Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1220–1234
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Advances in the analysis of phenolic compounds in products derived from bees

A.M. Gómez-Caravaca, M. Gómez-Romero, D. Arráez-Román, A. Segura-Carretero *, A. Fernández-Gutiérrez *

*Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain*

Received 30 December 2005; received in revised form 2 March 2006; accepted 4 March 2006
Available online 18 April 2006

**Abstract**

Honey and propolis are rich in phenolic compounds, which act as natural antioxidants, and are becoming increasingly popular because of their potential role in contributing to human health. These compounds can also be used as indicators in studies into the floral and geographical origin of the honey and propolis themselves. We present here an overview of current analytical methods for measuring polyphenols in honey and propolis. The analytical procedure to determine individual phenolic compounds involves their extraction from the sample, analytical separation and quantification. The techniques reviewed are based on spectrophotometry as well as analytical separation techniques such as gas chromatography, high-pressure liquid chromatography and capillary electrophoresis.


*Keywords:* Phenolic compounds; Honey; Propolis; Analysis

## 1. Introduction about interest of phenolic analysis

Quality control, nutritional value and the monitoring of hazardous residues in foodstuffs have all become major topics of public interest [1]. The effects of growing conditions, processing, transport, storage, genetics and other factors concerning chemical and biochemical components are also important issues in food science [2]. In recent years there has been growing interest in functional foods, i.e. foods that can provide not only basic nutritional and energetic requirements but also additional physiological benefits [3]. The term "functional food" was used for the first time in Japan in the 1980s and was applied to processed food which contained ingredients that conferred the benefits of some physiological functions. Nowadays a functional food can be defined as a food that produces a beneficial effect in one or more physiological functions, increases well-being and/or decreases the risk of suffering from a particular medical condition. The functionality of a food is usually related to some of the ingredients that it contains and at present consumers prefer these ingredients to have a natural rather than synthetic origin. Thus they are commonly extracted from plants, food by-products and other natural sources [4].

Among the functional ingredients the group most widely studied is the family of antioxidants. Traditionally, this kind of compounds have played an important role in food science and technology because of their usefulness in preserving foodstuffs against oxidative degradation [5]. Interest in antioxidant compounds has increased nowadays in the light of recent evidence regarding the important role of antioxidants in human health. In fact several preventative effects against different diseases such as cancer, coronary diseases, inflammatory disorders, neurological degeneration, aging, etc., have been related to the consumption of antioxidants [6,7].

Phenolic compounds or polyphenols, are one of the most important groups of compounds occurring in plants, where they are widely distributed, comprising at least 8000 different known structures [8]. Polyphenols are also products of the secondary metabolism of plants. These compounds are reported to exhibit anticarcinogenic, anti-inflammatory, anti-atherogenic, antithrombotic, immune modulating and analgesic activities, among others and exert these functions as antioxidants [9–13]. In general, phenolic compounds can be divided into at least 10 types depending upon their basic structure: simple phe-

* Corresponding authors. Tel.: +34 958 248593; fax: +34 958 249510.
*E-mail addresses:* ansegura@ugr.es (A. Segura-Carretero), albertof@ugr.es (A. Fernández-Gutiérrez).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.03.002

nols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids and lignins. Flavonoids constitute the most important polyphenolic class, with more than 5000 compounds already described [6].

Due to the importance of polyphenols in food this manuscript reviews their analysis in different products derived from bees. Several natural products are manufactured by bees to construct their hives and produce honey. These include beeswax, royal jelly, beebread, propolis and honey itself. There are no studies analysing the phenolic profile of beeswax, royal jelly and beebread and so this review confines itself to the analysis of polyphenols in honey and propolis.

In general, an analytical procedure for the determination of individual phenolic compounds involves three basic steps: extraction from the sample, analytical separation and quantification. Several methods have been developed to analyse polyphenols in honey and propolis: colorimetric reactions, thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and lately, capillary electrophoresis (CE).

## 2. Honey

### 2.1. Introduction

Honey has been used as a food since the earliest times. Only in recent years, however, has evidence emerged of its antioxidant capacity [14]. It is also used as a food preservative [15–17], preventing deteriorative oxidation reactions in foods, such as lipid oxidation in meat [18,19] and the enzymatic browning of fruits and vegetables [20–22]. Antioxidants specifically retard deterioration, rancidity or discoloration due to oxidation caused by light, heat and some metals. Nevertheless, the antioxidant activity of honey varies greatly depending on the floral source [23,24] and external factors such as the season and environment, and finally its processing.

Honey is reported to contain at least 181 substances [25] and is considered as part of traditional medicine. Apitherapy has recently become the focus of attention as a form of folk and preventive medicine for treating certain conditions and diseases as well as promoting overall health and well being [26]. It has been reported to be effective in gastrointestinal disorders [27,28], in the healing of wounds and burns [29,30], as an antimicrobial agent [28–32] and to provide gastric protection against acute and chronic gastric lesions [33,34].

Honey is a supersaturated solution of sugars, to which the main contributors are fructose (38%) and glucose (31%). It also has a wide range of minor constituents, many of which are known to have antioxidant properties [35,36]. These include flavonoids and phenolic acids [37,38], certain enzymes (glucose oxidase, catalase) [25], ascorbic acid [25], Maillard reaction products [25], carotenoid-like substances [42], organic acids [37] and amino acids and proteins [39]. The natural antioxidants, especially flavonoids, exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory actions [40].

The quality of honey is judged by its botanical or floral origin and chemical composition [37] and price of honey is based on its quality [41]. Traditionally, the floral source of a honey has been identified by the analysis of bee pollens present in the honey. Tan et al. however [42] have suggested that chemical approaches might be more accurate and easily undertaken in the characterisation of the floral source of a honey [43]. The analysis of their phenolic compounds, including flavonoids, has been suggested [44] and this technique tends to be used to study their floral and geographical origins. Before this, researchers tried to use the analysis of amino acids [38,45] to complement pollen analysis in the determination of the floral origins of honey. Even so, the analysis of phenolic compounds has been regarded as a very promising way of studying the floral and geographical origins of honeys [44–49]. In these studies, the flavanone hesperetin has been used as a marker for citrus honey [50–52], the flavonol kaempferol for rosemary honey [50,53] and quercetin for sunflower honey [54]. Some phenolic acids, such as ellagic acid in heather honey, have also been used as floral markers [37,55,56], and the hydroxycinnamates (caffeic, *p*-coumaric and ferulic acids) in chestnut honey [37]. Pinocembrin, pinobanksin and chrysin are the characteristic flavonoids of propolis and these flavonoid compounds have been found in most European honey samples [54]. In some honeys, such as those derived from lavender and acacia, no specific phenolic compounds have been found as suitable floral markers [54]. Other possible phytochemicals markers may be found, such as abscisic acid for heather honey [56]. Abscisic acid has also been detected in rapeseed, lime tree and acacia honeys [54]. A study of the phenolic contents of honey may also determine the presence of antimicrobial activity [57,58].

### 2.2. Sample preparation

Isolation of the phenolic compounds from the sample matrix is generally a prerequisite to any comprehensive analytic scheme, although enhanced selectivity in the subsequent quantification step may reduce the need for sample manipulation. The ultimate goal is the preparation of a sample extract uniformly enriched in all components of interest and free from interfering matrix components [59]. The extraction procedure used in most of the studies published is a solid phase extraction consisting of the following steps. The honey samples are mixed with five parts of water (pH 2 with HCl) until completely fluid and then filtered through cotton to remove solid particles. The filtrate is then passed through a column of Amberlite XAD-2 [60]. The phenolic compounds remain in the column while sugars and other polar compounds elute with the aqueous solvent, resulting in a flavonoid recovery of >95% [60,61]. The column is washed with acidic water (pH 2 with HCl) and subsequently with distilled water. The whole phenolic fraction is then eluted with methanol and dried under reduced pressure at 40 °C. There is a modification to this extraction in which the filtrate is mixed with Amberlite and stirred with a magnetic stirrer for 10 min before filling the column.

It is possible to carry out the next step, the clean-up, in two different ways. In the first one, the residue obtained after the evaporation of the methanol is resuspended in distilled water and extracted with diethyl ether. The ether extracts are combined and diethyl ether is removed by flushing with nitrogen. The dried residue is then redissolved in methanol and filtered [43,53,54,56,62–71]. In the second one, the residue is dissolved in methanol and the solution passed through a Sephadex LH-20 column. The phenolic fraction is evaporated to dryness under reduced pressure, redissolved in methanol and filtered [36,50,61,72,73].

Weston et al. [65] have demonstrated that phenolic acids seem to be eluted with the sugar fractions during the fractionation of honey on a XAD-2 column, as Ferreres et al. [74] mentioned that sugars and polar compounds were washed with water. In addition, by using diethyl ether, they aimed to eliminate the non-flavonoid phenolic compounds, which contaminated the flavonoid peaks; thus the main diethyl ether extract contents were flavonoids.

