Vol. 13(19), pp. 487-500, December, 2019
DOI: 10.5897/JMPR2019.6849
Article Number: 62E626462356
ISSN 1996-0875

http://www.academicjournals.org/JMPR

ACADEMIC JOURNALS
expand your knowledge

Journal of Medicinal Plants Research

Review

Recent advances in analytical approaches for the standardization and quality of polyphenols of propolis

Fabio Galeotti[1], Federica Capitani[1], Alfredo Fachini[2] and Nicola Volpi[1]*

[1]Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 213/D, Modena, Italy.
[2]B Natural R&D Unit, Via Gran Sasso 33. Corbetta (Milano), Italy.

Received 25 September, 2019; Accepted 28 October, 2019

Analytical approaches utilized for the characterization of polyphenols from propolis useful for the determination of its quality is investigated in this study. A qualitative and quantitative evaluation of propolis bioactive molecules is of interest in medicine and nutraceuticals. Recent powerful analytical techniques are of great utility to separate and quantify polyphenols in extracts and finished products due to their capacity to produce typical fingerprints and a reliable identification of many components. According to this, an HPLC-UV-MS procedure was validated and applied for the characterization and quantification of bioactive substances in propolis and for an accurate assessment of their content in extract samples. By using this analytical approach, we obtained specific compositions related to brown propolis acquired from different geographic areas (and preparations and treatment). This is more important by considering the scientific opinion of European Food Safety Authority (EFSA) which provided a negative response related to health claims of propolis and its polyphenols. These results prove that HPLC-MS is an attractive tool for the standardization and quality control of propolis and may be realistically applied to screen raw material and to evaluate finished commercial preparations and nutraceutical benefits.

Key words: Propolis, polyphenols, flavonoids, phenolic acids, high-performance liquid chromatography-mass spectrometry (HPLC-MS), quality control, standardization

INTRODUCTION

Propolis is a resin-like material from the buds of poplar trees and is rarely available in its pure form. It contains bee products, and has a long history of medicinal use, dating back to 350 B.C. Propolis is collected by honeybees from various plants and trees, in particular from the poplar (*Populus*) genus but also from beech, horsechestnut, birch and conifer. This resinous material is mixed with wax and used in the construction and adaptation of bee nests (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011). In spite of possible differences in the collected resins from various plant sources and their active molecular composition, most raw propolis looks quite similar consisting of resin, wax, essential oils and minor components such as pollen and organic compounds (Volpi, 2004; Volpi and Bergonzini, 2006). Resin is one of the most active

*Corresponding author. E-mail: nicola.volpi@unimore.it Tel: 0039 (0)59 2055543. Fax: 0039 (0)59 2055548.

fraction of propolis constituted of the polyphenolic component comprising flavonoids and related phenolic acids (Salatino et al., 2011; Viuda-Martos et al., 2008). Bees use propolis for mechanical aims and for its biological properties. In fact, its antibacterial capacity is an important property also used by human beings for therapeutic applications (Castaldo and Capasso, 2002; Sforcin, 2007; Watanabe et al., 2011). Moreover, many studies focused the attention on other possible beneficial activities of propolis such as anti-inflammatory, antiviral, antiulcer, local anaesthetic, hepatoprotective and immune-stimulating (Banskota et al., 2001; Castaldo and Capasso, 2002; Sforcin, 2007; Viuda-Martos et al., 2008; Salatino et al., 2011; Watanabe et al., 2011).

For the above-mentioned biological activities, propolis has been largely used in folk medicine due to the presence of more than 300 identified components, and many of them are biologically active, such as several aromatic compounds, flavonoids, prenylated *p*-coumarinic acids, acetophenone derivatives, caffeoylquinic acids, lignans, diterpenic acids, triterpenes as well as volatile compounds (monoterpenes, sesquiterpenes and aromatic compounds), sugars and derivatives (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011; Pasupuleti et al., 2017). Polyphenols are the most representative biomolecules possessing at least one aromatic ring with one or more hydroxyl functional groups. Flavonoids represent the most abundant group of phenolic compounds having their structures based on a C6-C3-C6 skeleton (Figure 1A) and classified into several classes such as chalcones, flavones, flavonols, flavanones, isoflavonoids, anthocyanidins and flavanols (catechins and tannins). Non-flavonoids species are composed of simple phenols, phenolic acids, coumarins, xanthones, stilbenes, lignins and lignans. Phenolic acids are further classified in benzoic acid derivatives, formed of a C6-C1 skeleton, and cinnamic acid derivatives, constituted of a C6-C3 skeleton (Salatino et al., 2011) (Figure 1B). Besides the presence of many of the above-mentioned compounds, the variability of propolis chemical composition is more complicated by several glycoside phenolic derivatives making the analysis of these molecules a very hard challenge.

Current applications of propolis include preparations mainly based on extracts (ethanolic, water, glygeric, glycolic, oily or mix of this) and specific components may be selected during the extractive and preparative processes (Galeotti et al., 2017; Galeotti et al., 2018). These processes remove wax and other inert material and enrich the polyphenolic active compounds. In fact, commercial and nutraceutical health care products contain propolis solubilized in various solvents such as organic, water, oily or mix (Galeotti et al., 2018).

As mentioned above, propolis is a complex and heterogeneous material possessing a variable physical consistence, color, fragrance and active components depending on many factors such as types of vegetable sources, geographic origin, season of collection as well as the type of collecting bees (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011). Moreover, its biological properties and antibiotic activity are the result of completely different chemical composition and content of the actives. On this basis, many analytical approaches have been developed to analyse the raw propolis as well as the many derived finished products in an effort to standardize the collecting material, the derivatives generated during the preparative processes and the products dissolved in many solvents and matrices for commercial purposes. In particular, many analytical methods are available with the aim to standardize the polyphenol active propolis component by a chemical point of view as well as biological activity. Thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the most applied analytical methods. Moreover, the advent of mass detectors and soft ionization approaches such as electrospray ionization (ESI) combined with HPLC and further tandem mass spectrometry (MS/MS) has facilitated the study of polyphenols, their structural determination and their quantitation in low concentration (Cuyckens et al., 2001; Cuyckens and Claeys, 2004). Due to its powerful capacity to separate single components in complex mixtures and molecular structural identification, liquid chromatography (LC)-MS has gained large interest as a reliable and successful technique for the characterization of active compounds in biological products such as propolis.

For the utilization in the many healthcare finished products, propolis needs accurate standardization of its active compounds to assure quality, safety and efficacy to the consumers. Related to this, the aim of this paper is to present and discuss new recent analytical techniques useful for the determination of propolis polyphenols and of its quality with special attention to HPLC-MS characterization. This is more important by considering that in a recent scientific report (European Food Safety Authority - EFSA Journal, 2010), EFSA provided negative response to the health claims of propolis and its polyphenolic component. In fact, two main key points have been considered important by EFSA: A) propolis is a highly heterogeneous biological product and its active compounds polyphenols may change for composition and content also related to the different kind of extraction process, and B) there is no clear relationship between propolis structure/composition and health claims in the absence of its fine structural characterization.

SAMPLE PROPOLIS PREPARATION

As mentioned above, more than 200-250 compounds, mainly polyphenols and related derivatives may be present in propolis (Volpi, 2004; Volpi and Bergonzini, 2006).

