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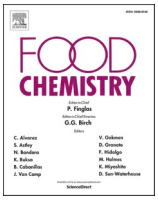

Review

Recent trends in the analysis of honey constituents

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ABSTRACT

The main goal of this article is to present an overview of the analytical methodologies employed in recent years (2015–2021) to determine several honey constituents, and, specifically, those with health-promoting effects and nutritional value, like phenolic compounds, sugars, amino acids and proteins, vitamins, lipids, minerals, and organic acids. The review is structured according to the different families of compounds, and they will be discussed along with the main extraction and analytical techniques used for their determination. Phenolic compounds, sugars and amino acids have been the main compounds determined in honey. The analytical methods (sample treatment and determination techniques) are strongly dependent on the compound. Nevertheless, it can be concluded that high-performance liquid chromatography was predominantly selected for determining honey constituents; while, in relation to the sample treatment, the preferred option was a dilution of the honey with water or a buffer.

1. Introduction

Honey is one of the most complex natural foods as it contains about 200 substances (da Silva, Gauche, Gonzaga, & Costa, 2016; Pita-Calvo, Guerra-Rodríguez, & Vázquez, 2017) being carbohydrates (as sugars) being the main constituents, especially reducing sugars like fructose and glucose (Trifković, Andrić, Ristovojević, Guzelmeric, & Yesilada, 2017). However, this bee product also contains proteins and amino acids (AAs), lipids, vitamins, phenolic compounds, minerals, and organic acids among other compounds (Afrin et al., 2020; Rahman et al., 2017; Trifković et al., 2017). The chemical composition of honey is strongly dependent on the botanical and geographical origin, together with other factors, such as climatic conditions or beekeeper strategies (da Silva et al., 2016). The relationship of the honey composition with its origin is a relevant issue, as it has been used for many years as a tool for assessing honey authenticity (Trifković et al., 2017). Moreover, the study of honey composition is also useful in its quality control, to verify the presence of the compounds responsible for some of the health-promoting and nutritional effects associated with this product (Pita-Calvo et al., 2017; Puścion-Jakubik, Borawska, & Socha, 2020). It should be mentioned that plant microribonucleic acids (miRNAs), which are fundamental for the modulation of gene expression in the cells, have been recently detected in honey (Gismondi, Di Marco, & Canini, 2017; Smith et al., 2021). They could be of great interest in the coming years since their

Abbreviations: AAs, amino acids; AAS, atomic absorption spectroscopy; ACN, acetonitrile; AE, anion exchange; AES, atomic emission spectrometry; AOAC, Association of Official Analytical Chemists; CE, capillary electrophoresis; DAD, diode array detector; DLLME, dispersive liquid–liquid microextraction; EA, elemental analyser; ECD, electrochemical detector; FAAs, free AAs; FAs, fatty acids; FCM, Folin-Ciocalteu method; F/G, fructose/glucose ratio; FLD, fluorescence detector; FMOC-Cl, 9-fluorenylmethyl chloroformate; GC, gas chromatography; G/W, glucose/water ratio; HPAEC, high-performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; IRMS, isotope ratio mass spectrometry; IT, ion trap; LLE, liquid-liquid extraction; MALDI, matrix-assisted laser desorption/ionization; MiRNA, microribonucleic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MTBE, methyl *tert*-butyl ether; MWCNTs, multiwalled carbon nanotubes; NMR, nuclear magnetic resonance; OES, optical emission spectroscopy; OH-FAs, hydroxy Fas; OPA, o-phthaldialdehyde; PAD, pulsed amperometric detector; AGE, polyacrylamide gel electrophoresis; PDA, photodiode array detector; QqQ, triple quadrupole; QTOF, quadrupole time-of-flight; QTRAP, triple quadrupole linear ion trap mass spectrometer; QuEChERS, quick, easy, cheap, effective, rugged, and safe; RID, refractive index detector; SBSE, stir bar sorptive extraction; SDS, sodium dodecyl sulphate; SE, solvent extraction; *SEC*, size exclusion chromatography; SPE, solid-phase extraction; SQ, single quadrupole; SULLE, sugaring-out assisted liquid-liquid extraction; TAAs, total AAs; TCARC, total carotenoid content; TERPRCs, terpenoids and related compounds; TOF, time-of-flight; UHPLC, ultra-high performance liquid chromatography.

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detection in honey could partially explain, some of the biological properties of this food. Moreover, miRNA profiles are specific to each honey, and subsequently, this finding suggest their potential as novel honey authentication tools.

As can be expected, numerous studies have been published in the last few years relating to the extraction and determination of honey constituents with potential health benefits or with nutritional value (see Fig. 1), and the worldwide interest in this topic is demonstrated by the large list of countries in which such studies were carried out (see Fig. 2), with large numbers of publications from Brazil, China, and Spain. As can be seen in Fig. 3, many of the studies were devoted to the analysis of phenolic compounds, carbohydrates, and AAs and proteins; other compounds such as vitamins, lipids, minerals, and organic acids have received less attention. It must be specified that the order of the constituents in Section 2 is considered following the data summarized in Fig. 3. The study of honey constituents has been subjected to an extensive study in the last years, and subsequently several interesting reviews have been published (Afrin et al., 2020; Balkanska, Stefanova, & Stoikova-Grigorova, 2020; Cianciosi et al., 2018; da Silva et al., 2016; da Silva et al., 2016; de Melo, de Almeida-Muradian, Sancho, & Pascual-Maté, 2017; Lewoyehu & Amare, 2019; Mărgăoan et al., 2021; Pascual-Maté, Osés, Fernández Muiño, & Sancho, 2018a; Pita-Calvo et al., 2017; Puścion-Jakubik et al., 2020; Rahman et al., 2017; Seraglio et al., 2019, 2021; Siddiqui, Musharraf, Choudhary, & Rahman, 2017; Trifković et al., 2017; Viteri, Zacconi, Montenegro, & Giordano, 2021). The attention of some of these works was mainly focused in discussing in a general way the chemical composition and the associated health-promoting properties (da Silva et al., 2016; de Melo et al., 2017; Viteri et al., 2021). Authors selected a specific type of honey, such as citrus honey (Seraglio et al., 2021a) or honeydew honey (Seraglio et al., 2019), a family of compounds, like phenolics (Cianciosi et al., 2018), aromatic compounds (Rahman et al., 2017) or minerals (Mărgăoan et al., 2021); while, in other cases, they decided to investigate in detail some of the potential biomedical activities, like antioxidant (Lewoyehu & Amare, 2019; Mărgăoan et al., 2021) or anti-cancer (Afrin et al., 2020). Other reviews focused on the analytical methodologies employed in authentication and quality control of honey (Balkanska et al., 2020; Pascual-Maté et al., 2018a; Pita-Calvo et al., 2017; Puścion-Jakubik et al., 2020; Siddiqui et al., 2017; Trifković et al., 2017). In one of these publications (Siddiqui et al., 2017), the main analytical techniques were reviewed, especially nuclear magnetic resonance (NMR) for the authentication of honey. The authors concluded that use of NMR techniques will be more common in the future when its cost decreases and the instruments will be easier to handle. The use of NMR to evaluate honey authenticity was also discussed by Trifković et al. (2017). However, in this case, authors included other relevant techniques such as infrared (IR), fluorescence spectrophotometry, chromatographic techniques like high-performance liquid chromatography (HPLC) or gas chromatography (GC), isotope ratio mass spectrometry (IRMS), and electrochemical techniques. The authors commented some of the most relevant applications to date for each technique, but the discussion of the analytical method was done in a general way, and no distinction was made according to the different families of compounds. A deeper insight of the analytic techniques commonly used to assess the quality control of honey was carried out by Pita-Calvo et al. (2017). Similar techniques (NMR, IR, GC, HPLC…) to those described by Trifković et al. (2017) were discussed, but more attention was paid to explain the experimental conditions and results. However, the attention was exclusively focused on carbohydrates and related compounds. Some families of compounds, including sugars, proteins, AAs, or minerals, were investigated in two recent publications (Balkanska et al., 2020; Puścion-Jakubik et al., 2020). In both cases, the authors commented in detail the results of some of the most relevant publications, but as happened in other of the discussed publications, little attention was paid to the experimental details. A more complete revision of the methods for analysing honey was carried out by Pascual-Maté et al. (2018a). Authors provided quite a detailed summary of the standardized and/or novel methods for determining the properties and the most important components of honey. However, the experimental conditions of the selected publications were not provided/discussed, and a section devoted to investigating the lipids was not included. In addition, most of the cited references in this article are prior to 2015. Thus, as it has been above discussed, several reviews have been published related to the honey composition and/or its health-promoting effects. In some of these, the different methodologies to determine the different families of honey compounds have been mentioned, and only few of them presented the discussion from an analytical perspective, including experimental conditions. Thus, we consider necessary to provide an update of the above-mentioned publications by discussing the recent trends in analysing honey constituents, paying special attention to the specific extraction and determination conditions, in order to propose the optimal conditions for each family of compounds.