Aljadi et al. [75,76] recover the phenolic compounds from honey using a C18-SPE cartridge. Honey samples are prepared, subjected to base hydrolysis and extracted with ethyl acetate (liquid–liquid extraction) as described by Wahdan [77]. The fraction extracted with ethyl acetate is evaporated under dryness, then the dry honey extract is redissolved in acidified deionised water and the phenolics are adsorbed onto preconditioned isolute C18 columns. The cartridges are preconditioned by passing methanol and acidified water. The adsorbed phenolics are then eluted from the cartridges by passing methanol–water solution 25% (v/v) at a drop wise flow rate. The recovered fractions are combined, dried under nitrogen and subjected to further analysis. Extraction using a SPE-C18 cartridge is a simple technique that employs inexpensive disposable extraction columns and provides many advantages, such as a reduction of solvent consumption and high recoveries of the analytes.

Another type of solid-phase extraction for phenolic compounds in honey, used by Inoue et al. [26], is a GL-Pak PLS-2 cartridge. Honey samples are dissolved in distilled water. The sample solution is transferred into an SPE cartridge preconditioned with methanol and distilled water. This is then washed with water and eluted with methanol. The solutions are evaporated to dryness under a stream of nitrogen. The samples are redissolved by adding methanol.

For the extraction of homogentisic acid from honey an aliquot of homogenized honey is dissolved in water in screw-capped tubes. Ethyl acetate is added to each tube and the mixture is agitated in a rotary shaker. The phases are allowed to separate and the organic extracts are centrifuged. After centrifugation, anhydrous sodium sulphate is added to the combined extracts and evaporated to dryness by a rotary evaporator. The residue is taken up with acetone and the acid isolated by preparative TLC using $H_2SO_4$ $10^{-2}$N ($R_f = 0.7$) [78].

To extract a similar quantity of honey an Amberlite XAD-2 column requires more solid phase than that used in the other different types of SPE but more phenolic compounds are identified than with the other types of SPE.

### 2.3. Spectrophotometric determination of phenolic compounds

The colorimetric assay based on the reaction of Folin-Ciocalteu reagent is a method widely used for the determination of total phenols in honey [17,75,79,80]. The method consists of calibration with a pure phenolic compound, extraction of phenols from the sample and the measurement of absorbance after the color reaction.

The main disadvantage of the colorimetric assay is its low specificity, as the color reaction can occur with any oxidizable phenolic hydroxy group. An interesting approach to the content of total extractable phenolic compounds in different food samples involving the comparison of chromatographic and spectrophotometric methods has recently been reported, accounting for the possible influence of other substances as interfering compounds [81].

A typical protocol using the Folin-Ciocalteu method could be as follows. Each honey sample is diluted with distilled water and filtered. This solution is then mixed with Folin-Ciocalteu reagent for 5 min and sodium carbonate is added. After incubation at room temperature the absorbance of the reaction mixture is measured at 760 nm against a methanol blank. Gallic acid is used as standard to produce the calibration curve. The mean of three readings is used and the total phenolic content is expressed in mg of gallic acid equivalents/100 g of honey [82]. A modification of the Folin-Ciocalteu method has been carried out by Vinson et al. [83].

### 2.4. Chromatographic determination of the phenolic profile of honey

The need for knowing the profiles and identifying individual honey compounds requires the replacement of traditional methods by separative techniques. High-performance liquid chromatography (HPLC) is without doubt the most useful analytical technique for characterizing polyphenolic compounds, though gas chromatography and capillary electrophoresis are used in some instances.

GC was employed in its beginnings in an attempt to facilitate the determination of polyphenolic compounds. It has been used to determine polyphenols in honey in some published studies [47,52,76]. GC–MS has also been employed for the analysis of flavonoids in honey and, in this case, the derivatization step was unnecessary [84].

Studies with HPLC are described in Table 1, giving mobile phases, type of elution employed, stationary phase, extraction system, detection system used, compounds identified and several pertinent observations. The HPLC mode most widely used has been reversed-phase HPLC. In this case the stationary phase consists of a non-polar octadecylsilane ($C_{18}$) bonded phase and the mobile phase is a polar solvent.

The majority of published chromatography studies describe the use of an elution mobile gradient phase in recognition of the complexity of the phenolic profile. Several mobile phases have been used but the most common are binary systems comprising an aqueous component and a less polar organic solvent.

Table 1
Separation of phenolic compounds of honey using HPLC methods

| Column | Mobile phases | Elution | Detection | Extraction system | Identified compounds | Observations | Reference |
|---|---|---|---|---|---|---|---|
| XTerra RP18 (15 cm × 0.39 cm, 5 μm) | A: water:formic acid (99.5:0.5); B: methanol | Gradient | DAD λ = 285 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | *p*-Hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *cis–trans*-abscisic acid, cinnamic acid, pinobanksin, quercetin, pinocembrin, kaempferol, chrysin, galangin | Antioxidants of honeys from various floral sources | [23] |
| Lichrocart RP-18 (18.1 cm × 0.4 cm, 5 μm) | A: water:formic acid (95:5); B: methanol | Gradient | DAD λ = 280 and 340 nm | SPE (Amberlite XAD-2) clean-up: Sephadex LH-20 | Ellagic acid, myricetin, chalcone, glycoside, quercetin, luteolin, 8-methoxikaempferol, kaempferol, apigenin, isorhamnetin, pinocembrin, chrysin, genkwanin, tectochrysin | Flavonoids in *Apis mellifera* and *Melipona* spp. honeys | [36] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (95:5); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, kaempferol 8-methyl ether, pinocembrin, quercetin 3,3′-dimethyl ether, isorhamnetin, chrysin, pinobanksin, tectochrysin | Flavonoids, phenolic acids and abscisic acid in Leptospermum honeys | [43] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Grandient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Pinocembrin, pinobanksin, chrysin, galangin, tecthochrysin, quercetin, kaempferol, 8-methoxykaempferol, caffeic acid, *p*-coumaric acid, *cis–trans*-abscisic acid, ferulic acid, apigenin, quercetin 3,7-dimethyl ether, quercetin 3,3′-dimethyl ether, hesperetin | Flavonoid profile of European unifloral honeys | [54] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | *Trans–trans*-abscisic acid, *cis*, *trans*-abscisic acid, pinobanksin, pinocembrin, chrysin, galangin | Analysis of abscisic acid and flavonoids in heather honey | [56] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Pinobanksin, quercetin, luteolin, 8-methoxykaempferol, kaempferol, apigenin, isorhamnetin, pinocembrin, chrysin, galangin, tectochrysin | Analysis of 15 flavonoids in rosemary honey | [62] |
| Lichrocart RP-18 (10 cm × 0.4 cm, 5 μm) | A: water:formic acid (95:5); B: methanol | Gradient | DAD λ = 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Quercetin glycoside, luteolin glycoside, 8-methoxykaempferol glycoside, kaempferol glycoside, quercetin, luteolin, methylated luteolin, 8-methoxykaempferol, isorhamnetin, genkwanin | Flavonoids in stinglessbee honey | [64] |
| LiChrospher 100 RP-18 (12 cm × 0.4 cm, 5 μm) | A: water:formic acid (95:5); B: methanol | Gradient | UV λ = 270 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Caffeic acid, phenyllactic acid, methyl syringate, cinnamic acid, pinobanksin, pinocembrin, chrysin, galangin | Antibacterial phenolic components of manuka honey | [65] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Myricetin, tricetin, quercetin, luteolin, kaempferol | Flavonoids markers of Eucalyptus honey | [66] |

Table 1 (*Continued*).

| Column | Mobile phases | Elution | Detection | Extraction system | Identified compounds | Observations | Reference |
|---|---|---|---|---|---|---|---|
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl eher, kaempferol, pinobanksin, pinocembrin, chrysin | Flavonoids in Eucalyptus Australian honeys | [67] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, *o*-coumaric, ferulic acid, ellagic acid, abscisic acid | Analysis of seven phenolic acids and two abscisic acid isomers in Eucalyptus honey | [68] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, 8-methoxy kaempferol, pinocembrin, quercetin 3,3′-dimethyl ether, isorhamnetin, chrysin, pinobanksin, genkwanin | Flavonoids in Melaleuca, Guioa, Lophostemon, Bansia and Helianthus honeys | [69] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, kaempferol 8-methyl ether, pinocembrin, quercetin 3,3,′-dimethyl ether, isorhamnetin, chrysin, pinobanksin | Quantitative analysis of Flavonoids in Australian Eucalyptus honeys | [70] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Gallic acid, chloroenic acid, coumaric acid, ferulic acid, ellagic acid, syringic acid | Phenolics acids in Melaleuca, Guioa, Lophostemon, Bansia and Helianthus honeys | [71] |
| $C_{18}$ (15 cm × 0.46 cm, 5 μm) | A: water:acetic acid (99:1); B: methanol:acetic acid (99:1) | Gradient | DAD λ = 280 nm | Extraction with ethyl acetate and SPE ($C_{18}$) | Gallic acid, caffeic acid, ferulic acid, benzoic acid, cinnamic acid | Isolation and identification of phenolic acids in Malaysian honey | [76] |
| Discovery RP Amide $C_{16}$ (15 cm × 0.46 cm, 5 μm) | A: water:acetic acid (95.5:0.5); B: methanol: acetic acid (95.5:0.5) | Gradient and isocratic | Multichannel Electrochemical detector and mass spectrometry | SPE: GL-Pak PLS-2 cartridge | Methyl syringate | Identification of phenolic compounds in manuka honey | [26] |
| Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290 and 340 nm, NMR | SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether | Ellagic acid, pinobanksin, hesperetin, quercetin, luteolin, 3-methylquercetin, 8-methoxykaempferol, kaempferol, apigenin, isorhamnetin, pinocembrin, phenylethyl caffeate, pinobanksin 3-acetate, dimethylallyl caffeate, quercetin 3,7-dimethyl ether, chrysin, galangin, galangin 3-methyl ether, myricetin 3,7,4′,5′-methyl ether, pinocembrin 7-methylether, tecthochrysin | Flavonoids in Tunisian honeys | [63] |
| Spherisorb ODS2 (25 cm × 0.46 cm, 5 μm) | Methanol/$H_2SO_4$ $10^{-2}$N (10:90, v/v) | Isocratic | DAD λ = 292 nm NMR, MS | Extraction with ethyl acetate and TLC | Homogentisic acid | Determination of homogentisic acid in strawberry-tree honey | [78] |

For example, a method available for the analysis of the phenolic fraction of honey is a reversed-phase HPLC using gradient elution with an aqueous solution of formic acid and methanol as solvents.

