Review

Extraction and determination of bioactive compounds from bee pollen

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A B S T R A C T

Since ancient times bee pollen has been considered a good source of bioactive substances and energy. Taking into account the current demand for healthy and natural foods, it is not surprising that bee pollen has been attracting commercial interest in recent years, making it one of the most widely consumed food supplements. It has been extensively reported that bee pollen contains several health-promoting compounds, such as proteins, amino acids, lipids, phenolic compounds, vitamins or minerals. Thus, this study aims to give an overview of the extraction and determination techniques of several of the above-mentioned compounds which have been published in the last few years (2011-2017). The design of the study is in accordance with the different families of bioactive compounds, and the extraction procedures together with the analytical techniques employed and their determination are discussed. A list of some of the most relevant applications is provided for each category, including a brief summary of the experimental conditions. The references included will provide the reader with a comprehensive overview of and insight into the analysis of bioactive compounds from bee pollen.

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Contents

1. Introduction

Apicultural products have long been used in phytotherapy as well as in diet for their positive health consequences. Nowadays, bee products (honey, royal jelly, propolis, bees wax or bee pollen) are gaining prominence due to the presence of bioactive compounds that are associated with beneficial properties to health [1,2]. Bee pollen in particular is gaining attention as functional food for human consumption due to its high content of compounds with health-promoting effects, such as essential amino acids, antioxidants, vitamins and lipids [3]. It is collected by foraging honey bees (*Apis* sp. including *Apis mellifera*) and stingless bees, and is a combination of mainly floral pollen with some nectar or honey, enzymes, wax and bee secretion. The pollen mixture is transported as a small pellet in the pollen basket of the beeís legs to the bee hive, where it is stored and used as food for all the developmental stages in the hive [4,5]. From the moment in which the bees add their secretions to this pollen, it acquires certain peculiar characteristics which make it different from pollen collected by hand or that which is dispersed by wind [6]. Bee pollen contains several nutrients and bioactive compounds: proteins, which are among the main components of bee pollen, include enzymes and both essential and nonessential amino acids. In fact, bee pollen is referred to as the "*only perfectly complete food*", as it contains all the essential amino acids needed for the human organism [5]. A large number of carbohydrates are also found in this substance, for instance, reducing sugars, polysaccharides, starch, and soluble and non-soluble fibers. In addition, lipids, such as fatty acids, sterols, and triglycerides, minority compounds, for example, minerals and vitamins (water and fat soluble), and several other bioactive compounds, like the phenolic type, are present [7,8]. However, the composition of bee pollen is particularly dependent on plant origin, together with other factors such as climatic conditions, soil type, beekeeper activities, and the different processes or storage treatments in commercial production [5,9]. It is worth mentioning that the bioactive quality of bee pollen declines over time, and that conditioning carried out on fresh pollen

Fig. 1. Evolution of the published works in the last years (2011–2017) related to extraction and determination of bioactive compounds from bee pollen (data up to May 2017). The sources of information were the databases: ISI-Web of Knowledge, Scirus, Scopus and Science Direct. The search has been done using as keywords [(bee pollen) or (honey bee collected pollen)] and [(bioactive) or (lipids) or (vitamins) or (proteins) or (phenolic) or (essential elements) 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.

before storage is influential in its nutritional and functional value [3]. Since bee pollen displays a high level of moisture in its composition, a dehydration process (artificial drying) is necessary to avoid rapid fermentation and spoilage, which is crucial in order to extend the shelf life time [10].

Over the past years, the rising interest in the extraction and determination of these beneficial bee pollen compounds has been demonstrated by the number of published research papers dealing with this issue (See Fig. 1), and the large list of countries in which such studies were carried out (See Fig. 2). As can be observed in Fig. 3, several of these compounds have been examined in this matrix during this period of time, most of the studies being devoted to the analysis of proteins, peptides and amino acids (31% of the

Fig. 2. Summary of the number of publications per country related to the analysis of bioactive compounds from bee pollen.