Moreover, the content of polyphenols may vary depending on the origin and treatment of the raw samples and these differences are able to influence biological properties and clinical activities (Banskota et al., 2001; Viuda-Martos et al., 2008; Salatino et al., 2011). In fact, propolis cannot be used as raw material but it needs to be purified by using different extractive procedures with the aim to purify the active plant molecules from inert and potentially dangerous material for further analytical evaluation and/or biological assays. Treatment of propolis with ethanol, usually at concentrations of 70-80%, is largely used for its capacity to produce wax-free extracts and tinctures very rich in polyphenols (Banskota et al., 2001; Castaldo and Capasso, 2002). Treatments with pure water (able to enrich the final extracts with phenolic acids that are highly soluble in water), methanol, acetone, hexane, chloroform and others have also been applied to the aim to purify propolis (Popova et al., 2004: Gómez-Caravaca et al., 2006; Stalikas, 2007). The quality control of raw propolis is essential to evaluate the characteristics of this material and, as a consequence, it is more analyzed than commercial finished products. The preparation of crude extracts is performed by dehydrating the propolis that can be obtained in a fine powder. Then, a weighted sample, at a frequently used concentration of 10 mg/mL, is dissolved in the solvent and left for 24 h at room temperature or at 70°C for 2-3 h under occasional mixer (Volpi and Bergonzini, 2006). After filtering, the procedure is repeated several times to assure a complete recovery of the active propolis component. The insoluble material is eliminated by filtration or centrifugation and the solvent is then evaporated to dryness under low pressure to reduce the extracted volume (Galeotti et al., 2017; Galeotti et al., 2018). The obtained solution is directly tested for molecular composition and biological activity.

SPECTROPHOTOMETRIC ASSAYS OF POLYPHENOLS

The increasing utilization of food supplements and nutraceuticals based on propolis preparations requires the application of reliable and reproducible techniques for the quantitation of their active compounds. Spectrophotometric assays are especially useful for the routine control of propolis products. In particular, quantitative methods are available for the determination of total flavonoids or total phenolics but also for specific classes of polyphenols such as for flavanones/ dihydroflavonols or flavones/flavonols (Popova et al., 2004: Gómez-Caravaca et al., 2006) (Figure 1A). Other advantages of these methods are rapidity, simplicity, good repeatability, acceptable accuracy and low costs of the reagents and equipment. On the other hand, these assays are unable to give a specific, reliable and complete characterization of the active species as well as to evaluate their possible changes due to working processes or intentional adulteration (Popova et al., 2004: Gómez-Caravaca et al., 2006; Galeotti et al., 2017).

The total polyphenols content is generally determined by using the Folin-Ciocalteu assay, that is the most largely used spectrophotometric method utilized for the total quantification of these compounds (Popova et al., 2004). Quantitative assay of flavonoids in propolis is carried out by two different colorimetric approaches. Flavone and flavonol quantification is performed by using the aluminium chloride assay based on the formation of a complex between the ion Al(III) and carbonyl and hydroxyl groups of the flavonoid (Bonvehi and Coll, 1994). On the contrary, flavanones and dihydroflavonols interact with 2,4-dinitrophenylhydrazine (DNP) forming in acidic media phenylhydrazones that are spectrophotometrically quantified (Nagy and Grancai, 1996; Popova et al., 2004). Finally, the real content of total flavonoids is obtained by the sum of the results obtained by the two above assays.

CAPILLARY ELECTROPHORESIS (CE) SEPARATION OF PROPOLIS SAMPLES

Different CE separations are available such as the capillary zone electrophoresis (CZE) able to separate the various molecules on the basis of their charge and size. The micellar electrokinetic chromatography (MEKC) is capable of also separating neutral compounds by using a differential partitioning between a surfactant added to the separation buffer (Volpi, 2004). This last approach is useful for the analysis of flavonoids due to their weakly acidic nature that permits their separation in the presence of buffer having basic pH values. However, flavonoids may be degraded under alkaline conditions making CZE the most suitable CE approach for the separation of polyphenols of propolis and of other biological products (Gómez-Romero et al., 2007). In fact, twelve different flavonoids, two phenolic acids and one stilbene derivative, resveratrol, were separated and quantified by CZE by means of a sodium tetraborate buffer on an uncoated fused-silica capillary using normal polarity (Volpi, 2004).

CHROMATOGRAPHIC DETERMINATION OF THE PROPOLIS POLYPHENOLS

The complete characterization of the active compounds of propolis requires both their identification and quantitation. Chromatographic techniques such as gas chromatography (GC) and in particular high-performance liquid chromatography (HPLC), assure specific profile, identification and quantification of the total as well as individual polyphenolic species (Gómez-Caravaca et al., 2006; Stalikas, 2007). Detection systems of polyphenols are of paramount importance due to their capacity to

quantify and identify these complex biomolecules. To this aim, polyphenols are mainly detected by ultraviolet (UV) absorption, often using a photodiode array detector (PAD) (Gómez-Caravaca et al., 2006; Stalikas, 2007). However, coupled off-line and recently on-line techniques, in particular with mass spectrometry (MS), are being routinely used for propolis analysis and standardization (Cuyckens et al., 2001; Cuyckens and Claeys, 2004).

Thin-Layer Chromatography (TLC)

TLC is able to analyze specific polyphenols by using suitable stationary phases and solvents depending on the structure of the molecular species needing to separate. Silica gel is a classical stationary phase widely used to separate the more apolar flavonoids such as flavonols and isoflavonoids (Figure 1A) by using different mobile phases, generally constituted of mixing of solvents such as ethanol/water, petroleum ether/ethyl acetate, petroleum ether/acetone/formic acid, chloroform/ethyl acetate, toluene/chloroform/acetone (de Rijke et al., 2006). Visualization and quantification of the separated molecular species are carried out by using short- and long-wavelength UV-light but also, in some specific application, by spraying on the plate different reagents. Due to its complexity and time-consuming characteristics along with its poor resolution, TLC is now rarely applied even if it may be of some utility in specific applications (Milojković Opsenica et al., 2016; Tang et al., 2014).

Gas Chromatography (GC)

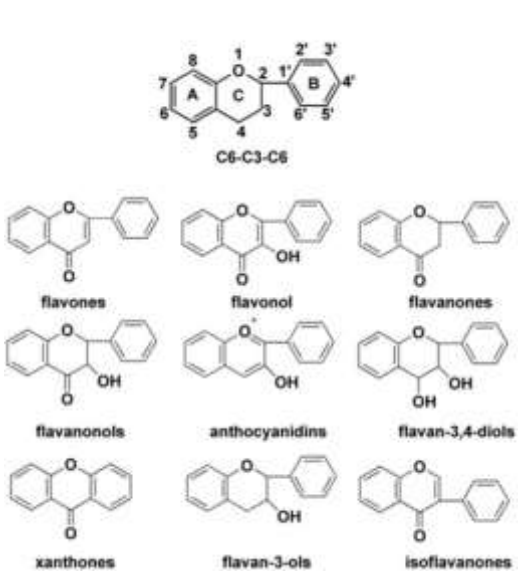
GC-FID (flame ionization detector) is applied for the analysis of polyphenols after their derivatization to make them volatile and suitable for chromatographic separation. GC coupled on-line with MS is now largely used thanks to the capacity of MS to detect the molecular mass values and structural information useful for the identification of unknown compounds. However, the majority of polyphenols from propolis are not volatile enough for GC-MS separation even after derivatization. As a consequence, GC-MS is generally used for the analysis of propolis volatile molecules that are mainly responsible for its specific aroma although their amount is generally very low (Mohtar et al., 2018; Pellati et al., 2013).