With regard to the detection system employed in HPLC, it should be emphasised that UV–vis detection with a diode array detector is undoubtedly the most common, although electrochemical detection systems [26,47,85–87] and mass detectors [26,78] have been used to a lesser extent.

The most frequent wavelengths used have been 290 and 340 nm. Because some phenolic compounds show several absorption maxima, the use of simultaneous multiple UV (photodiode array) is recommended for identification purposes, and also because this detector offers chromatograms at any wavelength accompanied by the absorption spectrum of each eluted band. In this way the absorption spectrum can be combined with retention parameters for the possible identification of an unknown compound as well as to measure the purity of the elution band in question. At 290 nm it is known that all polyphenolic compounds absorb, but nonetheless, some studies recommend using different wavelengths to achieve maximum sensitivity, and also, if possible, a suitable selectivity depending upon the polyphenolic compounds contained in the honey in question.

Polyphenols are usually identified by comparing retention times, UV spectra and chromatograms. NMR spectrometry is often also used as a complementary technique for structural assignment [56,63,66,78].

### 2.5. Electrophoretic determination of the phenolic profile

Capillary electrophoresis has also been used as an alternative technique to HPLC for the analysis of phenolic compounds in honey. CE combines short analysis times and high separation efficiency of polyphenols in honey. The use of this rapid analytical technique, allowing a faster screening of phenolic compounds, is highly recommended. The union of speed, resolution, simplicity and low operating costs make the technique an attractive option for the development of improved methods for determining phenolic compounds in honey.

Despite of the advantages that CE seems to have, there are few publications dealing with the determination of polyphenols in honey by this technique.

The operative modes used are borate-based CZE and borate-based micellar electrokinetic chromatography (MECK) with sodium dodecylsulfate (SDS) as micellar agent. The MECK methods study honey flavonoids [50,88] and the CZE method analyzes the whole polyphenolic fraction of honey [73].

A summary of optimized conditions of capillary electrophoresis methods (effective length of capillary, internal diameter of capillary, wavelength of detection, voltage, temperature, injection time, buffer concentration) where honey samples are analysed as well as the phenolic compounds studied are set out in Table 2.

## 3. Propolis

### 3.1. Introduction

Propolis, or bee glue, is a dark-coloured resinous substance collected by honeybees from leaf buds and cracks in the bark of various tree species [89]. Bees may also use material actively secreted by plants, or exuded from wounds in plants (lipophylic material on leaves, mucilages, gums, resins, lattices, etc.). Once collected, this material is enriched with salivary and enzymatic secretions. The resulting substance is used by bees to seal holes in their hives, strengthen the thin borders of the comb, exclude

Table 2
Summary of optimized conditions of capillary electrophoresis methods where honey samples are analized

| Instrumental variables | | | | | | Experimental variables | | | Identified compounds | References |
|---|---|---|---|---|---|---|---|---|---|---|
| $L_{ef}$ (cm) | i.d. (μm) | $\lambda_d$ (nm) | $V$ (kV) | $T$ (°C) | $t_{inj}$ (s) | Type of buffer | [Buffer] (mM) | pH | | |
| 63 | 70 | 280 | 20 | 30 | 2 | Sodium borate/SDS + 10% methanol | 200/50 | 8 | Pinobanksin, naringenin, hesperetin, 8-methoxykaempferol, myricetin, quercetin, luteolin, eriodictyol, pinocembrin, kaempferol, apigenin, chrysin, galangin | [50] |
| 63 | 50 | 340 | 21 | 25 | 2 | Boric acid/SDS | 200/50 | 8.5 | Eriodictyol, naringenin, hesperetin, pinobankin, pinocembrin, myricetin, quercetin, kaempferol, luteolin, apigenin, chrysin, galangin, genkwanin, tectochrysin | [88] |
| 50 | 50 | 280 | 20 | 30 | – | Sodium borate + 20% methanol | 100 | 9.5 | Hydroxymethylfurfural, phenylethylcaffeate, dimethylallylcaffeate, pinobanksin, naringenin, hesperetin, cinnamic acid, chlorogenic acid, *m*-coumaric acid, quercetin, luteolin, syringic acid, ferulic acid, pinocembrin, *o*-coumaric acid, kaempferol, *p*-coumaric acid, apigenin, vanillic acid, chrysin, galangin, ellagic acid, rosmarinic acid, *p*-hydroxybenzoic acid, caffeic acid, gallic acid, 2,4-dihydroxybenzoic acid | [73] |

$L_{ef}$, effective length of capillary; i.d., internal diameter of capillary; $\lambda_d$, wavelength of detection; $V$, voltage; $T$, temperature; $t_{inj}$, injection time; [Buffer] buffer concentration.

draught and make the entrance of the hive weathertight or easier to defend. Propolis is also used as an "embalming" substance to cover hive invaders which the bees have killed but cannot transport out of the hive [90].

Propolis has been used extensively in folk medicine since it possesses various biological activities such as antiseptic, antifungal, antibacterial, antiviral, anti-inflammatory, anaesthetic and antioxidant properties [89,91,92] among others. It can increase the body's natural resistance to infections and lower blood pressure and cholesterol levels. Applied externally, propolis relieves various types of dermatitis. In addition, it is used in mouthwashes and toothpastes to prevent caries and treat gingivitis and stomatitis [93] and it is claimed to be useful in cosmetics and as a constituent of health foods [94].

The plant origin of propolis determines its chemical diversity. Bee glue's chemical composition depends on the species of local flora present at the site of collection and thus in the geographic and climatic characteristics at the site [95]. In the world's temperate zones the dominant propolis source is the bud exudate of poplar (*Populus*) [90,96] whereas in the tropical regions there are no poplars and bees have to find different plant sources for bee glue. In spite of possible differences in composition due to the different plant sources, most propolis samples share considerable similarity in their overall chemical nature. It is made up of 50% resin (composed of flavonoids and related phenolic acids), 30% wax, 10% essential oils, 5% pollen and 5% other organic compounds [97]. Polyphenols (including flavonoids, phenolic acids and their esters), due to their proven ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters and to scavenge free radicals, are considered to be the main pharmacologically active molecules in propolis [98].

More than 180 compounds, mainly polyphenols, have been identified as constituents of propolis [94]. As mentioned already, the concentration of phenolic compounds may vary substantially according to the origin of the samples and such differences are likely to affect its biological activities and consequently its clinical properties [99]. Therefore the assay of these components is of great importance.

### 3.2. Sample preparation

Propolis cannot be used as a raw material; it must be purified by extraction with solvents. This process should remove the inert material and preserve the polyphenolic fractions. Extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenolic components [100] and this is the most commonly used solvent, especially at concentrations of 70% [101–108] and 80% [109–114], although other concentrations have also been used, such as 95% [115] and absolute ethanol [116,117]. Compared with absolute ethanol, extraction with aqueous ethanol results in wax-free tinctures, containing higher amounts of phenolic substances [118]. Park and Ikegaki [119] used various concentrations of ethanol as solvent and measured the absorption spectra of the different extracts. The 80% ethanolic extract showed highest absorption at 290 nm, which means that the highest concentration of flavonoids (especially of kaempferide, acacetin and isorhamnetin) was liberated from the propolis when using this solvent. With other ethanol concentrations, however, it is possible to extract higher quantities of other flavonoids, for example, with 60% ethanol the most extracted compounds were isosakuranetin, quercetin and kaempferol; and with 70% ethanol, pinocembrin and sakuranetin.

Extraction with pure water [101,119,120] (these extracts are likely to contain phenolic acids which are very soluble in water), methanol [62,63,100,121], hexane and acetone [122,123] and chloroform [124] has also been used.

The analysis of raw propolis is more frequent than the analysis of commercial propolis preparations [100,114,121,125]. The preparation of crude propolis begins by dehydrating the sample so that the dried propolis (cooled) can be ground into a fine powder. Then, in one procedure, a weighted sample is dissolved in the solvent (the most frequently used proportion is 1:10, w/v) and left for 24 h at room temperature [63,105,107,108,111,114,117,126]. It is then filtered and the procedure repeated several times [103,104,106,121] as successive extractions ensure the complete recovery of the phenolics. Alternatively, the sample is dissolved by shaking at 70 °C for 30 min [112,113,119]. After dissolution the insoluble portion is separated by filtration and the solvent is then evaporated to dryness under reduced pressure [63,103,104,107,108] and redissolved. Instead of this, the mixture can be centrifuged to obtain the supernatants [111,112,114,115,119,121,126], which can then be directly used for analysis.