Fig. 3. Summary of the bioactive compounds analyzed in bee pollen in the last years.

publications) and lipids (25%); while, carbohydrates and phenolic compounds (~15%) have also been widely studied. Several interesting reviews and research studies focusing on the general health benefits of bee pollen and its related compounds have been published [8,11,12]. For example, an interesting article was published in 2015, in which the literature regarding the macro- and microscopic composition of pollen was revised [12]. The authors have indicated in some tables the techniques employed to determine each bee pollen component, although they have not provided any further discussion or explanation. In addition, the different phenolic compounds that cause its antioxidant activity have been identified. In a more recent publication [11], there was a review of the research on the bioactivity of bee pollen compounds in order to explain their potential medicinal properties and action. The purpose of this study was also to demonstrate a possible therapeutic value of bee pollen. The authors mainly focused their attention on discussing the health-promoting effects of bee pollen compounds, and included only a short paragraph on the techniques used to study bee pollen composition. Mention should also be made of an interesting review studying the characteristics of bee pollen and its aptitude as a raw material for a solid-state fermentat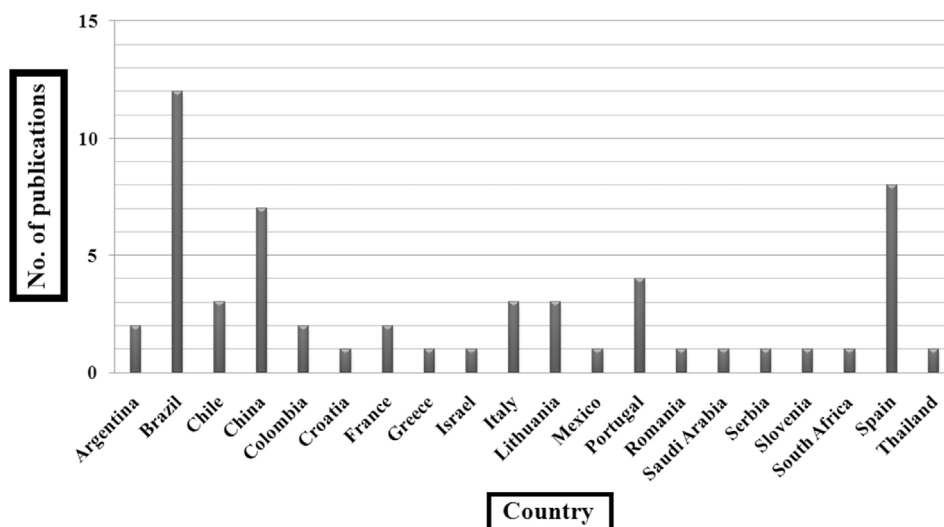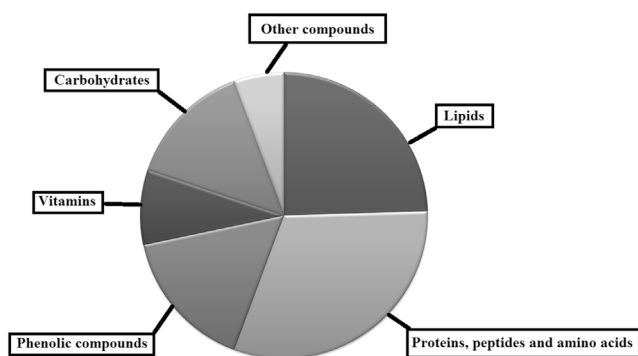ion process [8]. The authors have produced a detailed account of the different compounds encountered in bee pollen, their relation with some of the beneficial properties (nutritional and therapeutic) associated with the latter, and finally have commented on the bioactive and nutritional characteristics of fermented bee pollen. Yet information related to the quantification methods employed to determine each component was limited to a comment in the tables. Finally, bee pollen, apart from bioactive compounds, may also contain contaminants (heavy metals, pesticides, herbicides, mycotoxins, bacteria and antibiotics), as it is exposed to different contaminating sources in its environment [11,13], and for that reason it should be also analyzed for the monitoring of potential contaminants.