High-Performance Liquid Chromatography (HPLC)

To date, HPLC is the most suitable and reliable analytical approach for the identification and quantification of propolis active biomolecules. In fact, the relatively polar nature of polyphenols possessing several hydroxyl groups in the structure, combined with the UV adsorption of the aromatic rings and with the soft ionization techniques compatible with chromatography, make HPLC-UV(DAD) and HPLC-MS the most useful and reproducible methods for the characterization and determination of propolis active compounds (Table 1). As a consequence, HPLC coupled to UV and MS detectors has improved the analysis of non-volatile polyphenols allowing us to establish their quantification and structural identification (Table 1). The ion trap is the most recommended MS approach to characterize the propolis bioactives for its multiple fragmentation steps (MS^n) (Cuyckens et al., 2001; Cuyckens and Claeys, 2004). The structural identification of different classes and singular species of propolis polyphenols is obtained by comparing their chromatographic behavior and retention times, UV adsorption spectra and MS information to those of reference molecules. When standards are not commercially available, the structural nature of the unknown polyphenol can be obtained by comparing UV data and MS fragmentation pattern with those available in the literature (Cuyckens et al., 2001; Cuyckens and Claeys, 2004; Gardana et al., 2007). In fact, the pattern of fragmentation obtained by tandem mass belongs to a given molecule or class of molecules and, as concern flavonoids, their distinct classes differ in the presence of substitution groups strongly influencing the fragmentation pathway (Gardana et al., 2007). The interpretation of tandem mass and further MS^n data provides specific information permitting to identify the structure of the biomolecule of interest.

In a previous study, Volpi and Bergonzini (2006) developed a reliable HPLC-UV-ESI-MS analysis for the characterization of the chemical structure and therefore for the quality control of propolis applied to samples of various origin. We now report a further detailed characterization of brown propolis collected in different countries with the aim to observe or differentiate possible common features useful for the evaluation of its quality and to predict the biological activity.

We analyzed samples belonging to Europe from Italy, Spain, France, Romania, Bulgaria, Ukraine and Macedonia (Figure 2) while HPLC-MS profiles of brown propolis from Turkey and China are illustrated in Figure 3. Finally, samples collected from America, in particular from Uruguay and Brazil are shown in Figure 4. The total ion chromatograms (TIC) of ethanolic propolis extracts acquired in negative ion mode from the various European countries show quite the same profile for the presence of the same molecular species (Figure 2) identified by the same mass values and therefore the same ion species. HPLC-MS and MS^2 of the European brown propolis assured the identification of many compounds in each sample (Table 2) accounting for ~77-97% of the total molecular species. The remaining not identified percentage could be mainly represented by the family of triterpenoids and a small fraction of glycosylated derivatives (Galeotti et al., 2017). In fact, triterpenoids are



Hydroxybenzoic acids

Name	R1	R2	R3	R4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H

Hydroxycinnamic acids

Name	R1	R2	R3	R4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

B

Figure 1. Classification and structure of (A) flavonoids and (B) hydroxybenzoic and hydroxycinnamic acids derivatives.

abundant and very common in all vegetable forms and they are generally present in any kind extract (Connolly and Hill, 2010). Quantitative data reported in Table 2 were performed by MS detection versus a unique standard of galangin due to the absence of commercially available standard related to all polyphenol species identified. Anyway, this approach gives us the possibility to have a clearer picture of the different propolis samples along with the identification of the main polyphenols and quantification of the various classes useful to have a specific fingerprint. In fact, according to Figure 2 and Table 2, propolis from the various European countries are quite similar each other showing a common polyphenolic composition also with a comparable percentage. The ethanolic extracts of brown European propolis are rich in phenolic acid derivatives (in particular from caffeic acid), between ~5 and ~16%, and all samples show the presence of pinobanksin and derivatives (pinobanksin-3-*O*-acetate and pinobanksin-3-*O*-butyrate), chrysin, pinocembrin and galangin as main bioflavonoid species. Overall, flavones and flavonols are from ~22 and ~29%, flavanones and dihydroflavonols are between ~28 and ~48% while glycosilated species and terpenes are ~4 - 17%.

Propolis from Turkey and China (Figure 3) has a greater percentage of glycosilated derivatives and terpenes as also evident from the HPLC-MS profile accounting for ~20 - 21%. This is more evident for propolis from Uruguay very rich in glycosilated and terpenes species, ~34% with a lower percent of flavones and flavonols (13.6%) (Table 2).

Contrary to the brown propolis samples illustrated earlier, the two samples from Brazil show a different HPLC-MS profile (Figure 4) and composition (Table 3) accounting for a very low percent of the above identified polyphenols. In fact, we detected the presence of low percentages of chrysin, pinocembrin, galangin, pinobanksin-3-*O*-acetate and CAPE compared to the other brown propolis. Additionally, artepillin C, a molecular species specific for brown propolis from Brazil, was observed even if in percentages very lower than green propolis collected from Brazil (Cheung et al., 2011). Moreover, this peculiar composition was observed in two propolis samples of Brazil in different periods and seasons. This repeatability of propolis collected in various periods was also observed for different samples from China (Supplemental Figure S1) demonstrating that HPLC-MS is capable of giving a polyphenols fingerprint and composition specific for areas of production independently from the season of collection.

On the other hands, a simple HPLC profile acquired with UV/DAD detector (Figure 5) is unable to distinguish between samples from different geographic areas for the absence of UV chromophores in many of the molecular species present in propolis. This is evident also having standard commercially available due to the complexity of the propolis HPLC profile.

NMR ANALYSIS OF PROPOLIS

^{13}C- and ^{1}H-NMR and their bidimensional maps may provide specific fingerprints of propolis useful for obtaining global information in particular of complex samples. In fact, a recent study demonstrates that it is possible to use ^{1}H-NMR for the simultaneous recognition of propolis

Table 1. The most common HPLC conditions and detectors for the separation and characterization of polyphenols in propolis.

Column	Mobile phase	Detector	Application	Year	References
LiChrocart RP18	Gradient separation with water:formic acid 19:1 to methanol	DAD	Rosemary Propolis	1995	Gil et al. (1995)
LiChrospher 100 RP18	Gradient separation with formic acid to methanol	PAD	New Zealand Propolis	1996	Markham et al. (1996)
LiChrocart RP18	Gradient separation with water/formic acid 19:1 to methanol	DAD	Tunisian propolis	1997	Martos et al. (1997)
YMC Pack ODS-A	Isocratic separation with acetic acid/methanol/water 5:75:60	DAD	Brazilian Propolis	1998	Park et al. (1998)
YMC Pack ODS-A	Isocratic separation with water/methanol	DAD	Brazilian Propolis	2002	Park et al. (2002)
Capcell Pak ACR 120 C18	Gradient separation with 0.1% formic acid/water to B: 0.1% formic acid/acetonitrile	PAD	Korean Propolis	2004	Ahn et al. (2004)
Synergi Fusion-RP18	Gradient separation with 0.25% acetic acid and methanol	UV-VIS and ESI-MS	Propolis from Argentina, Azerbaijan, China, Ethiopia, Kenya, Italy, Spain	2006	Volpi and Bergonzini (2006)
Symmetry C18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and triple quadrupole MS	Propolis form various geographic regions	2007	Gardana et al. (2007)
Symmetry C18	Isocratic separation with methanol/0.4% phosphoric acid 60:40	UV-VIS and PDA	Propolis form various geographic regions	2008	Zhou et al. (2008)
Ascentis C18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS/MS	Italian Propolis	2011	Pellati et al. (2011)
Zorbax SB-C18	Gradient separation with 0.05% acetic acid and acetonitrile	LTQ Orbitrap XL MS	Iraqi propolis	2011	Sulaiman et al. (2011)
Nucleosil C18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS/MS	Portuguese Propolis	2013	Falcão et al. (2013)
Sepax HP-C18	Gradient separation with 1% acetic acid and methanol	UV	Chinese Propolis	2014	Cui-ping et al. (2014)
Hypersil gold C18	Gradient separation with 1% formic acid and acetonitrile	Linear ion trap and Orbitrap hybrid MS	Serbian Propolis	2015	Ristivojević et al. (2015)
MultoHigh 100 RP18	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS/MS	Bolivian Propolis	2016	Nina et al. (2016)
LiChroCART Purospher StaR RP18	Gradient separation with 5% aseptic acid and methanol	DAD	Propolis from different geographic regions of Brazil	2016	Machado et al. (2016)
Zorbax Eclipse Plus	Gradient separation with 0.1% formic acid and acetonitrile	DAD and ESI-MS	Greek Propolis	2017	Kasiotis et al. (2017)
Tecnokroma C18	Gradient separation with 5% formic acid and acetonitrile	UV-VIS	Propolis form various geographic regions	2018	Escriche and Juan-Borrás (2018)