It must be stressed that, as in any analytical study, sampling is extremely important, because this procedure determines the final result.

### 3.3. Spectrophotometric determination of phenolic compounds

The increasing use of propolis preparations in medicine requires the development of suitable approaches for the quantitative determination of their active components. Rapid spectrophotometric methods are assumed to be especially useful for the routine control of propolis [111,118,127,128]. These methods are aimed at the determination of either total flavonoids and total phenolics [118,127] or total flavanones/dihydroflavonols and total flavones/flavonols [128].

Popova et al. [106] pointed out that the quantification of the active compounds within groups with the same or close chemical structures correlates better with their biological activity and is more informative than the quantification of individual components. They assume, therefore, that this is a correct approach to characterising and standardising propolis preparations. Other advantages are its simplicity, good repeatability and acceptable accuracy.

Quantitative determinations of flavonoids in propolis are conducted by two colorimetric methods [129]. The aluminium chloride method is used to determine the flavone and flavonol content; it is based on the formation of a complex between the aluminium ion Al(III) and the carbonyl and hydroxyl groups of the flavonoid. The test solution, methanol and aluminium chloride in methanol (w/v) are mixed and left for 30 min. The

absorbance is then measured at 425 nm [106,127]. To quantify flavanones and dihydroflavonols the 2,4-dinitrophenylhydrazine (DNP) method is used. This is based upon the interaction of these compounds with DNP in acidic media to form coloured phenylhydrazones. The test solution and DNP solution diluted with methanol are heated at 50 °C for 50 min in a water bath. After cooling to room temperature, the mixture is diluted with potassium hydroxide in methanol (w/v). The resulting solution is diluted with methanol. Absorbance is measured at 486 nm [128,129]. The sum of flavonoid contents determined by the above two methods closely represents the real content of total flavonoids [129].

It is possible to determine the total flavonoid content with the Folin-Ciocalteu method, which is the most widely used for the spectrophotometric quantification of total phenolics [130]. Briefly, the test solution, distilled water, Folin-Ciocalteu reagent and sodium carbonate solution are mixed. The sample is left for incubation and the absorbance is measured at 760 nm [106,117].

### 3.4. Chromatographic determination of the phenolic profile of propolis

The complete characterization of propolis activity involves both qualitative and quantitative chemical analysis. Chromatographic techniques such as fine chromatography, gas and, in particular, HPLC provide the profile and identification of the individual phenolic compounds. They are discussed in this section. Special attention is given to detection systems, due to their importance in the characterization of polyphenolic compounds. Detection is routinely achieved by ultraviolet absorption, often involving a photodiode array detector. Coupled techniques, particularly mass spectroscopy, are being used increasingly for routine work.

#### 3.4.1. Thin-layer chromatography

In TLC, the choice of stationary phase as well as a suitable solvent depends upon the polyphenolic structures being studied.

A classical stationary phase of silica gel (precoated plates) is widely used [108–113,131,132] to separate more apolar flavonoids such as flavonols and isoflavonoids [81]. Samples are eluted with different mobile phases: ethanol/water (55:45, v/v) [110,112], petroleum ether/ethyl acetate (70:30) [108], petroleum ether/acetone/formic acid (35:10:5) [132], chloroform/ethyl acetate (60:40) [113], toluene/chloroform/acetone (40:25:35) [111], *n*-hexane/ethyl acetate/acetic acid (31:14:5) [111,132,133] or (60:40:3) [111] and chloroform/methanol/formic acid (44.1:3:2.35) [131].

Medic-Saric et al. [133] used two-dimensional TLC with densitometric evaluation 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.

Visualization is performed in short- and long-wavelength UV light and in some cases spraying with different reagents. A common wavelength is 366 nm [108,110,112,132].

#### 3.4.2. Gas chromatography

GC determines phenolic compounds both qualitatively and quantitatively. It is usually necessary, however, to derivatize the compounds to make them suitable for GC analysis. During the last 10 years GC has been extensively employed by several researchers [103–105,108,124,134–147]. An alternative is high-temperature, high-resolution gas chromatography (HT-HRGC) [107,112,122,123,148], which is an established technique for separating complex mixtures and identifying high-molecular-weight compounds that do not elute when analysed on ordinary GC columns.

GC coupled with mass spectrometer (MS) is the method most widely used, since MS allows the acquisition of molecular mass data and structural information together with the identification of compounds. Propolis, however, contains components that are not volatile enough for direct GC–MS analysis even upon derivatization or HT–GC–MS [116]. Table 3 shows some GC temperature ranges used, characteristics of the column employed, analysis time, detection system, type of derivatization, compounds identified and several observations about a few notable published works.

#### 3.4.3. Liquid chromatography and high-performance liquid chromatography

High-performance liquid chromatography (HPLC) currently represents the most popular and reliable analytical technique for the characterization of polyphenolic compounds, as witnessed by the number of papers published on the subject. HPLC coupled to MS, and even to nuclear magnetic resonance spectroscopy (RMN), has improved the analysis of non-volatile species and allows us to establish definitive structures [149].

Electrospray ionisation (ESI) [150] permits the direct ionisation and transference of molecules to mass spectrometers and has extended the applicability of MS for a variety of new classes of molecules with thermal instability, high polarity and high mass.

Mirodikawa et al. [120] have established a suitable LC–MS method for the determination of the chemical constituents and therefore the quality of propolis.

Several authors have analysed polyphenolic compounds in propolis of diverse origins using different HPLC methods with different extraction systems and coupling diverse detector systems [62,63,100,106,109,110,112,113,115,117,119,126,134,146,151–157]. Table 4 summarises the information provided by some representative papers and gives the characteristics of the column employed, the mobile phases, the type of elution employed, the detection and extraction systems used, compounds identified and several pertinent observations.

### 3.5. Capillary electrophoresis analysis of phenolic compound in propolis

Because of the previously mentioned characteristics of capillary electrophoresis, this technique could well prove to be an interesting choice for the analysis of phenolic compounds. Nevertheless, to our knowledge, there are few reports about its use

Table 3
GC conditions for determination of phenolic compounds in propolis

| Temperature range (°C) | Column | Analysis time (min) | Detection | Derivatization | Identified compounds | Observations | Reference |
|---|---|---|---|---|---|---|---|
| 85–310 | DB1 column (30 m × 0.32 mm i.d.) | 85 | MS | Pyridine + BSTFA | Pinostrobin chalcone, hexamethoxy flavone, pinostrobin, pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin, naringenin, dihydrocinnamic acid, cinnamic acid, *p*-coumaric acid, isoferulic acid, ferulic acid, caffeic acid | Composition and activities of Egyptian propolis | [103,104] |
| 100–310 | HP5-MS capillary column (23 m × 0.25 mm i.d.), 0.5 mm film thickness | 42 | MS | Pyridine + BSTFA | Pinocembrin, pinobanksin, pinobanksin *O*-acetate, chrysin, galangin, pentenyl caffeates, benzyl caffeates, phenethyl caffeate | Composition of European propolis | [105] |
| 100–310 | HP5-MS capillary column (23 m × 0.25 mm i.d.), 0.5 μm film thickness | 42 | MS | Pyridine + BSTFA | Cinnamic acid, benzyl cinnamate, cinnamyl cinnamate, pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin, phenylethyl caffeate, cinnamyl caffeate, vanillin, *p*-coumaric acid, ferulic acid, caffeic acid, dehydroabietic acid | Composition and antibacterial activity of Turkish propolis TLC analysis too | [108] |
| 50–285 | HP1 methyl silicone capillary column (25 m × 0.25 mm i.d.) | 55 | MS | Methylation | Cinnamic acid, vanillin, ethyl cinnamate, vanillic acid, *p*-coumaric acid, ferulic acid, ethyl ferulate, 3-methylbut-2-enyl ferulate, 3-methylbut-3-enyl ferulate | Major organic constituents in New Zealand propolis. HPLC analysis too | [134] |
| 40–390 | Borosilicate capillary column (20 mm × 0.3 mm i.d.) coated with PS-086[a] ($d_f$ = 0.1 μm) connected to a 2 m piece of 0.25 mm i.d., high-temperature fused silica (which served as an interface) | 54 | MS | BSTFA | Ethyl hydrocinnamate, hydrocinnamic acid, inositol, cinnamic acid, ferulic acid, caffeic acid, pinostrobin | Composition and microbicidal activity of Brazilian and Bulgarian propolis | [107] |
| 50–285 | CBP5 column (30 m × 0.25 mm i.d.) | 55 | MS | Methylation | Coumaric acid, ferulic acid, pinobanksin, kaempferol, apigenin, isosakuranetin, pinocembrin, dimethylallyl caffeic acid, pinobanksin 3-acetate, chrysin, galangin, kaempferide, tectochrysin | Propolis and plant resins HPLC and TLC analysis too | [112] |
| 40–390 | Glass column (22 m × 0.2 mm i.d.) coated with PS-086[a] | 54 | FID MS | Trimethylsilylation: bis (trimethylsilyl)-trifluoro acetamide (BSTFA) | Inositol, *p*-cinnamic acid, ferulic acid, isoferulic acid, caffeic acid | Flavonoids in acetone no derivatizated, derivatization of methanol extract | [122] |
| 40–380[b] | Fused silica capillary (10 m × 0.3 mm i.d.) coated with 0.1 μm film of Silaren-30[c] | 55 | FID MS | No | Hydrocinnamic acid, vanillin, cinnamic acid, benzyl cinnamate, naringenin 3′,4′-dimethoxy, betuleol, kaempferid | Hexane and acetone crude extracts | [123] |
| 40–370[d] | | 50 | | | | | |

[a] 15% phenyl, 85% methyl polysiloxane.
[b] Program A.
[c] 30% diphenylpolysiloxane, 40% sildiphenylene ether, 30% dimethyl polysiloxane.
[d] Program B.