As has been previously seen, several reviews have been published with the aim of revealing the biological activity and therapeutic properties of several of the major constituents of bee pollen. In some of these, the different methodologies to determine each component have been mentioned, but to the best of our knowledge they have never been discussed from an analytical perspective. In view of all these aspects, the aim of this study is to present and discuss the main extraction and analytical techniques used to obtain, identify, characterize and/or quantify the bioactive compounds of bee pollen in the period 2011–2017, although in some specific cases previous studies will also be considered. Attention will mainly be paid to those in which advanced analytical techniques (chromatography, spectroscopy, spectrometry, etc) were employed. The review is structured according to the different families of bioactive compounds: **i**) lipids; **ii**) phenolic compounds; **iii**) proteins, peptides and amino acids (AAs); **iv**) vitamins; **v**) carbohydrates; **vi**) other bioactive compounds (minerals, glucosinolates

(GLSs) and related compounds). Readers interested in more specific details concerning biological activity, therapeutic properties or publications from earlier years can refer to the above-mentioned reviews.

2. Bioactive compounds of bee pollen

2.1. Lipids

Lipids are important as nutritious molecules of reserve and energy, and as such they are necessary for a suitable performance and development of the pollen grain. Some kinds of pollen are especially rich in lipid components and, depending on the botanical species to which they belong, their content can range between 1% and 20% of the bee pollen dry weight [6]. Humans use various biosynthetic pathways to both break down and synthesize lipids, but some essential lipids cannot be obtained in this way and must be obtained from diet; bee pollen could be a potential source of some of these compounds. The concentration of lipids varies greatly in different pollen plant species, as occurs with other bioactive compounds. The total lipid fraction of honeybee-collected pollen contains carotenoids, sterols and fatty acids as the main constituents [9], which is in agreement with the data summarized in Table 1 [1–4,7,9,14–33]. However, most of the research work on pollen lipids has focused on determining total lipid content (See Table 1). In these studies, bee pollen lipids have usually been isolated by using a Soxhlet extractor and different solvents like petroleum ether [1,7,14], ethyl ether [2], diethyl ether [16–18,20] and hexane [15]; however, in certain publications the Soxhlet device was not employed, and the lipids were extracted with a simple solvent with chloroform and methanol mixtures [3,19,21,23], petroleum ether [22,24] or a chloroform, methanol and water mixture [25]. Nevertheless, it should be added that sometimes [22–24], a previous hydrolysis step was required, and total lipid content was obtained by weighing (gravimetric method) in all cases. Carotenoids, belonging to the chemical classification of terpenoids, have a technological and nutritional importance as they are natural colorants and act in some cases as pro-vitamin A (β-carotene), the dietary intake of which could lower the risk of different types of cancer or cardiovascular disease [9]. Largely speaking (See Table 1), extraction with different solvents has been used to isolate these compounds from bee pollen: a petroleum ether [2], acetone [26,27], or different mixtures (chloroform and methanol mixture [3]; acetone and petroleum ether [18,26]; methanol, ethyl acetate, petroleum ether and butylated hydroxytoluene (BHT) [19]; and ethanol, hexane and BHT [26]). In one publication, however, individual carotenoid content (neoxantin, anteraxantin, lutein, zeaxantin, α-carotene and β-carotene) was determined on an unsaponified fraction of total lipids following a cold saponification with KOH [3], which was also required for β-carotene determination in other studies [19]. Regarding the extraction of β-carotene from bee pollen, a more complex procedure (open column chromatography, OCC) has been employed in two cases [27,28]. The name given to this particular chromatography (OCC) comes from its open setup. This is a good technique for determining carotenoids at relatively high concentrations, and it is also very useful for separation and purification of carotenoids that could be used as standards when determining those compounds by liquid chromatography (LC). In one of the studies [27], the OCC and LC methodologies were compared to determine β-carotene in Brazilian sampled bee pollen, and the results showed that the content of β-carotene obtained by OCC and LC did not differ significantly, thereby proving the reliability of both methods for quantifying β-carotene. Finally, quantification of total carotenoid content was performed spectrophotometrically (UV–vis; diode

Table 1
Applications in the analysis of lipids in bee pollen samples.