DAD = Diode Array Detector. PAD = Pulsed Amperometric Detector.

polyphenols by means of specific tools for spectra pre-treatment and analysis (Bertelli et al., 2012). However, the use of NMR requires very expensive equipment and highly expert operators in the field. In fact, in particular for propolis, the utilization of the NMR technique produces very complicated spectra that need to be further analyzed by chemometric methods. Finally, contrary to HPLC-MS, NMR is capable of just identifying a limited number of polyphenol species compared to the high complexity of propolis composition.

PROPOLIS FOR STANDARDIZATION AND QUALITY CONTROL

To obtain an accurate total polyphenols and flavonoids content, all assays need of specific calibration curves made of suitable standards. As a consequence, what are the most reliable standards to produce accurate and specific total polyphenols content of propolis? Many quality control (CQ) laboratories generally adopt a single commercially available polyphenol, such as galangin, pinocembrin, myricetin or others, or a limited mixture of these compounds. This is necessary due to the lacking of these compounds.

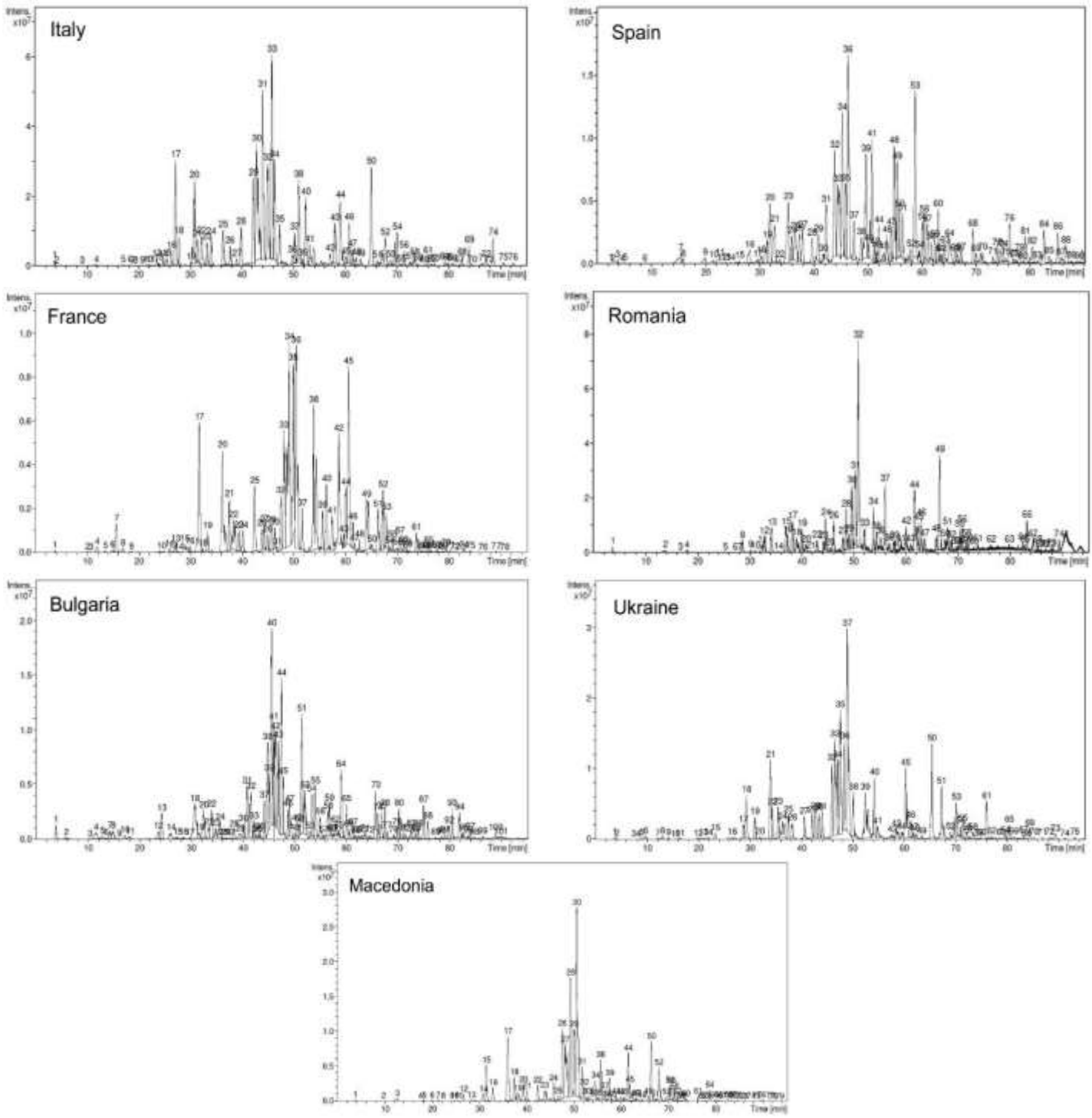


Figure 2. Total ion chromatograms (TIC) of ethanolic extracts of propolis of European origins.

This is necessary due to the lacking of a suitable propolis reference standard. However, as evident, the content of total polyphenols is highly underestimated by using a single standard due to the elevated complexity of propolis polyphenols and derivatives. The correct solution to this problem would be the adoption of “house propolis standards” constituted of highly purified and well characterized propolis samples. Finally, house standards possessing an overall structural composition and chemical profile similar to the samples to be analyzed

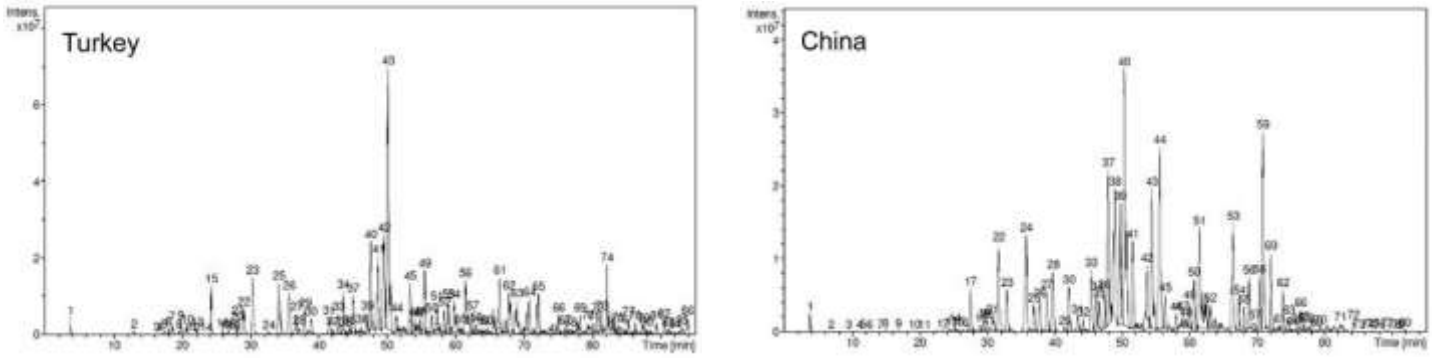


Figure 3. Total ion chromatograms (TIC) of ethanolic extracts of propolis from Turkey and China.