Table 4
HPLC conditions for determination of phenolic compounds in propolis

| Column | Mobile phase | Elution | Detection | Extraction system | Identified compounds | Observation | References |
|---|---|---|---|---|---|---|---|
| LiChrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290, 340 nm | In MeOH for 2 h at room temperature | Pinobanksin, quercetin, 8-methoxykaempferol, kaempferol, apigenin, isorhamnetin, quercetin 3,3′-dimethyl ether, pinocembrin, quercetin 7,3′-dimethyl ether, chrysin, galangin, techtochrysin | Flavonoids in rosemary nectar, honey and propolis | [62] |
| LiChrocart RP-18 (12.5 cm × 0.4 cm, 5 μm) | A: water:formic acid (19:1, v/v); B: methanol | Gradient | DAD λ = 290, 340 nm | In MeOH for 24h at room temperature, evaporated and redissolved in MeOH | Pinobanksin, pinocembrin, phenylethyl caffeate, pinobanksin 3-acetate, dimethylallyl caffeate, chrysin, galangin, myricetin 3,7,4′,5′-methyl ether, pinocembrin 7-methyl ether | Phenolics in Tunisian honey and propolis | [63] |
| Spherisorb ODS-2 (25 cm × 0.7 cm, 5 μm) | Methanol:water (58:42, v/v) | Isocratic | UV, EIMS, NMR | In a Soxhlet apparatus with MeOH, centrifuged, evaporated, clean-up | Myricetin 3,7,4′,5′-methyl ether, pachypodol | One fraction of the chromatographed sample is purified by semipreparative HPLC | [63] |
| Symmetry $C_{18}$ column (22 cm × 0.46 cm, 5 μm) | A: 30 mM $NaH_2PO_4$ (pH 3); B: acetonitrile | Gradient | PAD λ = 265, 290, 360 nm | Commercial preparations diluted in MeOH | 3,4-Dihydroxy-cinnamic acid, 4-hydroxy-cinnamic acid, 3-hydroxy-cinnamic acid, quercetin, kaempferol, galangin, naringenin, pinocembrin, chrysin | Quality control of commercial propolis APCI mass spectra obtained | [100] |
| Intersil 5 ODS-2 column (25 cm × 0.46 cm i.d.) with a Chromosphere ODS guard column (1 cm × 0.3 cm i.d.) | A: water: acetic acid (95:5, v/v); B: methanol | Gradient | UV λ = 290 nm | In 70% EtOH for 24 h at room temperature (×2) | Flavones and flavonols, flavanones and dihydroflavonols and total phenolics (caffeic acid, *p*-coumaric acid, ferulic acid, kaempferol, pinocembrin, phenethyl caffeate, isopentyl caffeate, chrysin, galangin, pinostrobin, benzyl caffeate) | Active constituents of poplar-type propolis; verification of the spectrophotometric quantification results. | [106] |
| YMC Pack ODS-A (RP) | Acetic acid:methanol:water (5:75:60, v/v/v) | Isocratic | DAD λ = 254 nm | In 80% EtOH for 30 min at 70 °C, centrifuged | Quercetin, kaempferol, apigenin, isorhamnetin, rhamnetin, pinocembrin, sakuranetin, isosakuranetin, chrysin, acacetin, galangin, kaempferide, tectochrysin | Antimicrobial activity of Brazilian propolis; TLC analysis too | [110] |
| YMC Pack ODS-A RP-18 (25 cm × 0.46 cm, 5 μm) | A: water; B: methanol | Gradient | DAD λ = 268 nm | In 80% EtOH for 30 min at 70 °C, centrifuged | Coumaric acid, ferulic acid, pinobanksin, kaempferol, apigenin, isosakuranetin, pinocembrin, dimethylallyl caffeic acid, pinobanksin 3-acetate, chrysin, galangin, kaempferide, techtochrysin | Botanical origin and composition of Brazilian propolis; TLC, GC analysis too | [112] |
| ODS column (25 cm × 0.4 cm i.d., 5 μm) | A: $H_2O$:0.1% $H_3PO_4$; B: $CH_3CN$:0.1% $H_3PO_4$ | Gradient | UV λ = 254 nm | Aqueous-ethanolic extract and partition between inmiscible solvents | Evidenced the presence of phenolic compounds by the intense fluorescence | Antibacterial activity of Brazilian propolis. TLC analysis too | [113] |
| Superspher 100 RP-18 (12.5 cm × 0.4 cm, 4 μm) | A: Methanol:acetic acid, 1 M (50:50); B: Methanol:acetic acid, 1 M (40:60); C: acetonitrile | Gradient | UV λ = 254 nm | In 95% EtOH for 7 days at room temperature, centrifuged, evaporated and redissolved | Pinocembrin, galangin | Activity against *Streptococcus pyogenes* of Italian propolis | [115] |

Table 4 (*Continued*).

| Column | Mobile phase | Elution | Detection | Extraction system | Identified compounds | Observation | References |
|---|---|---|---|---|---|---|---|
| YMC Pack ODS-A (RP) | Acetic acid:methanol:water (5:75:60, v/v/v) | Isocratic | DAD λ = 254 nm | In 10–95% EtOH for 30 min at 70 °C, centrifuged; in water too | Isosakuranetin, sakuranetin, quercetin, kaempferol, pinocembrinm, kaempferide, acacetin, isorhamnetin | Evaluation of the preparations to see which have maximum absorption | [119] |
| Capcell Pak ACR 120 $C_{18}$ column (25 cm × 0.2 cm i.d., 5 μm) | A: 0.1% formic acid:water; B: 0.08% formic acid:acetonitrile | Gradient | PAD λ = 195–650 nm MS (ESI) | In EtOH for 24 h at room temperature, centrifuged | Caffeic acid, *p*-coumaric acid, 3,4-dimethoxycinnamic acid, quercetin, pinobansin 5-methyl ether, apigenin, kaempferol, pinobanksin, cinnamylideneacetic acid, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, phenethyl caffeate, tectochrysin, artepillin C | Antioxidant activity of propolis of various geographic origins | [126] |
| Capcell Pak ACR 120 $C_{18}$ column (25 cm × 0.2 cm i.d., 5 μm) | A: 0.1% formic acid:water; B: 0.1% formic acid:acetonitrile | Gradient | PDA λ = 195–650 nm MS (ESI) | In EtOH for 24 h at room temperature, centrifuged | Caffeic acid, *p*-coumaric acid, 3,4-dimethoxycinnamic acid, pinobansin 5-methyl ether, apigenin, kaempferol, pinobanksin, cinnamylideneacetic acid, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, phenethyl caffeate, cinnamyl caffeate, tectochrysin | Antioxidant activity of propolis from Korea; colorimetric measurements too | [117] |
| LiChrospher 100 RP-18 (11.9 cm × 0.4 cm, 5 μm) | (1) A: formic acid; B: methanol; (2) A′: $H_3PO_4$ (pH 2.0); B′: MeCN | Gradient | PAD λ = 268 nm | Samples supplied as ethanolic tinctures | Cinnamic acid, pinobanksin, pinocembrin, pinobanksin 3-acetate, 1,1′-dimethylallylcaffeic acid, chrysin, galangin, pinocembrin 7-methyl ether, chrysin 7-methyl ether, galangin 7-methyl ether | Major organic constituents in New Zealand propolis; GC–MS analysis too | [135] |
| YMC PACK ODS column (25 cm × 2 cm) | 0.1% trifluoroacetic acid in $CH_3CN$:$H_2O$ (6:4) | Isocratic | UV, MS, 2D NMR | In EtOH for 12 h at room temperature, concentrated | Isonymphaeol-B, nymphaeol-A, nymphaeol-B, nymphaeol-C | New prenylflavonoid isolated from propolis from Okinawa; structure determined; extract previously chromatographed | [149] |
| Chromsep RP-18 (25 cm × 0.46 cm i.d., 5 μm) | A: methanol; B: water:acetonitrile (97.5:2.5, v/v) | Gradient | UV λ = 310 nm | – | Chrysin and others | Establishing ideal conditions for analysis | [155] |