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
TL	Different botanical origins (NS [14],3 [1], 5 [16],9 [17],11 [15], 20 [28]) (Italy [1], Brazil [14,17,28], Portugal and Spain [15], Saudi Arabia[16])	Soxhlet extraction (petroleum ether [1,14], hexane [15], diethyl ether [16,17,28])	GVM	[1,14–17,28]
TL, TC and IC (2)	32 different botanical origins (Brazil)	i) Intermittent Soxhlet extraction (ethyl ether)[TL] ii) Solvent extraction (petroleum ether)[TCIC]	GVM[TL] UV–vis[TC] RPLC-PDA[IC]	[2]
TL, FAMEs (25), IC (6) and ST (12)	Chesnut (*Castanea sativa*), willow (*Salix alba*) (Italy)	i) Solvent extraction (chloroform/methanol)[All] ii) SPE (NH₂ cartridges)[FAME] iii) Transesterification[FAME] iv) Saponification[ST,IC,TP] v) Silylation[ST]	GVM[TL], GC-FID[FAME,ST] RPLC-DAD[IC]	[3]
TL	Corn (*Zea mays* L.) (Thailand)	NS	NS	[4]
TL and FAMEs (7)	10 different botanical origins (Portugal)	i) Soxhlet extraction (petroleum ether)[All] ii) transesterification[FAME]	GVM[TL] GC-FID[FAME]	[7]
TL, TC and β-carotene	10 different botanical origins (Brazil)	i) Soxhlet extraction (diethyl ether)[TL], ii) Solvent extraction (acetone/petroleum ether)[TC] and saponification[TC,β–carotene]	GVM[TL] OCC + UV–vis[TC,β–carotene]	[18]
TL, FAMEs (14), TC and IC (4)	16 different botanical origins (Romania)	i) Solvent extraction (chloroform/methanol)[TL,FAME] ii) Transesterification[FAME]; iii) Solvent extraction (mixture of methanol/ethyl acetate/petroleum ether containing BHT) [TC,IC] iv) Saponification[TC,IC]	GVM[TL], GC–MS[FAME] UV–vis[TC] RPLC-DAD[IC]	[19]
TL and FAMEs (10)	NS (Colombia)	i) Soxhlet extraction (diethyl ether)[TL] ii) Solvent extraction (petroleum ether)[FAME] iii) Transesterification[FAME]	GVM[TL] GC-FID[FAME]	[20]
TL and FAMEs (18)	*Aloe greatheadii* var. *davyana* (South Africa)	i) Solvent extraction (chloroform/methanol)[All] ii) Transesterification[FAME]	GVM[TL] GC-FID[FAME]	[21]
TL	Different botanical origins (NS [25],11 [22],15 [24],30[23]) (Brazil [22,25], Serbia [24], France [23])	i) Hydrolysis (thermal [22], acid [23,24]) ii) Solvent extraction (petroleum ether [22,24], chloroform/methanol [23], chloroform/methanol/water [25])	GVM	[22–25]
Licopene and β-carotene	12 different botanical origins (Chile)	Solvent extraction (BHT, ethanol and hexane)[Licopene] or (acetone and petroleum ether)[β–carotene]	UV–vis	[26]
β-carotene	NS (Brazil)	i) Solvent extraction (acetone)[All] ii) Partition (petroleum ether)[All] iii) Washing (water) and evaporation[All] iv) Dilution (petroleum ether)∘[CC] v) Evaporation and dilution (acetone)[RPLC]	UV–vis RPLC-PDA	[27]
β-carotene	20 different botanical origins (Brazil)	i) Solvent extraction (acetone) ii) Washing (water) and evaporation iii) Dilution (light petroleum/acetone) iv) OCC	UV–vis	[28]
FAMEs (7 [29],12 [9],15 [30])	Different botanical origins (NS [30],9[29],12 [9]) (Brazil [30], China [9], Portugal [29])	i) Soxtec™ extraction (diethyl ether [9], petroleum ether [29,30]) ii) Transesterification	GC-FID	[9,29,30]
FAMEs (8)	NS (Israel)	i) Acid hydrolysis ii) Solvent extraction (petroleum ether) iii) Transesterification	GC-FID	[31]
FAMEs (42)	Red clover (*Trifolium pratense* L.) (Lithuania)	i) Solvent extraction (chloroform/methanol/water), ii) Hydrolysis iii) Transesterification	GC-FID	[32]
FFAs (28)	Rape (*Brassica napus* L.) (China)	i) SFE (using CO₂) or solvent extraction (petroleum ether) ii) Pre-column derivatization (using TSPP)	RPLC-FLD	[33]