In view of all these aspects, the aim of this study is to present and discuss the main extraction and analytical techniques that were used to obtain, identify, characterize and/or quantify honey constituents with potential health and nutritional effects in the period 2015–2021. Attention will mainly be paid to those in which advanced analytical techniques were employed. Readers interested in more specific details

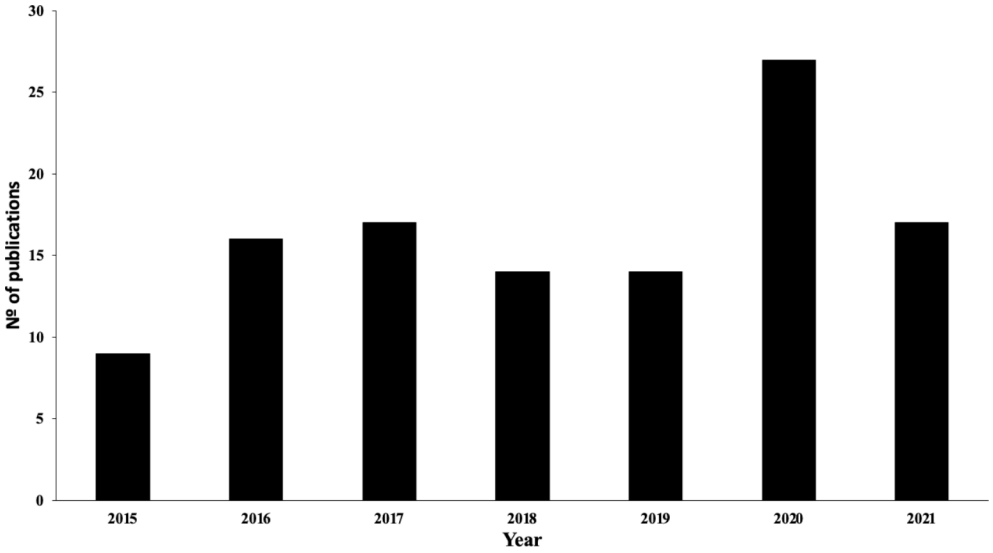

Fig. 1. Evolution of the published works in the last years (2015–2021) related to extraction and determination of honey constituents, which present health promoting effects and nutritional value (data up to December 2021). The sources of information were the databases: ISI-Web of Knowledge, Scopus and Science Direct. The search has been done using as keywords [(honey)] and [(composition) or (constituents) or (compounds) or (nutrients) or (bioactive) or (health-promoting) or (lipids) or (vitamins) or (proteins) or (phenolic) or (essential elements) or (minerals) or (sugars) or (amino acids) or (carbohydrates) or (peptides) (extraction) or (isolation) or (quantification) or (separation) or (determination) or (analysis) or (chromatography)] among several others.

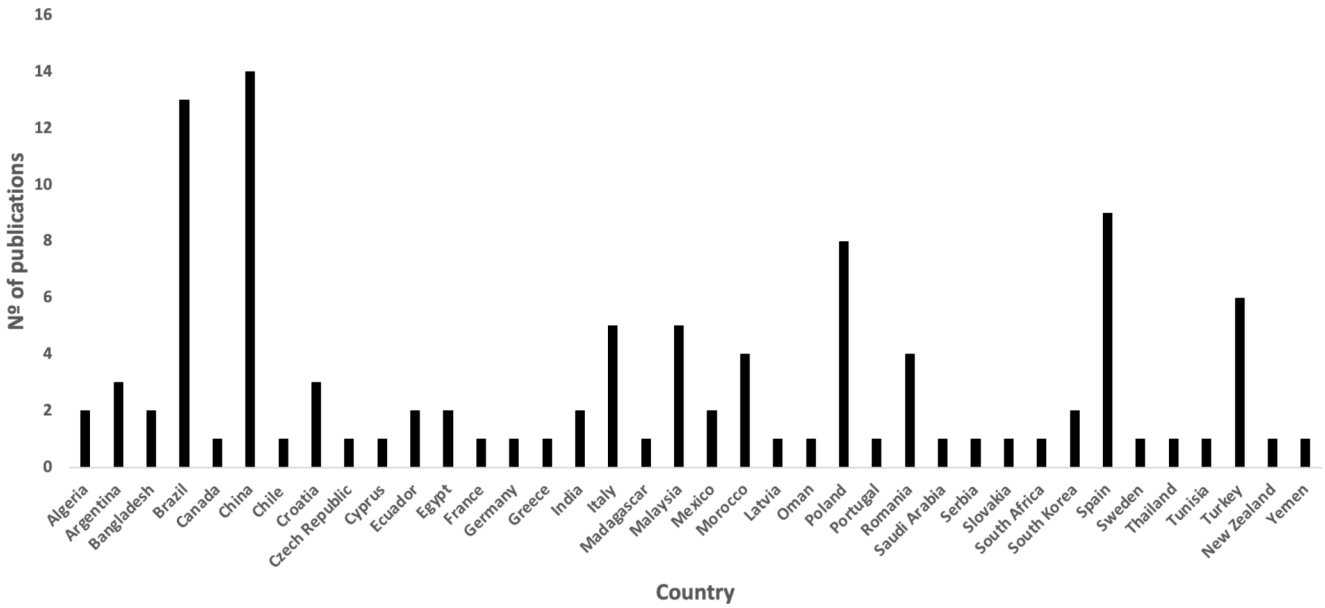

Fig. 2. Summary of the number of publications per country of origin of the analyzed honey samples in the last years (2015–2021). The search has been done as in Fig. 1.

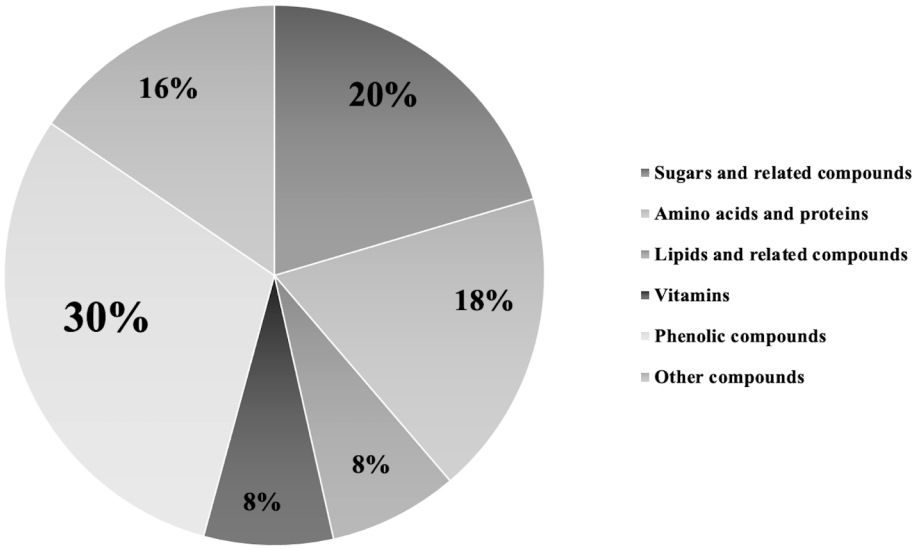

Fig. 3. Summary of the honey constituents, which present health-promoting effects and nutritional value, in the last years (2015–2021). The search has been done using as in Fig. 1.

concerning other constituents, like for starch, compounds derived from honey process such as Maillard reaction products (5-hydroxymethylfurfural), toxic or poisonous constituents, biological activity, therapeutic properties, and specific data can refer to the above-mentioned reviews and to the related literature.

2. Honey constituents

2.1. Phenolic compounds

Phenolic compounds comprise one (phenolic acids) or more (polyphenols) aromatic rings with attached hydroxyl groups in their structures (Minatel, Vanz Borges, Ferreira, Gomez Gomez, Chen, & Pereira Lima, 2017), and receive much attention for their wide range of health-promoting functions, especially antioxidant. They can be classified into two main groups: those that are not flavonoids (phenolic acids, stilbenes, and lignans) and flavonoids (flavonols, flavones, anthocyanidins, flavanones, isoflavones, and others; Ares, Valverde, Bernal, Nozal, & Bernal, 2018). As can be seen in Table 1S (see Supplementary Material), flavonoids and phenolic acids are the main groups of phenolic compounds detected in honey. Flavonoids are the most common group of polyphenolic compounds in the human diet, and they have a significant contribution as an antioxidant source to human diet; while PHAs are related to the protection of deoxyribonucleic acid (DNA) and cell membrane lipids against reactive oxygen species (Ares, Bernal, Nozal, & Bernal, 2021). The phenolic compound composition in honey depends on several factors, such as the botanical and geographic origins or weather conditions, and for that reason, phenolic compounds have been proposed as markers to determine the origin of honey (Soares, Amaral, Oliveira, & Mafra, 2017).