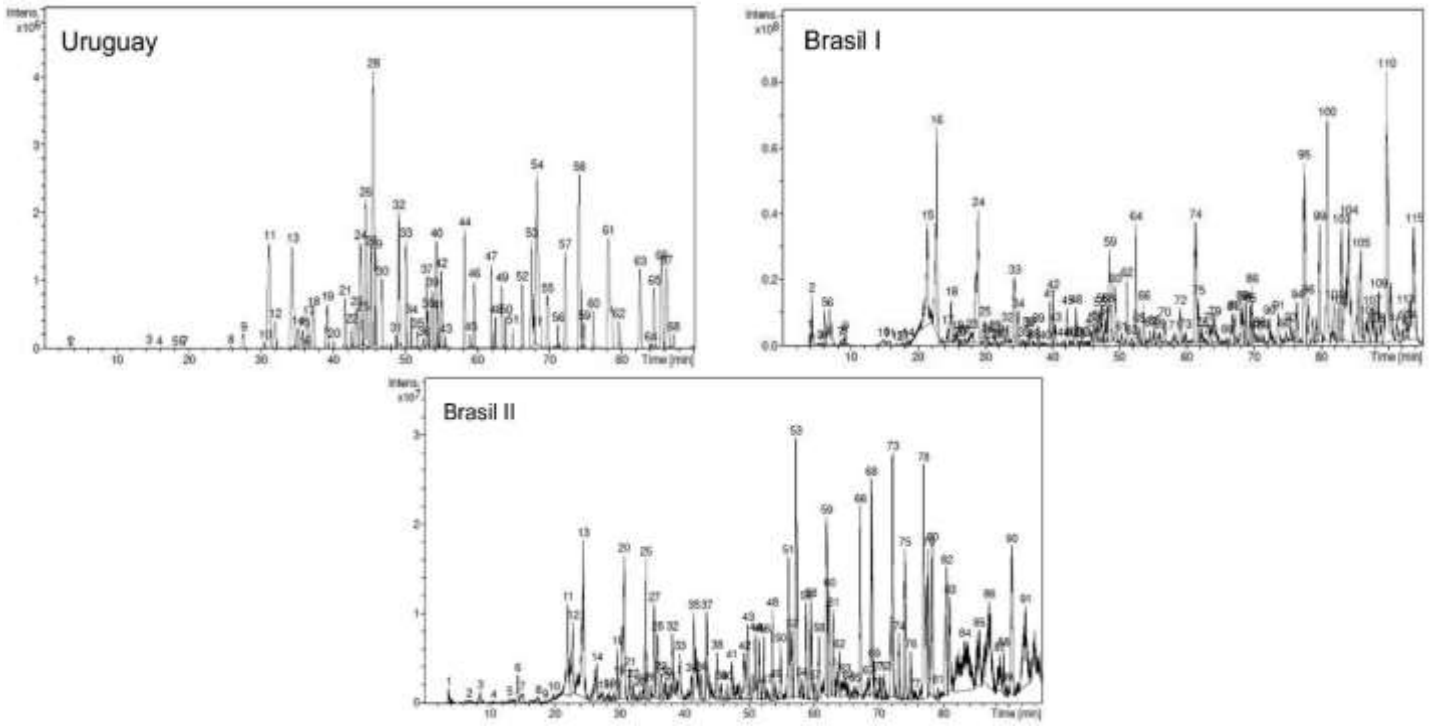


Figure 4. Total ion chromatograms (TIC) of ethanolic extracts of propolis from Uruguay and Brazil.

should be utilized to obtain more accurate and suitable quantitative determinations.

CONCLUSIONS

It is well known that polyphenols and flavonoids constitute the most important and active biomolecules of propolis responsible for the great part of its biological, nutraceutical and therapeutic effects. In this regard, HPLC-MS represents a powerful analytical technique capable of differentiating among various propolis samples. Thus, it could provide an effective alternative to classical analytical phytochemistry useful for the screening of commercial preparations of propolis and to evaluate their specific therapeutic benefits. In fact, due to its capacity to identify and quantify individual compounds from all the constituents present, also in the presence of overlapping signals, HPLC-MS method should be applied for the characterization of the main bioactives and for the evaluation of molecular markers useful for the identification of each propolis type.

By considering the results illustrated above and the many scientific studies available in the literature, we can

Table 2. Molecular composition of the propolis from different countries determined by HPLC-UV-ESI-MS.

Molecular Species		*m/z*	Italy	Spain	France	Romania	Bulgaria	Ukraine	Macedonia	Turkey	China	Uruguay
			European Propolis									
1	Dicaffeic acid	341	Np	np	np	np	np	np	np	np	np	np
2	Caffeic acid	179	Np	np	np	np	np	np	np	np	np	np
3	p-coumaric acid	163	Np	np	np	np	np	np	np	np	np	np
4	Ferulic acid	193	Np	np	np	np	np	np	np	np	np	np
5	Isoferulic acid	193	Np	np	np	np	np	np	np	np	np	np
6	3,4-dimethyl-caffeic acid (dmca)	207	Np	np	np	np	np	np	np	np	np	np
7	Quercetin	301	0.8	0.9	0.5	0.7	0.5	0.8	0.7	0.7	0,2	0,1
8	Pinobanksin-5-methyl-ether	285	1.5	3.0	5.5	1.4	1.7	2.6	3.4	1.4	3,1	4,1
9	Quercetin-3-methyl-ether	315	2.6	1.9	0.7	2.4	0.5	1.2	1.1	3.0	1,3	1,0
10	Cinnamic acid	147	Np	np	np	np	np	np	np	np	np	np
11	Chrysin-5-methyl-ether	267	Np	np	np	np	np	np	np	np	np	np
12	Apigenin	269	0.6	2.0	2.4	1.2	1.5	1.5	3.8	1.2	0,2	0,2
13	Kaempferol	285	2.2	0.9	1.7	2.7	1.7	1.7	1.8	2.3	0,8	0,5
14	Pinobanksin	271	1.6	1.1	3.0	2.3	2.0	4.9	3.0	1.9	4,1	3,9
15	Isorhamnetin	315	2.0	1.6	1.9	1.8	1.9	1.7	0.8	1.0	1,0	0,4
16	Luteolin-methyl-ether	299	1.2	1.0	0.6	1.8	0.5	1.1	1.3	1.2	1,3	0,8
17	Quercetin-dimethyl-ether	329	1.1	1.3	0.8	0.6	1.0	0.1	0.7	0.7	2,0	1,2
18	Galangin-5-methyl-ether	283	0.9	1.0	2.5	0.8	0.4	0.9	1.2	0.8	0,5	1,2
19	Pinobanksin-5-5methyl-ether-3-o-acetate	327	Np	np	np	np	np	np	np	np	np	np
20	Cinnamilidenacetic acid	173	Np	np	np	np	np	np	np	np	np	np
21	Quercetin-7-methyl-ether	315	0.7	0.9	0.7	0.5	0.6	1.2	1.4	1.0	0,3	0,1
22	Quercetin-methyl-x-methylether	329	2.6	1.0	0.7	1.8	0.6	1.3	1.3	1.7	2,1	1,3
23	Caffeic acid isoprenyl ester	247	0.9	2.1	1.7	0.8	1.6	3.7	5.5	0.8	nd	nd
24	Chrysin	253	5.3	4.3	5.5	2.4	3.7	6.7	6.9	4.0	5,3	2,8
25	Caffeic acid benzyl ester	269	0.2	4.6	0.1	0.8	0.9	1	0.3	0.2	0,3	0,2
26	Caffeic acid isoprenyl ester	247	0.1	0.1	0.2	0.1	0.8	2.1	0.1	0.1	0,1	0,1
27	Pinocembrin	255	4.5	5.5	10.3	4.8	3.0	6.7	10.4	3.6	5,9	4,5
28	Galangin	269	5.9	2.8	7.2	6.9	4.0	5.2	6.2	5.7	4,5	2,4
29	Caffeic acid phenylethyl ester (cape)	283	1.3	1.2	1.3	1.5	2.1	2.1	2.4	1.5	2,0	1,8
30	Pinobanksin-3-o-acetate	313	13.1	9.1	8.9	13.3	7.0	12.7	17.2	12.7	8,9	7,9
31	Methoxy-chrysin	283	1.0	0.8	0.6	1.2	0.9	4.8	0.8	0.6	2,9	1,6
32	p-coumaric prenyl ester	231	Np	np	np	np	np	np	np	np	np	np
33	p-coumaric benzyl ester	253	Np	np	np	np	np	np	np	np	np	np
34	Caffeic acid cinnamyl ester	295	0.4	4.6	1.0	0.5	0.5	0.7	2.9	0.4	5,2	3,5
35	p-coumaric prenyl ester	231	Np	np	np	np	np	np	np	np	np	np
36	Pinobanksin-3-o-propionate	327	4.3	4.8	3.1	5.4	5.2	5.1	3.2	3.1	6,8	2,6
37	p-coumaric cinnamyl ester	279	0.5	1.0	0.6	0.3	0.2	0.9	0.2	0.4	1,3	1,1

