is a good example of this, since its detection is unique to honeys of *Citrus* spp. origin [208, 209]. The concentration of this compound in Italian *Citrus* spp. honey has been pointed to reach 4.09 µg/100g [102], thought in general, its concentrations can reach about 8 µg/100g.

CONCLUSION

In summary, since honeybee-derived products, in particular honey, propolis and bee pollen, have been showing to possess a series of valuable health benefiting compounds, including phenolic compounds, researchers interest have grown in order to identify, characterize and comprehend their individual and/or synergistic contributions for the health benefits claimed in these three bee products. Therefore, advances in separative and chromatographic techniques, together with the improvement of the detection methods, have greatly contributed to the progress and production of very rich data in this field.

Despite the attemps to find patterns in the phenolic composition of honey, propolis and bee pollen, they exhibit such an immense rich and diversified composition wich are very likely to variations according to their botanical and geographical origin. Still, it is possible to distinguish some phenolics that are ubiquitous among these products such as the non-flavonoids caffeic, *p*-coumaric, cinnamic, benzoic, vanillic acids and their derivatives, and four distinct groups of flavonoids including flavonols, flavones, flavanones and dihydroflavonols.

CONFLICT OF INTEREST

The author confirms that author has no conflict of interest to declare for this publication.

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