Phenolic compounds have been extensively determined in honey (see Supplementary Material, Table 1S), and different strategies have

been followed depending on the final goals of the studies. In some cases, it was decided to evaluate the total phenolic (TPC; Agdaba et al., 2020; Biluca et al., 2019; Bonhevi, Coll, & Bermejo, 2019; Boussaid et al., 2018; Can et al., 2015; Cebrero et al., 2020; Das et al., 2015; da Silva et al., 2016, 2020; Dżugan et al., 2020; Fröschle, Horn, & Spring, 2018; Gašić et al., 2015; Guo, Deng, & Lu, 2019; Gulzemeric, Ciftci, Yuksel, & Yesilada, 2020; Halouzka, Tarkowski, & Zeljković, 2016; Kováčik, Grúz, Biba, & Hedbavny, 2016; Leyva-Daniel et al., 2020; Nayik & Nanda, 2016; Ranneh et al., 2018; Sawicki, Bączek, & Starowicz, 2020; Valdés-Silverio et al., 2018; Vasić et al., 2019; Vazquez, Armada, Celeiro, Dagnac, & Llompart, 2021; Cebrero et al., 2020; Wang et al., 2021) or total flavonoid (TFC; Boussaid et al., 2018; Can et al., 2015; Das et al., 2015; Dżugan et al., 2020; Gašić et al., 2015; Guo et al., 2019; Gulzemeric et al., 2020; Halouzka et al., 2016; Nayik & Nanda, 2016; Ranneh et al., 2018; Sancho et al., 2016; Sawicki et al., 2020; Valdés-Silverio et al., 2018; Wang et al., 2021) content. These are predominantly determined by employing Folin-Ciocalteu method (FCM) and $AlCl_3$ colorimetric assay, respectively. In both cases, the sample treatment generally consists of a simple dilution with water or with water and an acid (HCl or formic acid), although in one work a solid-phase extraction (SPE) with polymeric (Strata-X) cartridges was selected to obtain the phenolic extracts prior to perform the FCM (Sancho et al., 2016). Phenolic compounds react with the Folin-Ciocalteu reagent, at basic pH, giving rise to a blue coloration that can be determined spectrophotometrically. This reagent has a specificity problem, as it not only reacts with phenols but with any reducing substance (Ares et al., 2018). Meanwhile, the basis of the $AlCl_3$ method is that the aluminium cation forms stable complexes with flavonoids in methanol. That is how it is possible to determine flavonoids, avoiding the interference of other phenolic compounds, especially phenolic acids (Ares et al., 2021). Another way to estimate TFC employs a modification of the Glorieś method (Can et al., 2015). This is also a spectrophotometric method, but in this case, honey is mixed with ethanol and HCl, and quercetin is used as a standard. Meanwhile, in one publication (Cebrero et al., 2020), TFC and TPC contents were determined using a different approach. In this case, they were characterised through total fluorescence spectroscopy and parallel factor analysis.

For the determination of the individual phenolic compounds of the honey samples, the analysis is more complex. After the previously mentioned dilution, an extraction/clean-up step is often required, which is usually performed by solid-phase extraction (SPE) with ion-exchange sorbents, followed by a separation, which usually comprises a high-performance liquid chromatography/ultra-high-performance liquid chromatography (HPLC/UHPLC) analysis in reverse-phase mode with different detectors (UV, diode array (DAD), electrochemical (ECD), mass spectrometry (MS), tandem MS (MS/MS); Campillo, Viñas, Férez-Melgarejo, & Hernández-Córdoba, 2015; Can et al., 2015; Das et al., 2015; da Silva et al., 2020; Dżugan et al., 2020; Elamine et al., 2021; Fröschle et al., 2018; Gao et al., 2020; Gašić et al., 2015; Guo et al., 2019; Gulzemeric et al., 2020; Halouzka et al., 2016; Kováčik et al., 2016; Mannina et al., 2015; Mattonai, Parri, Querci, Degano, & Ribechini, 2016; Nayik & Nanda, 2016; Oroian & Sorina, 2017; Ouchemoukh et al., 2017; Rusko, Vainovska, Vilne, & Bartkevics, 2021; Ranneh et al., 2018; Seraglio et al, 2016, 2017, 2021a,2021b; Silva, Gonzaga, Fett, & Costa, 2019; Sun, Tan, Zhang, & Zhang, 2016; Vasić et al., 2019; Wabaidur et al., 2015; Wang et al., 2021; Zhao et al., 2016; Zhu et al., 2019). However, it must be remarked that current trends in HPLC that reduces the length of the chromatographic run, like UHPLC (Dżugan et al., 2020; Gašić et al., 2015; Kováčik et al., 2016; Ranneh et al., 2018; Seraglio et al., 2017, 2021a; Vasić et al., 2019; Wabaidur et al., 2015) or that enhances the method sensitivity and selectivity such as the use of MS/MS detectors (ion trap (IT), Mannina et al., 2015; Orbitrap, Gašić et al., 2015; Rusko et al., 2021; Vasić et al., 2019; triple quadrupole (QqQ), Dżugan et al., 2020; Halouzka et al., 2016; Kováčik et al., 2016; Valdés-Silverio et al., 2018; Vasić et al., 2019; Vazquez et al., 2021; quadrupole time-of-flight (QTOF), Campillo et al., 2015; Elamine et al., 2021; Mattonai et al., 2016; Sun et al., 2016; triple quadrupole linear ion trap mass spectrometer (QTRAP), Ranneh et al., 2018; Seraglio et al., 2017, 2021a; and time-of-flight (TOF), Ouchemoukh et al., 2017) have been widely employed in recent years. However, it should be mentioned one study in which phenolic acids were determined in honey powders by using GC–MS/MS (triple quadrupole; Kozłowicz et al., 2020). Powdered honey is an attractive substitute for liquid honey, and in order to obtain this product, different strategies have been proposed like spray, vacuum, and microwave-vacuum drying. Authors proposed a novel spray-drying method that allowed the retention of the bioactive compounds and the related health-promoting effects. Sample treatment consisted of a SPE (C_{18}), and 3-phenyllactic and ferulic acids were found at the highest concentrations (greater than3 mg/kg) in the honey powders.

The most employed sample treatment for determining individual phenolic compounds consists of two steps: i) a dilution with water or a mixture of water with an acid (HCl or formic acid); ii) SPE with an anion-exchange sorbent (see Supplementary Material, Table 1S), usually XAD-2. This is a well-known hydrophobic copolymer of styrene–divinylbenzene resin, which sorbs organic compounds, like phenolic compounds (Avino, Cinelli, Notardonato, & Russo, 2011). However, other SPE sorbents have been also chosen, such as C_{18} (Dżugan et al., 2020; Gašić et al., 2015), polymeric (Oasis HLB; Gao et al., 2020), polymeric anion-exchange (Strata-X-A; Sun et al., 2016) and multiwalled carbon nanotubes (MWCNTs; Wabaidur et al., 2015). In one of these publications (Sun et al., 2016), eight different SPE sorbents (reverse phase (RP) and reverse phase-anion exchange (RP-AE)) were evaluated for their suitability for the concentration of flavonoids and phenolic acids in honey prior to their determination by HPLC-PDA-MS (QTOF). Results showed that the RP-AE sorbents generally provided a better performance than the RP ones, and more flavonoids and phenolic acids were found and in a higher content when using RP-AE. Meanwhile, resveratrol, which is the most representative member of the stilbenes and is related to several health-promoting effects (antioxidant, antimicrobial, and antiaging; Ares et al., 2018), was determined among other phenolic compounds like flavonoids and phenolic acids in Croatian sage honey by using SPE (C_{18}) combined with UHPLC-MS/MS (Orbitrap; Gašić et al., 2015). Resveratrol was detected only in four of the 18 analyzed honey samples in a concentration range between 0.08 and 0.46 mg/kg.

However, SPE was not always used when determining phenolic compounds in honey. Conventional solvent extraction (SE) was chosen in some cases (Can et al., 2015; Gulzelmeric et al., 2019), but nowadays, miniaturized preconcentration methodologies are preferred. They fulfil better the principles of green analytical chemistry than SE and SPE, such as simplicity and a reduced consumption of solvents. For instance, dispersive liquid–liquid microextraction (DLLME) was selected as sample treatment when investigating the content of flavonoid aglycone compounds in Spanish honeys (Campillo et al., 2015). Authors optimised the most relevant DLLME conditions, and they were able to determine eight flavonoid aglycones with an HPLC-DAD-MS/MS (QTOF) instrument. A different liquid–liquid extraction approach, which is called sugaring-out assisted liquid–liquid extraction (SULLE) was proposed for the determination of phenolic compounds in Chinese honeys (Zhu et al., 2019). SULLE belongs to the so-called homogeneous liquid–liquid extraction (LLE) methods in which acetonitrile (ACN) is mixed with water to form a homogenous solution to facilitate the extraction of the analytes, and in the case of SULLE, sugars trigger the phase separation in ACN–water mixtures (Chen et al., 2019). In the previously mentioned study (Zhu et al., 2019), seventeen different phenolic compounds (flavonoids and phenolic acids), were determined by HPLC-ECD in honeys from different origins, and it was found that the analytical performance of this method was quite similar or even better that those obtained with more conventional approaches (SPE, LLE). SULLE was also selected as sample treatment for investigating the phenolic profile of Latvian honeys (Rusko et al., 2021). In this case, authors developed a targeted multi-class UHPLC-HRMS (Orbitrap)

method in which a pentafluorophenyl stationary phase was selected that allowed the detection of 11 phenolic acids and 18 flavonoids. The selection of the column was justified by the better chromatographic resolution and peak efficiencies that were obtained when using pentafluorophenyl-based column compared to more conventional C_{18} columns.