Table 5
CE conditions for determination of phenolic compounds in propolis

| Instrumental variables | | | | | | Experimental variables | | | Identified compounds | References |
|---|---|---|---|---|---|---|---|---|---|---|
| $L_{ef}$ (cm) | i.d. (μm) | $\lambda_d$ (nm) | $V$ (kV) | $T$ (°C) | $t_{inj}$ (s) | Type of buffer | [Buffer] (mM) | pH | | |
| 56 | 50 | 200 (DAD) | 30 | 25 | 2 | (a) Sodium phosphate | 25 | (a) 7 | (a) 3,4-Dimethoxycinnamic acid, *p*-coumaric acid, cinnamic acid, benzoic acid | [102] |
| | | | | | | (b) Sodium borate | | (b) 9.3 | (b) Methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, *p*-coumaric acid, cinnamic acid, benzoic acid | |
| 50 | 75 | 214 | 18 | 25 | 4 | Borate + 0.5%MeOH | 100 | 9.5 | Rutin, chrysin, myricetin, kaempferol, hesperetin, daidzein, genistein, apigenin, quercitrin, luteolin, galangin | [114] |
| 50 | 75 | 262 | 23 | 25 | 12 | $H_3BO_3$–$Na_2B_4O_7$ | 40–60 | 9.2 | Rutin, ferulic acid, apigenin, luteolin, quercetin, caffeic acid | [121] |
| 50 | 50 | 254 | 15 | 25 | – | Sodium tetraborate | 30 | 9 | Pinocembrin, acacetin, chrysin, catechin, naringenin, galangin, luteolin, kaempferol, apigenin, myricetin, quercetin, cinnamic acid, caffeic acid, resveratrol | [125] |
| 56 | 50 | 200 (DAD) | 30 | 25 | 2 | Borate/SDS + 10% (v/v) acetonitrile | 25/50 | 9.3 | Pinocembrin, chrysin, galangin | [102] |
| 50 | 75 | 214 | −15 | 25 | 4 | Sodium borate/SDS | 30/50 | 8.5 | Unsatisfactory conditions in the separation of some flavonoids | [114] |
| 55 | 50 | 214 | 14 | – | 8 | Borax/SDS + 5% (v/v) EtOH | 30/12 | 9 | Hesperetin, cinnamic acid, nicotinic acid | [158] |

$L_{ef}$, effective length of capillary; i.d., internal diameter of capillary; $\lambda_d$, wavelength of detection; $V$, voltage; $T$, temperature; $t_{inj}$, injection time; [Buffer] buffer concentration.

with propolis and so far its applications are basically on the determination of flavonoids.

Different modes of operation are applicable with CE. Capillary zone electrophoresis (CZE) [102,114,121,125] is based on differences in the electrophoretic mobility of compounds caused by their charge and size. Micellar electrokinetic chromatography (MEKC) [102,114,141,158], in which surfactants such as sodium dodecyl sulphate (SDS) are added to the separation buffer, is also capable of separating neutral compounds.

The effects of some of the variables, such as buffer pH, buffer concentration, separation voltage and injection time, are studied in order to optimize the analytical conditions [114,121,158]. A summary of the optimized conditions of CE methods is provided in Table 5.

As flavonoids are weakly acidic their separation requires a buffer of pH > 10 to be successful. Chi et al. [99] determined flavonoids and phenolic acids in propolis by CZE using a buffer with pH 10.1. Nonetheless, important flavonoids such as myricetin and quercetin, for instance, may decompose in such an alkaline medium [50]. Therefore, if possible MEKC is used for the determination of flavonoids in natural samples [102].

## 4. Conclusions

The quality of honey and propolis depends on its chemical composition and floral origin. Their polyphenolic content is strongly affected by the floral, geographical origin and climatic characteristic of the site. For this reasons, the identification and quantification of the polyphenols of honey and propolis are of great interest.

Furthermore, they have a very important antioxidant capacity that is provided by polyphenols such as flavonoids and phenolic acids. These antioxidants report beneficial effects in human health. It has been commented that consumption of these bee products contributes to the treatment of stomach ulcer, sore throat and wounds and burns. Numerous studies have proven their versatile pharmacological activities: antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant, antitumor, etc.

As a result, many analytical procedures have been carried out directed towards the determination of the complete phenolic profile of honey and propolis. The techniques employed in the last years have been GC, HPLC and CE, mainly combined with diode array detection and mass spectrometry

## Acknowledgements

The authors gratefully acknowledge contract C019 of the Consejería de Agricultura y Pesca of the Junta de Andalucía. They also gratefully acknowledge the financial support of Project CTQ2005-01914/BQU and the Ministerio de Educación y Ciencia for the grant AP-2004-5488.

## References

[1] E. Ibáñez, A. Cifuentes, Crit. Rev. Food Sci. 41 (2000) 413–450.