BHT: butylated hydroxytoluene; **DAD**: diode array detector; **FAMEs**: fatty acids methyl esters; **FFAs**: free fatty acids; **FID**: flame ionization detector; **GC**: gas chromatography; **GVM**: gravimetric method; **IC**: individual carotenoids; **NS**: not specified; **OCC**: open column chromatography; **PDA**: photodiode array detector; **RPLC**: reverse-phase liquid chromatography; **SFE**: supercritical fluid extraction; **SPE**: solid-phase extraction; **ST**: sterols; **TC**: total carotenoid; **TL**: total lipids; **TP**: tocopherols; **TSPP**: 1-[2-(*p*-toluenesulfonate) ethyl]-2-phenylimidazole [4,5-f]9,10-phenanthrene.

(DAD) or photo diode (PDA) array detectors) in all cases, although when individual determination was required a previous separation by reverse-phase LC (RPLC) was carried out.

Sterols are an important group of organic molecules that can be found in plants, animals and fungi, cholesterol being the most popular animal sterol. Sterols from plants, which are known as phytosterols, have been shown in clinical trials to block cholesterol absorption sites in the human intestine, and decrease the level of plasma cholesterol associated with low density lipoproteins, thus helping to reduce cholesterol in humans [34]. As can be seen in Table 1, sterols have been scarcely investigated in bee pollen [3]. These were extracted with a chloroform and methanol mixture, after which the resulting extract was cold saponified and the sterols, including cholesterol among a further eleven compounds, were determined as trimethylsilyl derivatives by gas chromatography equipped with a flame ionization detector (GC-FID). It can be also observed in Table 1 that GC coupled to an FID or mass spectrometry (MS) was the preferred technique when determining fatty acids [7,9,20,21,29–32]; the latter, however, should be converted into their corresponding methyl esters (FAMEs) by a transesterification procedure prior to GC analysis, as more robust and reproducible chromatographic data are obtained in this way [35]. Fatty acids are important as nutritional substances in living organisms, especially those of the ω-3 series, which are essential for human metabolism, in view of the direct relationship with the functional role of pollen as anti-atherogenic food [3]. They have usually been extracted from bee pollen by means of a solvent with petroleum ether [20,31], or chloroform and methanol mixtures [3,19,21,32], but in some cases Soxhlet [7] and SoxtecTM [9,29,30] systems were used to isolate them prior to their conversion to FAMEs. The main difference between SoxtecTM and conventional Soxhlet is the addition of a previous step in which the sample, introduced into a cellulose thimble, is completely submerged in the extraction solvent in order to facilitate the rapid solubilization of the analyte in boiling solvent. This boiling step allows for a more efficient extraction and greatly reduces extraction time and solvent volume [36]. Here, diethyl and petroleum ether were the solvents chosen. Finally, mention should be made of a study in which fatty acids were successfully determined in bee pollen with a totally different methodology, consisting of a supercritical fluid extraction (SFE) with CO$_2$ and analysis by RPLC with a fluorescence detector (FLD) [33]. As can be observed in Table 1, fatty acids from bee pollen lipids have usually been isolated with organic solvents. These techniques have some disadvantages such as the loss of volatile compounds, long extraction times, toxic solvent residues and degradation of unsaturated compounds due to heat [33], which can be resolved in certain cases by SFE. In recent years, the use of this extraction method has attracted increasing interest from researchers in the food sector since supercritical fluids provide high solubility and improved mass transfer rates. In addition, CO2 is the most commonly used fluid because of its properties (non-toxic, non-explosive, relatively cheap, and easily available experimental conditions). On the other hand, the use of LC allows fatty acids to be converted after a derivatization process to a large number of different derivatives than those obtained with GC; this can overcome some problems, such as tailing peaks and low detector sensitivity, by the formation of fewer polar compounds which can be more easily analyzed by LC [33].