One of the most popular miniaturized extraction procedures, QuEChERS (quick, easy, cheap, effective, rugged, and safe), has also been employed (Silva et al., 2019). This is an extraction procedure that involves two stages: an extraction stage, which usually employs ACN, followed by a second stage devoted to cleaning the extract by means of dispersive SPE. In this study, authors proposed a modified QuEChERS method that in combination with HPLC-DAD allowed the determination of several phenolic compounds in Brazilian honeydew honeys. Honeydew honey is a natural product elaborated by *Apis mellifera* bees from plant secretions or excretions of plant-sucking insects, such as aphids. Syringic acid and rutin were the most detected compounds (>65% of samples; 5–65 μg/g), while caffeic and salicylic acids were only detected in one sample each (Silva et al., 2019). Finally, it should be commented a different approach in which a sample preparation strategy based on miniaturized vortex extraction followed by ultrasound assisted extraction employing aqueous-based solvents was proposed for determining phenolic compounds in Spanish honeys from different botanical origins by HPLC-MS/MS (Vazquez et al., 2021). The highest concentration was found in the heather honeys, with total phenolic compounds concentrations reaching 252 μg/g, especially significant was the high content of 3-hydroxyphenylacetic acid (242 μg/g) detected in one heather sample. Meanwhile, p-hydroxybenzoic acid was detected in all the honey samples.

It can be concluded that they are two preferred strategies for determining phenolic compounds in honey samples, but in both cases a dilution of honey with water is recommended (see Table 1). The first involves spectrophotometric-based assays (FCM and $AlCl_3$) and they are used when the objective is determining total flavonoid and phenolic content. Meanwhile, SPE with an anion-exchange sorbent and HPLC or UHPLC in reversed-phase mode combined with MS/MS is the most chosen option when determining individual phenolic compounds. These analyses are more complex than FCM and $AlCl_3$, but more information/data are obtained, and more importantly, it is possible to determine the specific compounds that are present in honey, which could be useful from a quality and nutritional point of view. However, it is not always necessary to use expensive and complicated MS/MS instruments, as due to high concentrations detected, it is enough to use a more routine detector, such as DAD and ECD. In these latter cases, good chromatographic separation of the analytes is necessary to quantify them, while this is not mandatory when using MS/MS.

Table 1
Recommended methodologies for determining specific honey constituents.

Compounds	Sample treatment	Determination technique
Phenolic compounds (TFC, TPC flavonoids and phenolic acids)	i) DI (Water/HCl/Buffer)ii) FCM ^{TPC}, $AlCl_3{}^{TFC}$ii) SPE (XAD-2 resin)iii) Elution (MeOH)	UV–Vis$^{TFC\ and\ TPC}$ HPLC/UHPLC-DAD-MS/MS$^{flavonoids,\ phenolic\ acids}$
Sugars (ISGRC, ISGRS and TSGRC)	DI (Water)	HPLC-RID
AAs and proteins	i) DI (Water)ii) BA (CBBS)^{TPROC}iii) DV (FDR)^{AAs}	UV–Vis^{TPROC} HPLC/UHPLC-DAD-FLD-MS/MS^{AAS}
Vitamins	DI (Water/acid) orSE (Water/acid)	UV–Vis HPLC/UHPLC-DAD
Lipids and related compounds (FAs, TCARC and TERPRCs)	DI (Water/acid) orSE (AC, EA or HX)	UV–Vis^{TCARC} GC–MS/MS$^{FAs,\ TERPRCs}$ HPLC/UHPLC-MS/MS^{TERPRCs}
Other compounds (minerals and organic acids)	i) AD (HNO_3)^{minerals}ii) DI (Water)^{All}	ICP-AES/MS or AAS^{minerals} HPLC/UHPLC-DAD-MS/MS$^{organic\ acids}$

AAs, amino acids; **AAS,** atomic absorption spectroscopy; **AC,** acetone; **AD,** acid digestion; **AES,** atomic emission spectrometry; **$AlCl_3$,** aluminun trichloride method; **BA,** Bradford assay; **DAD,** diode array detector; **DI,** dilution; **EA,** ethyl acetate; **EtOH,** ethanol; **EV,** evaporation; **FAs,** fatty acids; **FDR,** fluor-derived reagents; **FCM,** Folin Ciocalteu method; **FLD,** fluorescence detector; **GC,** gas chromatography; **HPLC,** high-performance liquid chromatography; **HX,** hexane; **ICP,** inductively coupled plasma; **ISGRs,** individual sugars; **MS/MS,** tandem mass spectrometry; **NMR,** nuclear magnetic resonance; **SE,** solvent extraction; **SFN,** sulforaphane; **SPE,** solid-phase extraction; **TCARC,** total carotenoid content; **TERPRCs,** terpenes and related compounds; **TFC,** total flavonoid content; **TPC,** total phenolic content; **TSGRC,** total sugar content; **UHPLC,** ultra-high performance liquid chromatography; **vitamins,** vitamins; **WAX,** weak anion exchange.

2.2. Sugars and related compounds

Sugars are the major component of honey (>85%; Elamine et al., 2021), and their main function in the organism of living beings is to act as a source of energy. More than 22 sugars have been found in honey, fructose and glucose being the most abundant sugars. The sum of fructose and glucose, fructose/glucose (F/G) and glucose/water (G/W) ratios are important factors related to honey quality. F/G ratio indicates the ability of honey to crystallize, although the G/W ratio may also predict honey crystallization (El Sohaimy, Masry, & Shehata, 2015). The specific sugar content should be considered to evaluate the possibility of adulteration of honeys. A high value of sucrose in honey means that beekeeper removed the honey from the hive too soon, as sucrose content should be not higher than 5% on a mass basis (Pereira et al., 2020).

Two main strategies have been followed for determining sugar content in honey (see Supplementary Material, Table 2S). Some authors have focused their attention in evaluating the total sugar content, and in particular, the reducing sugar content by titration (Ramón-Sierra, Ruiz-Ruiz, & Ortiz-Vázquez, 2015; Sanťana, De Carvalho, Oda-Souza, Souza, & Dias, 2020) or colorimetric assay by using dinitrosalicylic acid (Villacrés-Granda et al., 2021). The second approach is to measure the content of the different reducing sugars, like glucose, fructose and/or maltose (Al-Farsi et al., 2018; Bonhevi et al., 2019; Can et al., 2015; De Beer, Otto, Pretoruis, & Schönfeldt, 2021; Domínguez, Jacksén, Emmer, & Centurión, 2016; Dong, Xiao, Xian, & Wu, 2018; El Sohaimy et al., 2015; Geană, Ciucure, Costinel, & Ionete, 2020; Pascual-Maté et al., 2018b; Pereira et al., 2020; Villacrés-Granda et al., 2021). The latter approach is more complex, as sugars are individually determined. Sample treatment consisted in most cases of a dilution with water, except for the titration-based works (Ramón-Sierra et al., 2015; Sanťana et al., 2020) and those studies in which GC was selected as the separation technique (Kozłowicz et al., 2020; Pascual-Maté et al., 2018b; Przybylski & Bonnet, 2021). Nevertheless, GC was rarely used in comparison with HPLC, as non-volatile sugars need to be derivatised prior to GC and the overall analysis times are usually longer than for HPLC.

HPLC has been predominantly coupled to a refractive index detector (RID), and different stationary phases have been selected, like amide, or others specifically designed for carbohydrate analysis (Agdaba et al., 2020; Belay et al., 2017; Boussaid et al., 2018; Can et al., 2015; Chuttong, Chanbang, Sringarm, & Burgett, 2016; De Beer et al., 2021; Elamine et al., 2021; El Sohaimy et al., 2015; Kanbur, Yuksek, Atamov, & Ozcelik, 2021; Leyva-Daniel et al., 2020; Pereira et al., 2020; Se, Ibrahim, Wahab, & Ghosdal, 2018; Villacrés-Granda et al., 2021). Dong et al. (2018) investigated a few Chinese honey samples that were adulterated with sugar or sweeteners by using IRMS coupled to elemental analyzer and HPLC. The goal was to evaluate the potential of these techniques to supplement an official C-4 sugar method for detecting honey adulterations with sugar cane or corn syrups (Official Method (1999)). An evaporative light scattering detector was selected for determining the sugar composition of Romanian honeys (Geană et al., 2020). It allows

the detection of analytes without UV chromophore groups, while offers excellent sensitivity. The most dominant sugars in the analyzed honeys were fructose and glucose, with percentages ranging from 15% to 50%, while sucrose was not detected or detected at trace levels.

A different approach in which sugars were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) was followed in several publications (Al-Farsi et al., 2018; Gašić et al., 2015; Vasić et al., 2019). HPAEC is a is a well-established technique for carbohydrate determinations, and the reasons for this choice are related to the high resolution of the separations that are often not possible using other techniques, and that PAD is sensitive enough to allow the determination of lower concentrations of carbohydrates . In one of these HPAEC/PAD-based studies (Gašić et al., 2015), fructose and glucose were the predominant sugars in Croatian honeys, the sum of glucose plus fructose being similar or higher than 600 g/kg.

Capillary electrophoresis (CE) can also be used to determine sugars. This technique offers the advantages of low sample volume, low consumption of solvents, quick analysis, as well as high resolution with minimal sample preparation. Domínguez et al. (2016) achieved the separation and determination of glucose, fructose, and sucrose by CE-DAD in less than five minutes and with a single dilution of the honey as the sample treatment. Sucrose was not detected in any of the analyzed honeys (Argentina, Brazil, and Sweden). Finally, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) was employed for determining disaccharide isomers by comparing dissociation patterns (Lee et al., 2020). Graphene oxide was used as a MALDI-MS matrix for the analysis of seven disaccharides in honey samples from South America, South Korea and Vietnam, and the results demonstrated the potential of this technique for quantifying disaccharide isomers in complex matrices.