[2] F.J. Señorans, E. Ibáñez, A. Cifuentes, Crit. Rev. Food Sci. 43 (2003) 507–526.
[3] I. Goldberg, Fuctional Foods. Designer Foods, Pharmafood, Nutraceuticals, Chapman and Hall, London, UK, 1996.
[4] M. Herrero, E. Ibáñez, A. Cifuentes, J. Sep. Sci. 28 (2005) 883–897.
[5] D.L. Madhavi, R.S. Singhai, P.R. Kulkarni, in: D.L. Madhavi, S.S. Deshpande, D.K. Salunkhe (Eds.), Food Antioxidants, Marcel Dekker, New York, 1996.
[6] J. Wollgast, E. Anklam, Food Res. Int. 33 (2002) 423–447.
[7] D.V. Madhavi, S.S. Despande, D.K. Salunkhe, in: D.L. Madhavi, S.S. Deshpande, D.K. Salunkhe (Eds.), Food Antioxidants, Marcel Dekker, New York, 1996.
[8] L. Bravo, Nutr. Rev. 56 (1998) 317–333.
[9] A.L. Catapano, Angiology 48 (1997) 39–44.
[10] K. Loku, T. Tsushida, Y. Takei, N. Nakatani, J. Terao, Biochim. Biophys. Acta 1234 (1995) 99–104.
[11] N. Salah, N.J. Millar, G. Paganda, L. Tijburg, G.P. Bolwell, C. Rice-Evans, Arch. Biochem. Biophys. 322 (1995) 339–346.
[12] M. Serafín, A. Ghiselli, A. Ferro-Luzzi, Eur. J. Clin. Nutr. 50 (1996) 28–32.
[13] J.A. Vinson, Y. Hao, X. Su, L. Zubik, J. Agric. Food Chem. 46 (1998) 3630–3634.
[14] FAO, FOA Agricultural Services Bulletin, FAO, Rome, Italy, 1996.
[15] T. Cherbuliez, The Medicine from the Bees, CD-ROM, Apimondia, 2001.
[16] T. Cherbuliez, R. Domerego, Médecine des Abeilles, editions Amyris, 2003.
[17] A. Meda, C.E. Lamien, M. Romito, J. Millogo, O.G. Nacoulma, Food Chem. 91 (2005) 571–577.
[18] S.M. Anthony, J.R. Rieck, P.L. Dawson, Poult. Sci. 79 (2000) 1846–1850.
[19] J. McKibben, N.J. Engeseth, J. Agric. Food Chem. 50 (2002) 592–595.
[20] J. Oszmianski, C.Y. Lee, J. Agric. Food Chem. 38 (1990) 1892–1898.
[21] M.R. McLellan, R.W. Kime, C.Y. Lee, T.M. Long, J. Food Process. Preserv. 19 (1995) 1–8.
[22] L. Chen, A. Mehta, M. Berenbaum, A.R. Zangerl, N.J. Engeseth, J. Agric. Food Chem. 48 (2000) 4997–5000.
[23] N. Gheldof, N.J. Engeseth, J. Agric. Food Chem. 50 (2002) 3050–3055.
[24] S. Frankel, G.E. Robinson, M.R. Berenbaum, J. Apic. Res. 37 (1998) 27–31.
[25] J.W. White, in: E. Crane (Ed.), Honey. A Comprehensive Survey, Heinemann, London, 1975, pp. 157–207.
[26] K. Inoue, S. Murayama, F. Seshimo, K. Takeba, Y. Yoshimura, H. Nakazawa, J. Sci. Food Agric. 85 (2005) 872–878.
[27] I.E. Haffeejee, A. Moosa, BMJ 290 (1985) 1886–1887.
[28] S.P. Ladas, D.N. Haritos, S.A. Raptis, Am. J. Clin. Nutr. 62 (1995) 1212–1215.
[29] S.E. Efem, Br. J. Surg. 75 (1988) 679–681.
[30] M. Subrahmanyam, Br. J. Surg. 78 (1991) 497–498.
[31] K.L. Allen, P.C. Molan, G.M. Reid, J. Pharm. Pharmacol. 43 (1991) 817–822.
[32] A.T. Ali, M.N. Choudhury, M.S. Al-Humayyd, Trop. Gastroenterol. 12 (1991) 73–77.
[33] A.T. Ali, Scad. J. Grastoenterol. 26 (1991) 281–288.
[34] A.T. Ali, Trop. Gastroenterol. 16 (1995) 18–26.
[35] S.M. Antony, I.Y. Han, J.R. Rieck, P.L. Dawson, J. Agric. Food Chem. 48 (2000) 3985–3989.
[36] P. Vit, C. Soler, F.A. Tomás-Barberán, Z. Lebensm, Unters. Forsch. A 204 (1997) 43–47.
[37] A. Cherchi, L. Spanedda, C. Tuberoso, P. Cabras, J. Chromatogr. A 669 (1994) 59–64.
[38] A.M.C. Davies, R.G. Harris, J. Apic. Res. 21 (1982) 168–173.
[39] J.W. White, J. Apic. Res. 17 (1978) 234–238.
[40] N.C. Cook, S. Sammon, Nutr. Biochem. 7 (1996) 66–76.
[41] P. Andrade, F. Ferreres, M.T. Amaral, J. Liq. Chromatogr. Relat. Tech. 20 (1997) 2281–2288.
[42] S.T. Tan, A.L. Wilkins, P.T. Holland, T.K. McGhie, J. Agric. Food Chem. 37 (1989) 1217–1221.
[43] L. Yao, N. Datta, F.A. Tomás-Barberán, F. Ferreres, I. Martos, R. Singanusong, Food Chem. 81 (2003) 159–168.
[44] M.J. Amoit, S. Aubert, M. Gonnet, M. Tacchini, Apidologie 20 (1989) 115–125.
[45] A.M.C. Davies, J. Food Technol. 11 (1976) 515–523.
[46] F. Ferreres, A. Ortiz, C. Silva, C. Garcia-Viguera, F.A. Tomás-Barberán, F. Tomás-Lorente, Z. Lebensm, Unters. Forsch. 194 (1992) 139–143.
[47] S. Sabatier, M.J. Amoit, M. Tacchini, S. Aubert, J. Food Sci. 57 (1992) 733–734.
[48] F.A. Tomás-Barberán, F. Ferreres, C. García-Viguera, F. Tomás-Lorente, Z. Lebensm, Unters. Forsch. 196 (1993) 38–44.
[49] F.A. Tomás-Barberán, C. García-Viguera, P. Vit-Olivier, F. Ferreres, F. Tomás-Lorente, Phytochemistry 34 (1993) 191–196.
[50] F. Ferreres, M.A. Blázquez, M.I. Gil, F.A. Tomás-Barberán, J. Chromatogr. A 669 (1994) 268–274.
[51] F. Ferreres, C. García-Viguera, F. Tomás-Lorente, F.A. Tomás-Barberán, J. Sci. Food Agric. 61 (1993) 121–123.
[52] F. Ferreres, J.M. Giner, F.A. Tomás-Barberán, J. Sci. Food Agric. 65 (1994) 371–372.
[53] F. Ferreres, T. Juan, C. Perez-Arquillue, A. Herrera-Marteache, C. Garcia-Viguera, F.A. Tomás-Barberán, J. Sci. Food Agric. 77 (1998) 506–510.
[54] F.A. Tomás-Barberán, I. Martos, F. Ferreres, B.S. Radovic, E. Anklam, J. Sci. Food Agric. 81 (2001) 485–496.
[55] F. Ferreres, P. Andrade, M.I. Gil, F.A. Tomás-Barberán, Z. Lebensm, Unters. Forsch. 202 (1996) 40–44.
[56] F. Ferreres, P. Andrade, F.A. Tomás-Barberán, J. Agric. Food Chem. 44 (1996) 2053–2056.
[57] The National Honey Board, 2003. 390 Lashley Street Longmont, http://www.nhb.org/.
[58] M.J. Snow, M. Manley-Harris, Food Chem. 84 (2004) 145–147.
[59] D. Tura, K. Robards, J. Chromatogr. A 975 (2002) 71–93.
[60] F.A. Tomás-Barberán, M.A. Blázquez, C. García-Viguera, F. Ferreres, F. Tomás-Lorente, Phytochem. Anal. 3 (1992) 178–181.
[61] F. Ferreres, P. Andrade, F.A. Tomás-Barberán, Z. Lebensm, Unters. Forsch. 199 (1994) 32–37.
[62] M.I. Gil, F. Ferreres, A. Ortiz, E. Subra, F.A. Tomás-Barberán, J. Agric. Food Chem. 43 (1995) 2833–2838.
[63] I. Martos, M. Cossentini, F. Ferreres, F.A. Tomás-Barberán, J. Agric. Food Chem. 45 (1997) 2824–2829.
[64] P. Vit, F.A. Tomás-Barberán, Z. Lebensm, Unters. Forsch. A 206 (1998) 288–293.
[65] R.J. Weston, K.R. Mitchell, K.L. Allen, Food Chem. 64 (1999) 295–301.
[66] I. Martos, F. Ferreres, F.A. Tomás-Barberán, J. Agric. Food Chem. 48 (2000) 1498–1502.
[67] I. Martos, F. Ferreres, L. Yao, B. D'Arcy, N. Caffin, F.A. Tomás-Barberán, J. Agric. Food Chem. 48 (2000) 4744–4748.
[68] L. Yao, Y. Jiang, R. Singanusong, N. Datta, K. Raymont, Food Chem. 86 (2004) 169–177.
[69] L. Yao, Y. Jiang, R. Singanusong, B. D'Arcy, N. Datta, N. Caffin, K. Raymont, Food Res. Int. 37 (2004) 166–174.
[70] L. Yao, Y. Jiang, B. D'Arcy, R. Singanusong, N. Datta, N. Caffin, K. Raymont, J. Agric. Food Chem. 52 (2004) 210–214.
[71] L. Yao, Y. Jiang, R. Singanusong, N. Datta, K. Raymont, Food Res. Int. 38 (2005) 651–658.
[72] F. Ferreres, F.A. Tomás-Barberán, M.I. Gil, F. Tomás-Lorente, J. Sci. Food Agric. 56 (1991) 49–56.
[73] P. Andrade, F. Ferreres, M.I. Gil, F.A. Tomás-Barberán, Food Chem. 60 (1997) 79–84.
[74] F. Ferreres, F.A. Tomás-Barberan, C. Soler, Apidologie 25 (1994) 21–30.
[75] A.M. Aljadi, M.Y. Kamaruddin, Food Chem. 85 (2004) 513–518.
[76] A.M. Aljadi, K.M. Yusoff, Turk. J. Med. Sci. 33 (2003) 229–236.
[77] H.A. Wahdan, Infection 26 (1998) 167–176.
[78] P. Cabras, A. Angioni, C. Tuberoso, I. Froris, F. Reniero, C. Guillou, S. Ghelli, J. Agric. Food Chem. 47 (1999) 4064–4067.