Table 2. Contd.

38	Pinobanksin-3-o-butyrate	341	Np	np	np	np	np	np	np	np	np	np
39	Pinobanksin-3-o-pentanoate	355	Np	np	np	np	np	np	np	np	np	np
40	Pinobanksin-3-o-hexanoate	369	Np	np	np	np	np	np	np	np	np	np
41	p-methoxy cinnamic acid cinnamyl ester	293	Np	np	np	np	np	np	np	np	np	np
Totally identified species			**86.0**	**91.0**	**90.2**	**91.6**	**77.2**	**94.9**	**93.7**	**82.2**	**97.4**	**93.6**
Phenolic acids derivatives			5.1	16.5	7.3	5.9	9.3	12.6	12.8	5.9	10.2	6.9
Flavones and flavonols (%)			28.9	22.4	25.8	25.2	23.8	28.2	28.0	23.9	22.4	13.6
Flavanones and dihydroflavonols (%)			35.5	35.5	39.8	43.4	28.0	44.0	48.5	31.6	44.7	39.0
Glycosilated species and terpenoids (%)			16.5	16.6	17.3	17.1	16.1	10.1	4.4	20.8	20.1	34.1

np = not present.

Table 3. Molecular composition of two propolis samples from Brazil determined by HPLC-UV-ESI-MS.

Molecular species	***m/z***	**Sample 1**	**Sample 2**
		Brazil	
	547	0.5	1.4
	515-353	2.2	3.4
	515-353	5.0	4.8
	487	0.6	0.9
	677-515	2.5	4.4
	301	1.2	2.0
	301	0.2	0.7
	331	1.1	0.7
Chrysin	253	0.6	0.4
Pinocembrin	255	0.4	0.5
Galangin	269	1.2	0.4
Caffeic acid phenylethyl ester (Cape)	283	1.0	0.4
Pinobanksin-3-o-acetate	313	1.7	2.5
	315	1.0	1.2
	393	0.8	1.1
	615-379	1.2	1.9
	333	2.9	2.0
	319	5.8	2.1
	452-315	3.7	2.9

Table 3. Contd.

	299	2.0	3.3
	485	1.6	1.5
	613	3.7	0.8
	613	0.5	1.2
	597-319	0.4	1.1
	405	1.0	0.9
	613	1.3	1.0
	471	2.6	0.8
	471	3.8	4.4
Artepillin C	593-471	1.6	2.3
	613-469	1.0	5.5
	559-471	6.0	2.2
	525	2.0	5.8
	513-305	4.7	3.5
	701-455-417	1.1	0.9
	657-547-453	0.6	1.1
	455-369-325	3.1	1.2
	561-527	3.7	8.7
	455-371-327	1.2	4.5

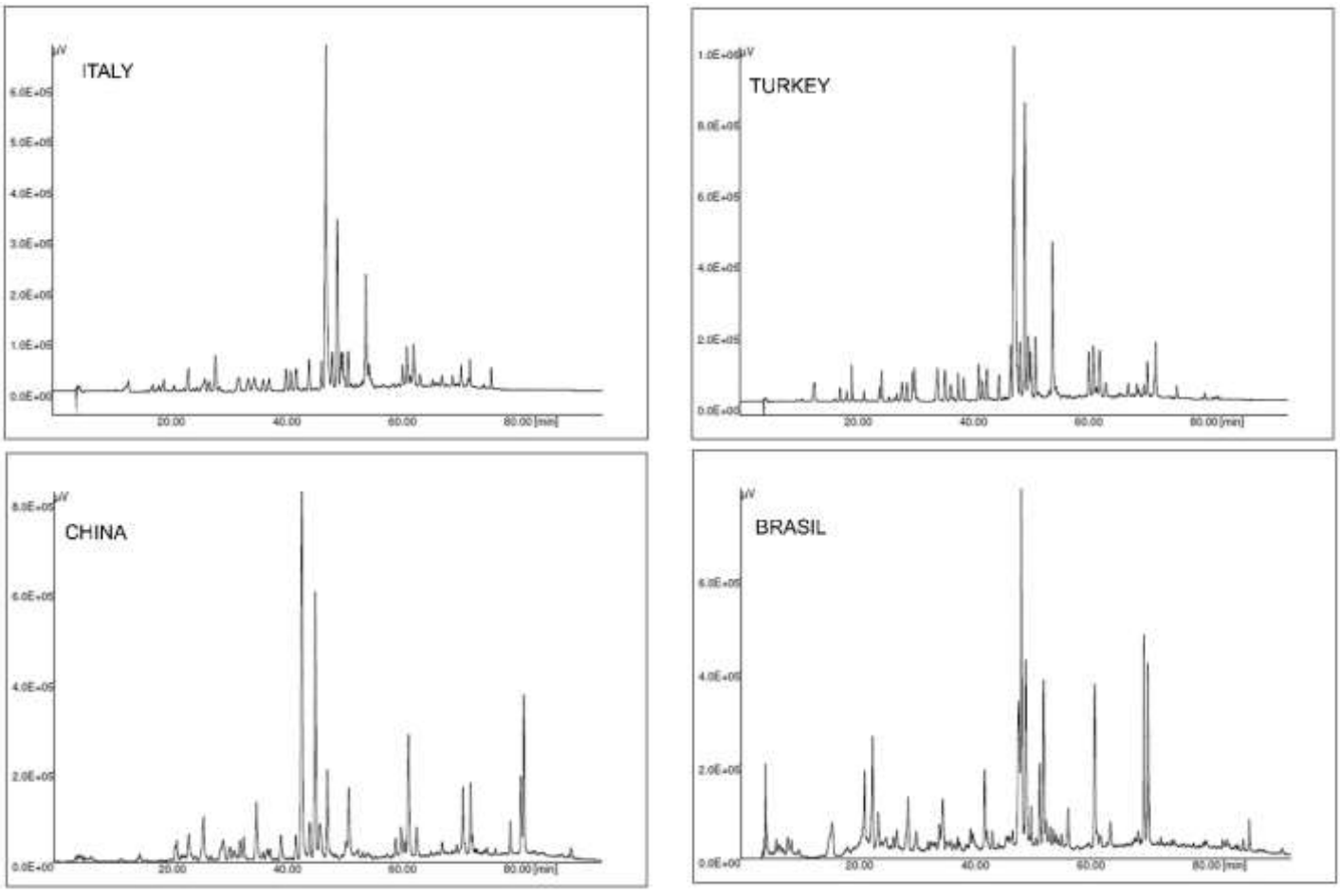


Figure 5. HPLC-UV chromatograms of ethanolic extracts of propolis from Italy, Turkey, China and Brazil.