To sum up, sugar analysis has been extensively performed during the last years, often for determining honey authenticity. Titration and colorimetric methods were used in some cases for evaluating the total sugar content, although the preferred option to calculate total sugars was to sum the individual components. HPLC-RID analysis of diluted (water) honey extracts seems to be the most widely used choice for determining individual sugars in honey (see Table 1), although it should not be forgotten the potential of HPAEC and CE for performing this task. HPAEC-PAD offers high resolution and excellent sensitivity while the benefits of CE with DAD include minimal consumption of solvents and small sample size. Finally, it should be remarked that the use of MS or MS/MS detectors for determining sugars was minimal, and it could be explained by the fact that those compounds are present in high concentrations in honey or because of the poor ionisation and nonlinear response of sugars in the mass spectrometer.

2.3. Amino acids and proteins

Proteins and AAs are present in the nectar or honeydew and in secretions of bees, bee pollen being the main source of AAs and proteins in honey (Kowalski, Kopuncová, Ciesarová, & Kukurová, 2017). Indeed, the AA content in honey has been used in several studies for determining the botanical origin of honey (Cebrero et al., 2020). It should be remarked that proline is the dominant free AA (FAA) in honeys, as it represents more than 50% of the total FAAs (Fröschle et al., 2018). Moreover, proline content of honey is a quality factor and an indicator of maturation, and in some cases, it allows detection of adulteration. Several works have been published in the last years in which AAs and proteins were investigated in honey (see Supplementary Material, Table 3S), and the methodologies were quite different depending on the compound.

For example, total protein content has been determined in several publications by using three different approaches, Kjeldahl (El Sohaimy et al., 2015), Bradford (Cebrero et al., 2020; da Costa & Toro, 2020; Kováčik et al., 2016; Ramón-Sierra et al., 2015; Valdés-Silverio et al., 2018; Zhang et al., 2019), and AOAC (Dong et al., 2018; Vasić et al., 2019). The Kjeldahl method has been used for more than 100 years for determining nitrogen in a wide range of samples and for the calculation of the protein content. This method can be basically divided into three stages: digestion or mineralisation, distillation, and titration (Ares et al., 2018). The protein content can be calculated in food matrices, assuming a ratio between the protein and nitrogen for the specific food being tested (Total Nitrogen × 6.25), although some authors stated that a 6.25 factor may overestimate protein content and preferred 5.60. This is explained because nitrogen might not only be of protein origin, but could be due to AAs (Rebelo, Ferreira, & Carvalho-Zilse, 2016). A different approach was proposed by Bradford (1976), and it is extensively used because it is faster and very reproducible. The determination of proteins in honey, which has been previously diluted with water or a buffer, by the Bradford method relates to the quantification of the binding of a dye, Coomassie blue G-250, to the protein, comparing this binding with that of different amounts of a protein standard (bovine serum albumin). The quantification is done by measuring the absorbance at 595 nm (Ares et al., 2018). The third method is based on the internationally accepted legislation (Official Method, 1999) for identifying the C-4 sugar adulteration of honey (Dong et al., 2018). It is based on the differences in the relationship between carbon-13 and carbon-12 of C4 plants from monocotyledonous species of cane sugar and corn, compared to dicotyledonous species (C3 plants). The carbon isotope ratios of honey samples and their extracted proteins are measured by elemental analyzer isotope ratio mass spectrometry (EA-IRMS) and LC-IRMS. There is an agreement that the difference between the value of $\delta^{13}C$ of the protein and the honey should not be higher than 1% (Dong et al., 2018; Vasić et al., 2019).

FAAs have been determined in honey by HPLC/UHPLC coupled to fluorescence detection (FLD; Biluca et al., 2019; Fröschle et al., 2018), DAD (Bonhevi et al., 2019; Fröschle et al., 2018; Zhao et al., 2016), MS (Biluca et al., 2019) or MS/MS (Kowalski et al., 2017; Mannina et al., 2015). Sample treatment consisted of a dilution with water, acidified water, or a buffer, although it must be also specified that FAAs have been usually derivatized when using FLD and DAD by employing fluoro-containing reagents (9-fluorenylmethyl chloroformate, FMOC-Cl; Fluor reagent) and *o*-phthaldialdehyde (OPA). Biluca et al. (2019) used two different detectors (FLD and MS) for determining FAAs in Brazilian honeys. Results showed that proline and phenylalanine were found in all samples over a wide concentration range (5–1231 mg/kg), while histidine was not detected in any of them. As it was previously mentioned, proline is the main FAA in honeys. Consequently, it is not surprising that proline was specifically determined in some publications (Al-Farsi et al., 2018; Domínguez et al., 2016; Fechner, Moresi, Ruiz Díaz, Pellerano, & Vázquez, 2016). In most of these studies, proline was determined by an official method (Official Method, 2005). This procedure consists of a honey dilution with a mixture of formic acid and water, and afterwards, a ninhydrin solution is added. The mixture is heated, and isopropanol added to the solution and mixed. The resulting product of the reaction is measured by UV–Vis at 520 nm (Fechner et al., 2016). A similar approach was followed by Valdés-Silverio et al. (2018) for determining the total FAA content, but in this case, a Cd-ninhydrin reagent was employed. An alternative to the official method could be CE-DAD (Domínguez et al., 2016). In this study authors analyzed six commercial honey samples from Argentina, Brazil and Sweden, and proline was detected in all samples in concentrations higher than 200 mg/kg.

An alternative methodology based on GC–MS (Azevedo et al., 2017) was also proposed for determining FAAs in Brazilian honeys. The first step of the sample treatment consisted of a dilution with water, but in this case, a derivatization was required prior to the GC analysis, consisting of SPE followed by the addition of an alkyl chloroformate reagent. Although the sample treatment was more complex than those required for HPLC-MS, the chromatographic run was shorter, as more than 30 FAAs were separated in<7 min. Glutamic acid was the FAA detected at the highest concentrations in most of the samples (110–1340

mg/kg). Finally, ^{1}H NMR has also been used for investigating the FAA content in honeys (del Campo, Zuriarrain, Zuriarrain, & Berregi, 2016; Mannina et al., 2015; Zheng, Zhao, Wu, Dong, & Feng, 2015). In the last decade, this technique has gained attention in food chemistry due to its advantages over other analytical techniques, as it is rapid, non-invasive, non-destructive, and it requires minimal sample preparation (Laghi, Picone, & Capozzi, 2014). In one these studies (Zheng et al., 2015), the NMR quantification of phenylalanine and tyrosine in Chinese honeys that were sampled at different times allowed the authors to point out a relationship between the Maillard reaction and a decrease of their content with the storage time. However, the highest concentrations in the honeys were observed for threonine, with values between 2.6 and 3.9 mg/g.

It can be concluded that different methodologies have been proposed depending on the compound to be analyzed (see Table 1). Proteins have been determined by three main methods (Kjeldahl, Bradford and IRMS), and according to the overall performance of these proposals, it seems that the simplest and most economical option is the Bradford method, although it should not be forgotten that the use of IRMS allows the detection of potential adulteration of honey. For FAAs, HPLC/UHPLC was predominantly selected for studying their content in honey. However, to use economical detectors, like DAD or FLD, requires a derivatization step; MS and MS/MS are a more expensive alternative, but they have better sensitivity, selectivity and they do not require derivatization of the analytes. Derivatization is also necessary when using GC–MS, but in this case, the analysis time was significantly shorter than that provided by HPLC. CE could be considered as a cheaper alternative to HPLC, and it should not be forgotten the potential of ^{1}H NMR for determining FAAs. The analysis is rapid and could be done with minimal sample treatment, but it is more expensive and complex than conventional HPLC.

2.4. Vitamins

Vitamins are complex organic substances, biologically active and with diverse molecular structure. As can be seen in Table 4S (see Supplementary Material), several vitamins have been found in honey samples (B, C, E and K), although most of the attention was focused on investigating vitamin C. This latter is a water-soluble nutrient found in certain foods. In the body, it acts as an antioxidant, helping to protect cells against damage caused by free radicals (Ares et al., 2021). Vitamin B2 (riboflavin), which is also a water-soluble compound, is important for the body's growth, helps in the production of red blood cells, and it also aids in the release of energy from proteins (Ares et al., 2018). Meanwhile, folic acid (vitamin B9), which is another water-soluble vitamin, helps the body in maintaining and creating new cells. Vitamin E and vitamin K2 are both fat-soluble vitamins with different functions. Vitamin E, which has a powerful antioxidant effect (Sawicki et al., 2020), is a family of eight structurally similar antioxidants that are divided into two groups (tocopherols and tocotrienols), while vitamin K2 is important in the body's use of calcium to help build bones and inhibit blood vessel calcification. All vitamins have been predominantly determined in honey by spectrophotometric/colorimetric and HPLC-based methods. The main differences between the proposed methods are related to the sample treatment, as several procedures were employed, such as a honey dilution with several water mixtures and different reagents/solvents (Álvarez-Suárez et al., 2018; Jahan, Islam, Alam, Gan, & Khalil, 2015; Leyva-Daniel et al., 2020; Majkut et al., 2021; Ranneh et al., 2018; Villacrés-Granda et al., 2021), SE (Guo et al., 2019; Mouhoubi-Tafinine, Ouchemoukh, & Tamendjari, 2016; Sawicki et al., 2020), SPE (Mannina et al., 2015) or a combination of size exclusion chromatography (*SEC*) with SPE (Kim & Brudzyski, 2018).