[79] M. Al-Mamary, A. Al-Meeri, M. Al-Habori, Nutr. Res. 22 (2002) 1041–1047.
[80] G. Beretta, P. Granata, M. Ferrero, M. Orioli, R.M. Facino, Anal. Chim. Acta 533 (2005) 185–191.
[81] A. Escarpa, M.C. González, Anal. Chim. Acta 427 (2001) 119–127.
[82] V.L. Singleton, R. Orthofer, R.M. Lamuela-Reventós, Methods Enzymol. 299 (1999) 152–178.
[83] J.A. Vinson, J. Proch, P. Bose, Methods Enzymol. 335 (2001) 103–114.
[84] T. Berahia, C. Cerrato, S. Sabatier, M.J. Amiot, Sci. des aliments 13 (1993) 15–24.
[85] H. Long, Y.X. Zhu, P.T. Kissinger, Chin. J. Anal. Chem. 31 (2003) 631–634.
[86] H. Long, Y.X. Zhu, L.A. Coury, C.T. Duda, C.B. Kissinger, P.T. Kissinger, LC GC North Am. Suppl. (2002) 61–64.
[87] E. Jorg, G. Sontag, Deutsche Lebensmittel-Rundschau 88 (1992) 179–183.
[88] C. Delgado, F.A. Tomás-Barberán, T. Talou, A. Gaset, Chromatographia 38 (1994) 71–78.
[89] E. Ghisalberti, Bee World 60 (1979) 59–84.
[90] V. Bankova, S.L. De Castro, M.C. Marucci, Apidologie 31 (2000) 3–15.
[91] M.C. Marcucci, Apidologie 26 (1995) 83–99.
[92] G.A. Burdock, Food Chem. Toxicol. 36 (1998) 347–363.
[93] H. Koo, P.L. Rosalen, J.A. Cury, Y.K. Park, M. Ikegaki, A. Sattler, Caries Re. 33 (1999) 393–400.
[94] S. Castaldo, F. Capasso, Fititoterapia 73 (2002) S1–S6.
[95] V. Bankova, eCAM 2 (2005) 29–32.
[96] B. Konig, Bee World 66 (1985) 136–139.
[97] J.W. Dobrowolski, S.B. Vohora, S.A. Kalpana, J. Ethnopharmacol. 35 (1991) 77–82.
[98] B.H. Havsteen, Pharmacol. Ther. 96 (2002) 67–202.
[99] H. Chi, A.K. Hsieh, C.L. Ng, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 680 (1994) 593–597.
[100] P.G. Pietta, C. Gordana, A.M. Pietta, Fitoterapia 73 (2002) S7–S20.
[101] V. Bankova, R. Christov, G. Stoev, S. Popov, J. Chromatogr. 607 (1992) 150–153.
[102] M.J. Hilhorst, G.W. Somsen, G.J. de Jong, J. High Resolut. Chromatogr. 21 (1998) 608–612.
[103] F.K. Abd El Hady, A.H. Hegazi, Z. Naturforsch. 57c (2002) 386–394.
[104] A.G. Hegazi, F.K. Abd El Hady, Z. Naturforsch. 57c (2002) 395–402.
[105] V. Bankova, M. Popova, S. Bogdanov, A.G. Sabatini, Z. Naturforsch. 57c (2002) 530–533.
[106] M. Popova, V. Bankova, D. Butovska, V. Petkov, B. Nikolova-Damyanova, A.G. Sabatini, G.L. Marcazzan, S. Bogdanov, Phytochem. Anal. 15 (2004) 235–240.
[107] K. Salomão, A.P. Dantas, C.M. Borba, L.C. Campos, D.G. Machado, F.R. Aquino Neto, S.L. de Castro, Lett. Appl. Microbiol. 38 (2004) 87–92.
[108] M. Popova, S. Silici, O. Kaftanoglu, V. Bankova, Phytomedicine 112 (2005) 221–228.
[109] Y.K. Park, M.H. Koo, M. Ikegaki, J.L. Contado, Arquivos Biol. Tecnol. 40 (1997) 97–106.
[110] Y.K. Park, M.H. Koo, J.A.S. Abreu, M. Ikegaki, J.A. Cury, P.L. Rosalen, Curr. Microbiol. 36 (1998) 24–28.
[111] M.I.N. Moreno, M.I. Isla, A.R. Sampietro, M.A. Vattuone, J. Ethnopharmacol. 71 (2000) 109–114.
[112] Y.K. Park, S.M. Alencar, C.L. Aguiar, J. Agric. Food Chem 50 (2002) 2502–2506.
[113] F.A. Santos, E.M.A. Bastos, M. Uzeda, M.A.R. Carvalho, L.M. Farias, E.S.A. Moreira, F.C. Braga, J. Ethnopharmacol. 80 (2002) 1–7.
[114] S.H. Fu, M.H. Yang, H.M. Wen, J.C. Chern, J. Food Drug Anal. 13 (1) (2005) 43–50.
[115] K. Bosio, C. Avanzini, A. D'Avolio, O. Ozino, D. Savoia, Lett. Appl. Mricrobiol. 31 (2000) 174–177.
[116] A.C.H.F. Sawaya, D.M. Tomazela, I.B.S. Cunha, V.S. Bankova, M.C. Marcucci, A.R. Custodio, M.N. Eberlin, Analyst 129 (2004) 739–744.
[117] M.R. Ahn, S. Kumazawa, T. Hamasaka, K.S. Bang, T. Nakayama, J. Agric. Food Chem. 52 (2004) 7286–7292.
[118] R.G. Woisky, A. Salatino, J. Apicult. Res. 37 (1998) 99–105.
[119] Y.K. Park, M. Ikegaki, Biosci. Biotechnol. Biochem. 62 (1998) 2230–2232.
[120] K. Mirodikawa, A.H. Banskota, Y. Tezuca, T. Nagaoka, K. Matsushige, D. Message, A.A.G. Huertas, S. Kadota, Phytochem. Anal. 12 (2001) 366–373.
[121] Y.H. Cao, Y. Wang, Q. Yuan, Chromatographia 59 (2004) 135–140.
[122] A.D. Pereira, M. Norsell, J.N. Cardoso, F.R.D. Neto, J. Agric. Food Chem. 48 (2000) 5226–5230.
[123] A.D. Pereira, A.C. Pinto, J.N. Cardoso, F.R.D. Neto, H.F.S. Ramos, G.M. Dellamora-Ortiz, E.P. dos Santos, J. High Resolut. Chromatogr. 21 (1998) 396–400.
[124] G. Negri, M.L.F. Salatino, A. Salatino, J. Apic. Res. 42 (4) (2003) 53–56.
[125] N. Volpi, Electrophoresis 25 (2004) 1872–1878.
[126] S. Kumazawa, T. Hamasaka, T. Nakayama, Food Chem. 84 (2004) 329–339.
[127] J.S. Bonvehi, F.V. Coll, R.E. Jorda, J. Am. Oil Chem. 71 (1994) 529–532.
[128] M. Nagy, D. Grancai, Pharmazie 51 (1996) 100–101.
[129] C.C. Chang, M.H. Yang, H.M. Wen, J.C. Chern, J. Food Drug Anal. 10 (2002) 178–182.
[130] P.G. Waterman, S. Mole, Analysis of Phenolic Plant Metabolites, Blackwell Scientific, Cambridge, MA, 1994.
[131] I. Jasprica, A. Smolcic-Bubalo, A. Mornar, M. Medic-Saric, J. Planar Chromatogr. 17 (2004) 95–101.
[132] M. Medic-Saric, L. Jasprica, A. Smolcic-Bubalo, A. Mornar, Croatica Chem. A 77 (2004) 361–366.
[133] M. Medic-Saric, L. Jasprica, A. Mornar, A. Smolcic-Bubalo, P. Golja, J. Planar Chromatogr. 17 (2004) 459–463.
[134] V. Bankova, R. Christov, A. Kujumgiev, M.C. Marcucci, S. Popova, Z. Naturforsch. 50c (1995) 167–172.
[135] K.R. Markham, K.A. Mitchell, A.L. Wilkins, J.A. Daldy, Y. Lu, Phytochemistry 42 (1996) 205–211.
[136] M.C. Marcucci, J. Rodríguez, F. Ferreres, V. Bancova, R. Groto, S. Popov, Z. Naturforsch. 53c (1998) 117–119.
[137] R. Christov, V. Bancova, A. Hegazi, F. Abd El Hady, S. Popov, Z. Naturforsch. 53c (1998) 197–200.
[138] V. Bankova, R. Christov, C. Marcucci, S. Popov, Z. Naturforsch. 53c (1998) 402–406.
[139] V. Bankova, G. Boudourova-Krasteva, S. Popov, J.M. Sforcin, S.R.C. Funari, Apidologie 29 (1998) 361–367.
[140] W. Maciejewicz, T. Meresta, Bull. Vet. Inst. Pulawy 41 (1999) 71–76.
[141] J.D. Fontana, M. Passos, M.H.R. dos Santos, C.K. Fontana, B.H. Oliveira, L. Schause, R. Pontarolo, M.A. Barbirato, M.A. Ruggiero, F.M. Lancas, Chromatographia 52 (2000) 147–151.
[142] A.G. Hegazi, F.K. Abd El Hady, F.A.M. Abd Allah, Z. Naturforsch. 55c (2000) 70–75.
[143] W. Maciejewicz, M. Daniewski, K. Bal, W. Markowski, Chromatographia 53 (2001) 343–346.
[144] W. Maciejewicz, J. Liq. Chromatogr. Relat. Tech. 24 (2001) 1171–1179.
[145] N. Keskin, S. Hazir, K.H.C. Baser, M. Kurkcuoglu, Z. Naturforsch. 56c (2001) 1112–1115.
[146] W. Maciejewicz, M. Daniewski, T.H. Dzido, K. Bal, Chem. Anal. 47 (2002) 21–30.
[147] G. Negri, M.L.F. Salatino, A. Salatino, J. Apic. Res. 42 (2003) 39–41.
[148] A.D. Pereira, H.F.D. Ramos, E.S.C. Pocas, P.C.M. Dias, E.P. dos Santos, J.F.M. da Silva, J.N. Cardoso, F.R.D. Neto, Z. Naturforsch. 54c (1999) 395–400.
[149] S. Kumazawa, H. Goto, T. Hamasaka, S. Fukumoto, T. Fujimoto, T. Nakayama, Biosc. Biotechnol. Biochem. 68 (2004) 260–262.
[150] R.B. Cole, Electrospray Ionisation Mass Spectrometry, John Wiley and Sons Inc., New York, 1997.
[151] Y.K. Park, M.H. Koo, H.H. Sato, J.L. Contado, Arquivos Biol. Technol. 38 (1995) 1253–1259.
[152] P. Menghinello, L. Cucchiarini, F. Palma, D. Agostini, M. Dacha, V. Stocchi, Arquivos Biol. Tecnol. 22 (1999) 3007–3018.

[153] M.C. Marcucci, F. Ferreres, A.R. Custodio, M.M.C. Ferreira, V.S. Bankova, C. Garcia-Viguera, W.A. Bretz, Z. Naturforsch. 55c (2000) 76–81.
[154] A.D. Pereira, A.F.D. Pereira, L.C. Trugo, F.R.D. Neto, Z. Naturforsch. 58c (2003) 590–593.
[155] M.L. Bruschi, S.L. Franco, M.P.D. Gremiao, J. Liq. Chromatogr. Relat. Tech. 26 (2003) 2399–2409.
[156] I.B.D.S. Cunha, K. Salomao, M. Shimizu, V.S. Bankova, A.R. Custodio, S.L. de Castro, M.C. Marcucci, Chem. Pharma. Bull. 52 (2004) 602–604.
[157] T. Hamasaka, S. Kumazawa, T.F. Fujimoto, T. Nakayama, Food Sci. Tech. Res. 10 (1) (2004) 86–92.
[158] Y. Lu, C. Wu, Z. Yuan, Fitoterapia 75 (2004) 267–276.