affirm that, concerning the points provided by EFSA, it is possible to have a full and clear characterization of propolis and its main bioactive compounds and to obtain specific fingerprints of raw samples of different origin and finished preparations by using on-line HPLC-MS (and tandem mass when necessary).

Therefore, by the very numerous scientific studies on its activities, the total propolis extract is considered the “active principle” and biomolecular marker must be used for quality control applying the powerful analytical approaches discussed above. In the case of propolis, the most important bioactive compounds have been demonstrated to be polyphenols that are known to change depending on type and external factors. By considering this, it is possible to standardize propolis quality and final preparations used in medicine and nutraceuticals according to the corresponding chemical profile obtained by HPLC-MS.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

ABBREVIATIONS

CE, Capillary electrophoresis; **CQ,** quality control; **CZE,** capillary zone electrophoresis; **DAD,** diode array detector; **DNP,** dinitrophenylhydrazine; **ESI,** electrospray ionization; **FID,** flame ionization detector; **GC,** gas chromatography; **HPLC,** high-performance liquid chromatography; **MEKC,** Micellar electrokinetic chromatography; **MS,** mass spectrometry; **NMR,** nuclear magnetic resonance; **PAD,** pulsed amperometric detector; **TIC,** total ion chromatogram; **TLC,** thin-layer chromatography.

REFERENCES

Ahn MR, Kumazawa S, Hamasaka T, Bang KS, Nakayama T (2004). Antioxidant activity and constituents of propolis collected in various areas of Korea. Journal of Agriculture and Food Chemistry 52:7286-7292.

Banskota AH, Tezuka Y, Kadota S (2001). Recent progress in pharmacological research of propolis. Phytotherapy Research 15:561-571.

Bertelli D, Papotti G, Bortolotti L, Marcazzan GL, Plessi M (2012). ¹H-NMR simultaneous identification of health-relevant compounds in propolis extracts. Phytochemical Analysis 23:260-266.

Bonvehi JS, Coll FV (1994). Phenolic composition of propolis from China and from South America. *Zeitschrift fur Naturforschung C* 49:712-718.

Castaldo S, Capasso F (2002). Propolis, an old remedy used in modern medicine. Fitoterapia 73 (Suppl 1):S1-6.

Cheung KW, Sze DM, Chan WK, Deng RX, Tu W, Chan GC (2011). Brazilian green propolis and its constituent, Artepillin C inhibits allogeneic activated human CD4 T cells expansion and activation. Journal of Ethnopharmacology 138:463-471.

Connolly JD, Hill RA (2010). *Triterpenoids*. Natural Products Report 27:79-132.

Cui-Ping Z, Shuai H, Wen-Ting W, Shun P, Xiao-Ge S, Ya-Jing L, Fu-Liang H (2014). Development of high-performance liquid chromatographic for quality and authenticity control of Chinese propolis. Journal of Food Science 79:C1315-1322.

Cuyckens H, Croley RJ, Metcalfe TR, March CDRE (2001). A tandem mass spectrometric study of selected characteristic flavonoids. International Journal of Mass Spectrum 210/211:371-385.

Cuyckens F, Claeys M (2004). Mass spectrometry in the structural analysis of flavonoids. Journal of Mass Spectromology 39:1-15.

de Rijke E, Out P, Niessen WM, Ariese F, Gooijer C, Brinkman UA (2006). Analytical separation and detection methods for flavonoids. Journal of Chromatography A 1112:31-63.

Escriche I, Juan-Borrás M (2018). Standardizing the analysis of phenolic profile in propolis. Food Research International 106:834-841.

Falcão SI, Vale N, Gomes P, Domingues MR, Freire C, Cardoso SM, Vilas-Boas M (2013). Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich in flavonoid glycosides. Phytochemical Analysis 24:309-318.

Galeotti F, Crimaldi L, Maccari F, Zaccaria V, Fachini A, Volpi N (2017). Selective treatment to reduce contamination of propolis by polycyclic aromatic hydrocarbons (PAHs) still preserving its active polyphenol component and antioxidant activity. Natural Products Research 31:1971-1980.

Galeotti F, Maccari F, Fachini A, Volpi N (2018). Chemical Composition and Antioxidant Activity of Propolis Prepared in Different Forms and in Different Solvents Useful for Finished Products. 7(3):41.

Gardana C, Scaglianti M, Pietta P, Simonetti P (2007). Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography-tandem mass spectrometry. Journal of Pharmacy and Biomedical Analysis 45:390-399.

Gil MI, Ferreres F, Ortiz A, Subra E, Tomas-Barberan FA (1995). Plant Phenolic Metabolites and Floral Origin of Rosemary Honey. Journal of Agriculture and Food Chemistry 43:2833-2838.

Gómez-Caravaca AM, Gómez-Romero M, Arráez-Román D, Segura-Carretero A, Fernández-Gutiérrez A (2006). Advances in the analysis of phenolic compounds in products derived from bees. Journal of Pharmacy and Biomedical Analysis 41:1220-1234.

Gómez-Romero M, Arráez-Román D, Moreno-Torres R, García-Salas P, Segura-Carretero A, Fernández-Gutiérrez A (2007). Antioxidant compounds of propolis determined by capillary electrophoresis-mass spectrometry. Journal of Separation Science 30(4):595-603.

Kasiotis KM, Anastasiadou P, Papadopoulos A, Machera K (2017). Revisiting Greek Propolis: Chromatographic Analysis and Antioxidant Activity Study. PLoS One 12:e0170077.

Machado BA, Silva RP, Barreto GA, Costa SS, Silva DF, Brandão HN, Rocha JL, Dellagostin OA, Henriques JA, Umsza-Guez MA, Padilha FF (2016). Chemical Composition and Biological Activity of Extracts Obtained by Supercritical Extraction and Ethanolic Extraction of Brown, Green and Red Propolis Derived from Different Geographic Regions in Brazil. PLoS One 11:e0145954.

Markham KR, Mitchell KA, Wilkins AL, Daldy JA, Lu Y (1996). HPLC and GC-MS identification of the major organic constituents in New Zeland propolis. Phytochemistry 42: 205-211.

Martos I, Cossentini M, Ferreres F, Tomás-Barberán FA (1997). Flavonoid Composition of Tunisian Honeys and Propolis. Journal of Agriculture and Food Chemistry 45:2824-2829.

Milojković OD, Ristivojević P, Trifković J, Vovk I, Lušić D, Tešić Ž (2016). TLC Fingerprinting and Pattern Recognition Methods in the Assessment of Authenticity of Poplar-Type Propolis. Journal of Chromatography Science 54:1077-1083.