The simplest procedures to determine vitamin C were those that involved a dilution with an aqueous solution (Álvarez-Suárez et al., 2018; Jahan et al., 2015; Ranneh et al., 2018; Villacrés-Granda et al., 2021). In one of these works (Jahan et al., 2015), a colorimetric method was employed, which was based on the conversion of ascorbic acid to dehydroascorbic acid by means of the interaction with a hydrazine derivative (to give a brownish red color). Vitamin C was detected in all samples in a concentration range between 80 and 200 mg/kg. Meanwhile, the same HPLC-DAD approach was selected in two other works (Álvarez-Suárez et al., 2018; Villacrés-Granda et al., 2021). Honey was diluted with metaphosphoric acid, but in one method (Álvarez-Suárez et al., 2018), the use of dithiothreitol was also required for the reduction of dehydroascorbate to ascorbate. Vitamin C was not detected in most of the Cuban honey samples studied, and when it was found, its content was quite variable (1–64000 μg/g). The effect of high hydrostatic pressure on the compound composition in honey, including vitamin C, has been also investigated (Leyva-Daniel et al., 2020). Results showed that this procedure did not provoke significant changes in the vitamin C content in comparison with unprocessed honeys. SE was used in two works (Guo et al., 2019; Mouhoubi-Tafinine et al., 2016). Citric and metaphosphoric acids were chosen as extractants, and the resulting extracts were mixed in all cases with 2,6-dichloroindophenol prior to measuring the absorbance of the solution. In one of these works (Guo et al., 2019), vitamin C was investigated in Chinese honeys not only for its antioxidant activity, but also because this vitamin could accelerate the removal of alcohol in the blood. Finally, vitamin C and vitamin E (tocotrienols and tocopherols) were determined in Polish honey by μHPLC-MS/MS (TOF) and HPLC-FLD, respectively (Sawicki et al., 2020). Sample treatment consisted of a simple extraction with metaphosphoric acid (vitamin C) or methanol (vitamin E). Authors concluded that the vitamin C content of the analyzed honey samples was much lower (2 μg/g) than previously reported values in honey from other countries (often greater than 50 μg/g), and they related this finding with differences in the extraction method. Meanwhile, tocopherols (430 μg/g) were mainly responsible for the level of vitamin E in honey (greater than95%).

Vitamin B2 and its metabolite (lumichrome) were investigated in Italian honey (Mannina et al., 2015). They were simultaneously studied with several other compounds, and this could explain the complexity of the analytical method in relation to those employed for vitamin C. Honey was diluted with formic acid, and then the resulting extract was passed through an SPE (C_{18}) cartridge. Compounds were eluted with methanol and after an evaporation step, they were determined by means of HPLC-PDA-MS/MS (IT).

Vitamin B9, was determined by reverse-phase HPLC-UV after being diluted with water, phosphate buffer and a sodium hydroxide solution (Álvarez-Suárez et al., 2018). Authors of this study concluded that there were no significant differences in the vitamin B9 content among the analyzed Cuban polyfloral honey samples, as the values were always close to 0.08 μg/g.

Vitamin K2 homologues (menaquinones) were identified for the first time in honey from New Zealand by using different analytical tools (UV–Vis, UHPLC-DAD, UHPLC-MS/MS; Kim & Brudzyski, 2018). The proposed sample treatment was complex, as it involves an *SEC* step, prior to determining the UV spectral profile of the collected fractions. It was followed by SPE with polymeric cartridges and a subsequent UHPLC analysis of the extracts. Results provided evidence of menaquinones as constituents of high molecular weight fractions of honeys, which displayed both antibacterial and antioxidant activities.

As can be deduced from the related literature, vitamins have been scarcely investigated in honey. Perhaps it could be due to the difficulty of their analysis as some are lipid-soluble while other are water-soluble. Among them, vitamin C attracted more attention than the others according to the number of publications. It is also remarkable that vitamins have been individually determined in honey with only one exception (Mannina et al., 2015). This makes quite difficult to propose a general methodology for determining vitamins. However, it can be postulated that a dilution with an aqueous solvent or a SE are good enough as sample treatments for most of vitamins, and that UV-based methods (colorimetric or HPLC) are also the simplest and cheapest option for quantifying vitamins in honey (see Table 1). Nevertheless,

HPLC-MS/MS could be an alternative when a structural confirmation is required, or vitamins are present at a low concentration.

2.5. Lipids and related compounds

Lipids are of crucial importance for the storage of energy and the development of the cell membrane. It must be specified that some essential lipids must be obtained from the diet, and honey could be considered as a potential source of lipids. The lipid fraction of honey mainly contains terpenes and related compounds (TERPRCs) that are usually known as terpenoids, including carotenoids (CARS), and fatty acids (FAs). TERPRCs, which are classified as non-saponifiable lipids or simple lipids, are aromatic and volatile organic compounds that are composed of different units of isoprene; for that reason, they are also known as isoprenoids. They give the organoleptic characteristics (aroma and flavor) of plants, and they exert several benefits for human health such as antibacterial and antioxidant effects (Ares et al., 2021). CARs, which belong to the tetraterpene family are organic pigments that are found naturally in plants, and they have some health-promoting effects, such as antioxidant or anticancer (Bernal, Mendiola, Ibáñez, & Cifuentes, 2011). Meanwhile, FAs are an essential part of the composition of most fats and oils, and a special class of FAs, hydroxy FAs (OH-FAs), can play important roles in many physiological processes of living organisms, such as indicating the status of lipid oxidative degradation pathways and exhibiting antibacterial and anticancer activities (Zhu, An, & Feng, 2020).

According to the examined literature (see Supplementary Material, Table 5S), lipids and related compounds have been investigated in several publications, and specifically, fatty acids (FAs; Karlidag, Keskin, Bayram, Mayda, & Ozkok, 2021; Leoni et al., 2021; Zhu et al., 2020), and terpenes and related compounds (TERPRCs), including terpenoids and carotenoids (CARs; Karlidag et al., 2021; Kim & Brudzyski, 2018; Leoni et al., 2021; Leyva-Daniel et al., 2020; Mannina et al., 2015; Mouhoubi-Tafinine et al., 2016; Ouradi et al., 2020; Petretto et al., 2017; Valdés-Silverio et al., 2018). As a difference with other honey constituents, a common analytical methodology for determining lipids cannot be found, as it is quite dependent on each family.

FAs have been determined in Turkish honeys with a methodology that consisted of a honey dilution with methanol and a further analysis by GC–MS (SQ; Karlidag et al., 2021). Several FAs were detected in the analyzed honeys, the highest concentrations being determined for stearic acid (320 mg/kg). OH-FAs have been also investigated in honey (Leoni et al., 2021; Zhu et al., 2020). In one of the studies, Italian honey samples were directly analysed by means of a headspace-GC–MS system (Leoni et al., 2021), which has the advantage of the lack of sample treatment. Authors related the highest antioxidant activity and cicatrising activity of the honeys with the presence of OH-FAs. On the contrary, a complex sample treatment was required in the other research (Zhu et al., 2020). It consisted of a dilution of the honey with water and formic acid, and after that, OH-FAs were sequentially extracted with ethyl acetate, methyl *tert*-butyl ether, and dichloromethane. The OH-FAs were first screened using chemical isotope labelling-assisted UHPLC-MS/MS (orbitrap), and then, OH-FAs were studied under collision-induced dissociation, which is helpful to discriminate and identify the hydroxyl position of OH-FAs isomers. Authors were able to annotate 97 of the potential 107 isomers detected in Chinese honeys, of which 23 are newly reported.

TERPRCs have been determined in honey samples by using quite different approaches of a variable complexity. The simplest procedure consisted of simple dilution with methanol, and a further analysis by GC–MS (SQ; Karlidag et al., 2021). A different approach was followed by Kim and Brudzyski (2018), as in this case, TERPRCs (monoterpenes) were extracted from an aqueous honey solution by using ethyl acetate as extractant. The isolated compounds were determined by UHPLC-MS/MS (QTOF) in South Korean honeys in a wide range of concentrations (0.1–100 μg/kg). In another study, a norisoprenoid (abscisic acid), which is widely distributed in many honey varieties, was identified among other bioactive compounds by HPLC-PDA-MS/MS (IT) in Italian honeys (Mannina et al., 2015). Sample treatment consisted of two steps, being the first one being a dilution of the honey with formic acid in water, and then SPE was performed with a C_{18} sorbent. In a further study, stir bar sorptive extraction (SBSE) combined with GC–MS (SQ) was selected (Petretto et al., 2017). SBSE requires the use of a magnetic stir bar coated with a film of stationary extraction phase, which is usually composed of on polydimethylsiloxane. Authors compared the performance of SBSE with other alternatives, such as solid-phase microextraction (SPME), and dynamic headspace, and the results showed that SBSE exhibited better sensitivity, although the extraction times were higher. Nevertheless, headspace-SPME coupled to GC–MS (SQ) has proven to be also useful for investigating the volatile, including TERPRCs, profile of Italian honeys (Leoni et al., 2021). Several terpenes were identified like (Z)-rose oxide, α-terpinene, linalool, and cymene, while the total terpene content ranged between 13 and 47 μg/kg. CARs have been also investigated in honey samples from different origins (see Supplementary Material, Table 5S). Total carotenoid content (TCARC; Mouhoubi-Tafinine et al., 2016, Ouradi et al., 2020; Valdés-Silverio et al., 2018) has often been determined. The procedure was quite similar in all cases. Firstly, CARs were extracted with a mixture of acetone and hexane (Valdés-Silverio et al., 2018) or also with ethanol under basic conditions (Mouhoubi-Tafinine et al., 2016; Ouradi et al., 2020). Then, TCARC was determined by measuring the absorbance of the extracts and using β-carotene for the calibration curve. Some CARs (epoxycarotenoids and carotenes) were determined by HPLC-PDA after being extracted from honey with hexane containing butylated hydroxytoluene (Leyva-Daniel et al., 2020). The highest CAR concentration was found for violaxanthin (1 mg/kg) in Mexican honeys.