It can be concluded that selecting the analytical methodology to determine bee pollen lipids is strongly dependent on its nature. Total lipid content has mostly been determined by Soxhlet extraction followed by gravimetry. Carotenoids have been mainly isolated from bee pollen by solvent extraction, whilst quantification has always been performed spectrophotometrically (UV–vis); however, separation (RPLC) has been required to ascertain individual content. On the other hand, GC is the technique of choice when establishing fatty acids and sterols following extraction with organic solvents and conversion to the corresponding derivatives.

2.2. Phenolic compounds

Phenolic compounds have recently received much attention for their wide range of functions, such as antimicrobial, anti-diabetic, anti-hyperlipidemia or anti-inflammatory effects; yet the main activity reported for these compounds has been as antioxidants. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. The number and arrangement of their carbon atoms are classified in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones, and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes, among others) [35]. Flavonoids are the most common group of polyphenolic compounds in the human diet. They have demonstrated certain pharmacological activity in in vitro studies: these include anti-allergic, anti-inflammatory, antioxidant, anti-microbial, anti-cancer, and anti-diarrheal properties. Bee pollen possesses a wide range of phenolic compounds, such as rutin, quercetin, vanillic acid, and protocatechuic acid; nevertheless, its composition varies because of its botanical and geographic origins as well as other factors, such as soil type, weather conditions, and beekeeper activities [37]. Total phenolic content (TPC) has been determined in most of the studies in which phenolic compounds were investigated in bee pollen (See Table 2). Phenolics were mainly isolated by means of a solvent extraction with an alcohol (ethanol or methanol) and water mixture [1,25,26,37,43,45], although methanol [5,28,30,32], water [38] and a water and acetone mixture [42] were also employed. It can also be deduced from the analysis in Table 2 that the Folin Ciocalteu method (FCM), a classic for TPC analysis, has been predominantly employed to assess total phenolic content in bee pollen. With this procedure, the measured color change, which is spectrophotometrically (UV–vis) monitored, is associated with the reduction of a molybdate-tungstate reagent induced by the phenols in the sample [35]. However, this is not a specific assay, as this reagent does not react only with phenols, but with any reducing sample substance. In other studies, total flavonoid content (TFC) was also determined [1,5,28,30,32,37,43,45]. The extraction procedure was exactly the same as the one for evaluating TPC (See Table 2), but the TFC of the bee pollen extracts was established (UV–vis) in most cases by means of the aluminium trichloride (AlCl$_3$) colorimetric assay. The principle of this method is that AlCl$_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it forms acid labile complexes with the orthodihydroxyl groups in some of the rings of the flavonoids [46]. In one study [1], the total content of two of the flavonoids classes, flavonol (TFLC) and anthocyanin (TANC), was determined. Flavonol content was measured with the same extracts as those used for assessing TFC and TFLC, but in this case absorbance was registered after incubation at room temperature of the mixture of these extracts and a solution of ethanol in HCl. Meanwhile, TANC was established according to the pH differential method, which is a spectrophotometric method based on the change in pigmentation of the pH-dependent of anthocyanins when different buffer solutions are added to the original extracts. Here it was necessary to perform a chromatographic (RPLC) [37,39,41,43–45,47] or electrophoretic (capillary electrophoresis, CE) [40] separation subsequent to a solvent extraction in order to individually determine the phenolic compounds. In most cases, the extracts obtained after a simple solvent extraction, which could be the same as those employed to measure TPC or TFC, were injected onto the LC system in reverse phase mode with C$_{18}$ analytical columns and coupled to a spectrophotometer (UV–vis, PDA or DAD) [37,39,41,43–45,47] or an MS detector [44,47]. In cer-