Mohtar LG, Rodríguez SA, Nazareno MA (2018). Comparative analysis of volatile compound profiles of propolis from different provenances. Journal of Science and Food Agriculture 98:3409-3415.

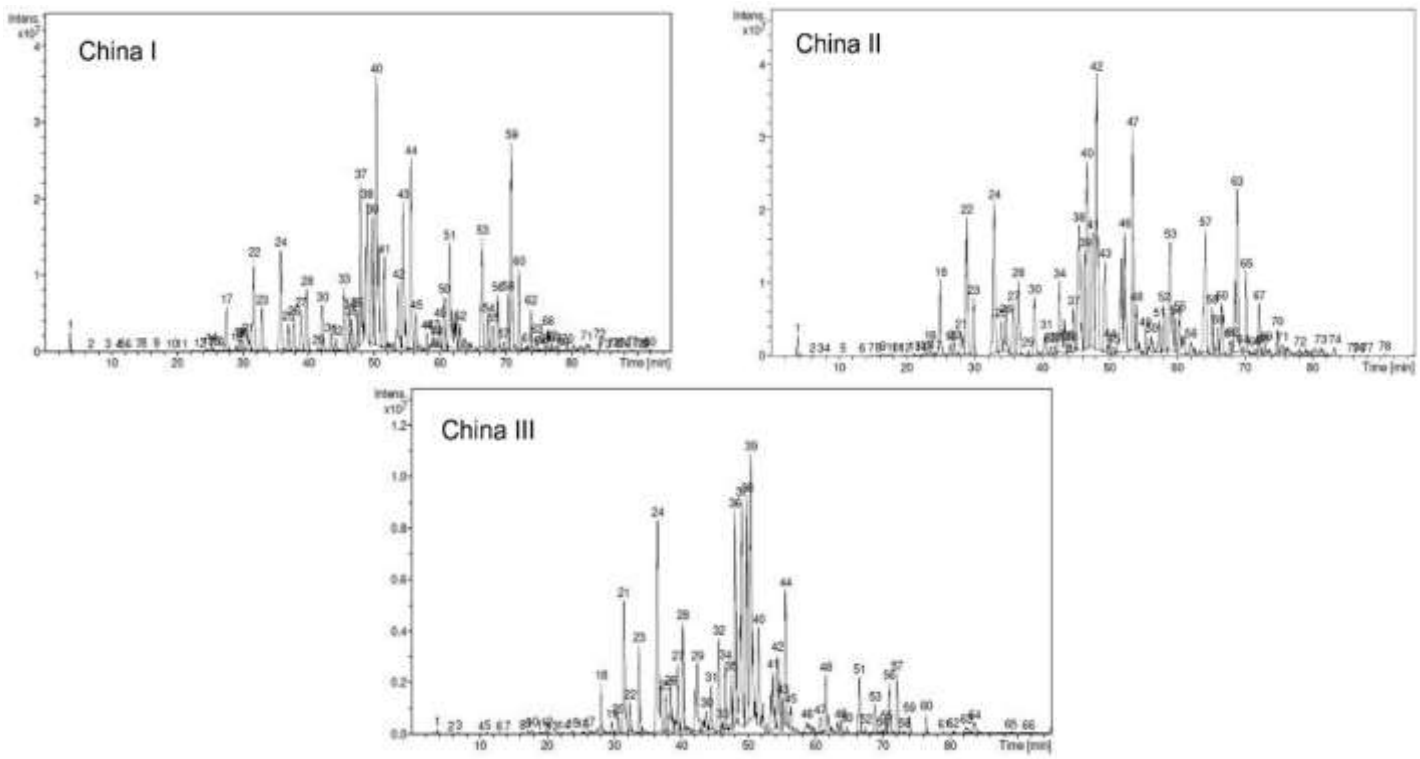
Nagy M, Grancai D (1996). Colorimetric determination of flavanones in propolis. Pharmazie 51:100-101.

Nina N, Quispe C, Jiménez-Aspee F, Theoduloz C, Giménez A, Schmeda-Hirschmann G (2016). Chemical profiling and antioxidant activity of Bolivian propolis. Journal of Science and Food Agriculture 96:2142-2153.

Park YK, Alencar SM, Aguiar CL (2002). Botanical Origin and Chemical Composition of Brazilian Propolis. Journal of Agriculture and Food Chemistry 50:2502-2506.

Park YK, Koo MH, Abreu JA, Ikegaki M, Cury JA, Rosalen PL (1998). Antimicrobial activity of propolis on oral microorganisms. Current Microbiology 36:24-28.
Pasupuleti VR, Sammugam L, Ramesh N, Gan SH (2017). Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits. Oxidative Medicine and Cellular Longevity 2017: 1259510.
Pellati F, Orlandini G, Pinetti D, Benvenuti S (2011). HPLC-DAD and HPLC-ESI-MS/MS methods for metabolite profiling of propolis extracts. Journal of. Pharmacy and Biomedical Analysis 55:934-948.
Pellati F, Prencipe FP, Benvenuti S (2013). Headspace solid-phase microextraction-gas chromatography-mass spectrometry characterization of propolis volatile compounds. Journal of Pharmcy and Biomedical Analysis 84:103-111.
Popova M, Bankova V, Butovska D, Petkov V, Nikolova-Damyanova B, Sabatini AG, Marcazzan GL, Bogdanov S (2004). Validated methods for the quantification of biologically active constituents of poplar-type propolis. Phytochemical Analysis 15:235-240.
Ristivojević P, Trifković J, Gašić U, Andrić F, Nedić N, Tešić Ž, Milojković-Opsenica D (2015). Ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC-LTQ/Orbitrap/ MS/MS) study of phenolic profile of Serbian poplar type propolis. Phytochemical Analysis 26:127-136.
Salatino A, Fernandes-Silva CC, Righi AA, Salatino ML (2011). Propolis research and the chemistry of plant products. Natural Products Report 28:925-936.
Sforcin JM (2007). Propolis and the immune system: a review. Journal of Ethnopharmacology 113:1-14.
Stalikas CD (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. Journal of separation science 30:3268-3295.
Sulaiman GM, Al Sammarrae KW, Ad'hiah AH, Zucchetti M, Frapolli R, Bello E, Erba E, D'Incalci M, Bagnati R (2011). Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials. Food Chemical Toxicology 49:2415-2421.
Tang TX, Guo WY, Xu Y, Zhang SM, Xu XJ, Wang DM, Zhao ZM, Zhu LP, Yang DP (2014). Thin-layer chromatographic identification of Chinese propolis using chemometric fingerprinting. Phytochemical Analysis 25:266-272.
Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Alvarez JA (2008). Functional properties of honey, propolis, and royal jelly. Journal of Food Science 73: R117-124.
Volpi N (2004). Separation of flavonoids and phenolic acids from propolis by capillary zone electrophoresis. Electrophoresis 25:1872-1878.
Volpi N, Bergonzini G (2006). Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. Journal of Pharmacy and Biomedical Analysis 42:354-361.
Watanabe MA, Amarante MK, Conti BJ, Sforcin JM (2011). Cytotoxic constituents of propolis inducing anticancer effects: a review. Journal of Pharmacy and Pharmacology 63:1378-1386.
Zhou J, Li Y, Zhao J, Xue X, Wu L, Chen F (2008). Geographical traceability of propolis by high-performance liquid-chromatography fingerprints. Food Chemistry 108:749-59.

Supplemental Figure



Supplemental Figure S1. Total ion chromatograms (TIC) of three ethanolic extracts of different propolis samples from China.