It can be concluded that selecting the analytical methodology to determine honey lipids and related compounds is strongly dependent on the lipid class. However, it is true that dilution of honey or a SE are usually employed to treat the honey samples for the different families of lipids, although the nature of the extractant differed (see Table 1). In addition, there are also differences in the determination method depending on the class of lipids and purpose of the study. TCARC has always been calculated spectrophotometrically (UV–Vis), which is a simple and relatively cheap procedure; however, separation has been required to ascertain individual components. GC is the technique of choice when investigating the FAs content, while HPLC or GC are equally selected for the determination of TERPRCs. In this case the choice depends on the nature (volatile or not) of the analytes and other compounds that were simultaneously analysed in some studies. On the other hand, there is no discussion about the selection of the detector, since the use of MS and MS/MS was ubiquitous. This could be directly related by the fact that lipids are usually present at lower concentrations, which requires the use of sensitive detectors. In addition, the main goal of some of the studies was to investigate the profile or to identify new compounds, and the best choice to achieve this objective is MS/MS.

2.6. Other compounds

2.6.1. Minerals

Minerals are present in honey at low percentages between 0.04% and 0.20%, depending on the botanical origin (Karabagias, Louppis, Kontakos, Papastephanou, & Kontominas, 2017). Some of these compounds have various beneficial functions for human health as they are fundamental for maintaining homeostasis and cell protection (Ares et al., 2018). On the other hand, some heavy metals like arsenic, cadmium, lead, and mercury are toxic if they are present in amounts that exceed the maximum tolerable limits. The mineral composition of honey also depends directly on the botanical origin of the honey, and subsequently, it can be also used to determine the geographical and botanical origin of honey. More than 54 different minerals have been determined in honey (Liu et al., 2021), potassium being the most abundant, representing

more than 30% of the total mineral content. Sodium, calcium, magnesium, and iron are also abundant. As can be seen in Table 6S (see Supplementary Material), a wet acid digestion with nitric acid alone (El-Haskoury, Kriaa, Lyoussi, & Makni, 2018; Karabagias et al., 2017, 2020; Laaroussi, Bouddine, Bakour, Oussaid, & Lyoussi, 2020; Louppis, Karabagias, Papastephanou, & Badeka, 2019), or in combination with perchloric acid (Kadri, Zaluski, & Orsi, 2017; Paul et al., 2017) or hydrogen peroxide (Di Rosa, Leone, Cheli, & Chiofalo, 2019; Gašić et al., 2015; Liu et al., 2021; Uršulin-Trstenjak et al., 2017; Voica, Iordache, & Ionete, 2020; Zerrouk, Seijo, Escuredo, & Rodríguez-Flores, 2018), was selected as sample treatment when determining minerals in honey, and it is predominantly followed by a dilution with water.

The determination techniques of choice were atomic absorption spectroscopy (AAS; El-Haskoury et al., 2018; Kadri et al., 2017; Paul et al., 2017; Zerrouk et al., 2018), or inductively coupled plasma (ICP), the latter linked to either optical emission spectrometry (OES; Gašić et al., 2015; Karabagias et al., 2017; Karlidag et al., 2021; Louppis et al., 2019; Liu et al., 2021), atomic emission spectrometry (AES; Laaroussi et al., 2020), or MS (Di Rosa et al., 2019; Uršulin-Trstenjak et al., 2017). Both determination methods (ICP and AAS) have their advantages and disadvantages. For example, AAS is faster and cheaper than ICP, although it has the disadvantage that only one element can be analyzed per analysis. On the contrary, ICP systems allow the simultaneous analysis of several elements, so that although the single analysis time is longer than for AAS, information can be obtained for many elements. ICP was used to characterize the botanical origin of Greek honeys on basis on the mineral content (Louppis et al., 2019). Authors determined the mineral content by ICP-OES and concluded that the use of different chemometric tools like factor analysis or linear discriminant analysis, among others, allowed the perfect classification of all the honeys (100%) according to the individual and total mineral content. In most samples the lead content was higher than the regulated limit (10 mg/kg), which implies that beekeeper's strategies should be improved. In other publication (Paul et al., 2017), different AAS modes were selected depending on the element. Arsenic was measured in honey using the graphite furnace technique, and mercury was measured using cold vapor AAS; while all the other elements (sodium, potassium, calcium…) were determined using a direct absorption technique (flame). Cold vapor AAS is the most common technique for mercury determination because the mercury compounds can be reduced to elemental mercury that occurs as vapor, while all other metals are solid at room temperature. Moreover, graphite furnace AAS was usually selected for determining arsenic as it provides excellent sensitivity and reduces the interference problems associated with other approaches. The most abundant elements were potassium, sodium, calcium, and magnesium (Paul et al., 2017), which were detected in all honey samples (Bangladesh) in a wide concentration range (0.13–2220 mg/kg), and potassium was usually detected at the highest concentrations (>550 mg/kg). Lead was found in all samples, but at low concentrations (<3 mg/kg).

Thus, it can be concluded that to determine minerals in honeys the best option would be to perform an acid digestion with nitric acid followed by an AAS or ICP analysis depending on whether the analysis is of a single mineral or group of minerals, and the nature of the element (see Table 1). However, the detector choice is an important factor when using ICP, as MS provides higher linear range and lower quantification limits but at a highest cost, while better results at higher concentration are usually obtained when using AES/OES. On the other hand, different atomization techniques could be used in AAS: flame atomizers are the simplest and fastest option; cold vapor, and graphite furnace AAS present a higher sensitivity, and in the case of graphite furnace, the interferences were reduced in comparison with other AAS techniques.

2.6.2. Organic acids

Many organic acids are metabolic intermediates and end products of microbial metabolism and are found in large quantities in fermented foods. They have some health-promoting properties such as antidiabetic, antimicrobial, and antioxidant (Ares et al., 2021). Organic acids determination in honey is not only justified by their bioactivity; they can be considered as markers of honey authenticity, and subsequently, as indicators of adulteration/fraud (Seraglio et al., 2021b). Several analytical techniques have been used to study organic acids in honey (see Supplementary Material, Table 6S), such as HPLC (An et al., 2020; Mannina et al., 2015; Suto, Kawashima, & Nakamura, 2020; Suto, Kawashima, & Suto, 2019; Villacrés-Granda et al., 2021), ion chromatography (IC; Matysiak, Balcerzak, & Michalski, 2018), ^{1}H NMR (del Campo et al., 2016), and capillary electrophoresis (CE; Seraglio et al., 2021b).

The sample treatment was quite similar in most cases. It consisted of a honey dilution with water or acidified water. Exceptions were An et al. (2020), who used solvent extraction, while Mannina et al. (2015) used SPE with a C_{18} sorbent. An et al. (2020) proposed a method in which three extractions were performed sequentially with different solvents, ethyl acetate, methyl-*tert*-butyl ether, and dichloromethane. This extraction method in combination with stable isotope labelling assisted HPLC-MS was an effective strategy for determining organic acids in Chinese honeys from different botanical origins as more than 490 potential organic acids were detected. The determination of such a high number of organic acids is not common, as usually, less than ten organic acids were simultaneously detected. For example, Seraglio et al. (2021b) employed a CE-DAD based method to evaluate the aliphatic organic acids content in Brazilian honeydew honeys. Authors detected 9 organic acids in the 60 analyzed samples, lactic, malic and gluconic acids being predominant, and the highest concentration was detected for gluconic acid (1432 mg per 100 g). A DAD was also selected in two other publications (Mannina et al., 2015; Villacrés-Granda et al., 2021), but in those works, reverse-phase HPLC was employed for separating the organic acids. MS detectors were also employed in two studies (Suto et al., 2019, 2020). In the first of the studies, Suto et al. (2019), the authors developed an interesting strategy based on heart-cutting two-dimensional liquid chromatography/isotope ratio mass spectrometry, to determine the honey authenticity/origin, based on their organic acidś stable carbon isotope ratios. This is challenge when using conventional HPLC as the isotope ratios are affected by the presence of carbohydrates. Hence, organic acids were first separated from the carbohydrates by a size-exclusion column (first dimension), and then, they were separated from each other by a reverse-phase column (second dimension). Authors focused their attention mainly on gluconic acid, although malonic and citric acid were also investigated. Gluconic acid, which is a glucose oxidation product, was also studied among other organic acids in the second of the works (Suto et al., 2020) with a more conventional HPLC-MS/MS (QQQ) approach. Results showed that gluconic acid was the predominant organic acid followed by citric and malic acids in 25 honeys from several countries. The average gluconic concentration was much higher (2995 mg/kg) than for the other organic acids (<150 mg/kg).

The quantitative analysis of organic acids has been also performed by employing other methods. For example, ^{1}H NMR has been employed to quantify organic acids among other compounds in Spanish honeys (del Campo et al., 2016). Results demonstrated that the composition of organic acids showed a great variability depending on the honey type, with concentrations ranged from 7 to 182 mg/kg, and the highest value was obtained for malic acid.

Finally, ion chromatography with conductimetric detection was employed for determining formic acid in Polish honeys (Matysiak et al., 2018). Formic acid content in foods like honey should be monitored as it could be toxic for humans at high amounts. Authors demonstrated that their procedure allowed rapid and selective evaluation of the content of formates in honey samples, using an anionic separation column, which was required to separate formate from other common honey ions (acetate), and conductimetric detection. Moreover, the formic acid content found was within the established levels (17–284 mg/kg) and subsequently did not represent a risk to human health.

To sum up, the determination of organic acids in honey has been

performed using several different methods, which presents some advantages and disadvantages depending on the technique of choice, but considering the simplicity, rapidness, and quality of the results, then an analytical methodology that involved a honey dilution and a further analysis by HPLC-DAD can be proposed (see Table 1). However, attention should be paid to the number of organic acids, expected concentration or research goals, because they could directly influence the decision about the most adequate analytical strategy. For example, the use of MS/MS has gained attraction in the last years for determining organic acids in honey due to its high selectivity and sensitivity, and the performance could be improved if it is employed in combination with UHPLC or two-dimensional HPLC, although both options are more complex and expensive. CE-DAD could be a cheaper alternative to HPLC-DAD, as lower solvent consumption is required, while ion chromatography has the attractive feature of allowing the direct analysis of organic acids in the presence of inorganic anionic species in complex samples. Both options have a similar cost and complexity to HPLC-DAD. Finally, the use of NMR is recommended when the objective is not only to quantify organic acids, but also to investigate their molecular structure, but NMR is complex and expensive than HPLC-DAD.

3. Conclusions

The determination of the honey constituents with potential health and nutritional benefits not only contributes to increasing the market value of honey or the consumerś preference but is also useful for establishing criteria for the discrimination of the geographical origin of honey by proposing markers of geographic origin that facilitate control/authentication tasks. According to the recent literature, phenolic compounds, sugars, and AAs have been predominantly determined in honey. The analytical methods (sample treatment and determination techniques) are strongly dependent on the compound, as for example, mineral analysis requires an acid digestion and a further analysis by ICP or AAS, while sugars were usually determined by HPLC-RID after diluting the honey with water. Nevertheless, it can be concluded that HPLC coupled to UV-based detectors was predominantly selected for determining honey constituents (see Fig. 4), although other techniques like GC, CE, NMR, ICP and AAS have been also successfully employed for performing this task. In relation to the sample treatment, the simplest option was a dilution of the honey with water or a buffer, followed by SPE and SE (see Fig. 5). Honey constituents continue to be of great interest for different sectors of the society (beekeepers, consumers, authorities). In the coming years, more compounds will be identified/investigated in honey, and particular attention may be paid to some of the most recently detected, such as miRNAs. The use of advanced analytical techniques, such as those described in this work, would be required. It is also expected that the use of MS/MS detectors will be extended due to their selectivity and sensitivity. Although keeping the cost of analysis as low as possible and proposing environmentally friendly methods should be also a priority.

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CRediT authorship contribution statement

Silvia Valverde: Conceptualization, Methodology, Investigation, Writing – original draft. **Ana M. Ares:** Conceptualization, Methodology, Investigation, Writing – original draft. **J. Stephen Elmore:** Conceptualization, Visualization, Writing – review & editing. **José Bernal:** Conceptualization, Project administration, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

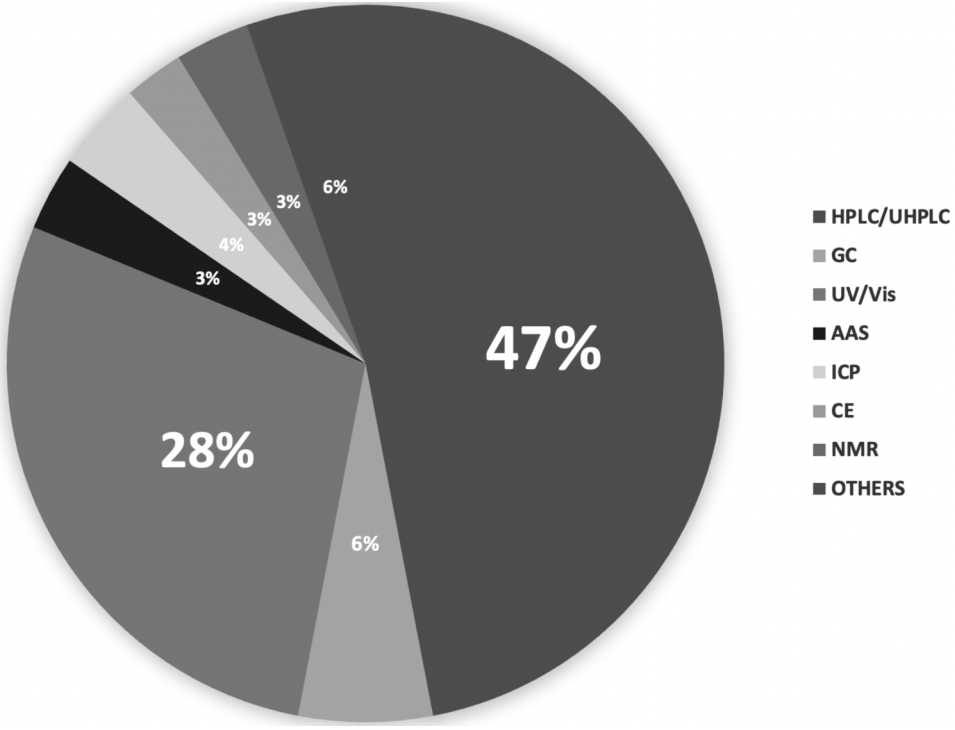

Fig. 4. Summary of the determination techniques used to analyze honey constituents, which present health-promoting effects and nutritional value (2015–2021). AAS: atomic absorption spectroscopy; CE: capillary electrophoresis; GC: gas chromatography; ICP: inductively coupled plasma; HPLC: high-performance liquid chromatography; NMR, nuclear magnetic resonance. The search has been done as in Fig. 1.

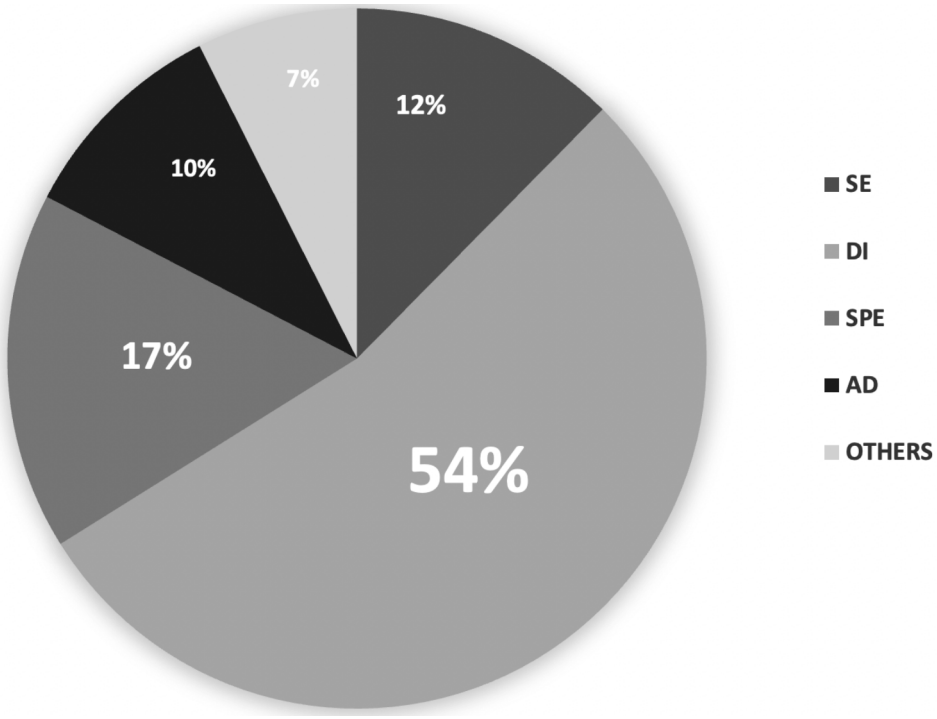

Fig. 5. Summary of the extraction techniques used when determining honey constituents, which present health promoting effects and nutritional value (2015–2021). AD, acid digestion; DI, dilution; SE, solvent extraction; SPE: solid-phase extraction. The search has been done as in Fig. 1.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132920.

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