Table 2
Applications in the analysis of phenolic compounds in bee pollen samples.

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
TPC, TFC, TFLC and TANC	3 different botanical origins (Italy)	i) Solvent extraction (ethanol/water)[All] ii) FCM[TPC] iii) AlCl$_3$[TFC] iv) ethanol/HCl[TFLC] v) Buffers[TANC]	UV–vis	[1]
TPC and TFC	Different botanical origins (NS [30],3 [5],9 [29]) (Portugal [5,29],Spain [5], Brazil[30])	i) Solvent extraction (methanol)[All] ii) FCM[TPC] iii) AlCl$_3$[TFC]	UV–vis	[5,29,30]
TPC	9 different botanical origins (Brazil)	i) Solvent extraction (ethanol/water) ii) ethanol iii) FCM	UV–vis	[25]
TPC	12 different botanical origins (Chile)	i) Solvent extraction (water) ii) FCM	UV–vis	[26]
TPC, TFC and IP (12)	Rape (*Brassica napus* L.) (China)	i) Cleaning (hexane)[All] ii) Solvent extraction (methanol/water)[FPE] iii) Solvent extraction (ethyl acetate)[FPE] iv) Evaporation and dilution(methanol)[FPE] v) Basic hydrolysis of the FPE residue vi) Solvent extraction (ethyl acetate)[BPE] vii) Evaporation and dilution (methanol)[BPE] viii) FCM[TPC,IP] ix) NaNO$_2$-Al(NO$_3$)$_3$[TFC,IP]	UV–vis[TPC,TFC] RPLC-PDA[IP]	[37]
TPC	5 different botanical origins (China)	i) Solvent extraction (water) ii) AlCl$_3$	UV–vis	[38]
IP (12 [35],13 [36])	Different botanical origins (Mesquite tree (*Prosopis juliflora*) [39], 10 [40]) (Mexico [39], China [40])	Solvent extraction (ethanol/water)	RPLC-DAD [39] CE-AD [40]	[39,40]
IPC (7)	Hairy rockrose (*Cystus incanus* L.) (Croatia)	NS	RPLC-UV–vis	[41]
TPC	4 different botanical origins (Chile)	i) Solvent extraction (acetone/water) ii) SPE (polymeric cartridges) iii) water	UV–vis	[42]
TPC, TFC and rutin	Chestnut (*Castanea sativa*) (Italy)	i) Solvent extraction (methanol/water)[All] ii) FCM[TPC] iii) AlCl$_3$[TFC]	UV–vis[TPC,TFC] RPLC-PDA[Rutin]	[43]
TPC and IF (13)	13 different botanical origins (Lithuania)	i) Solvent extraction (methanol/water)[All] ii) FCM[TPC]	UV–vis[TPC] UPLC-PDA-MS[IF]	[44]
TPC, TFC and IP (13)	NS (Lithuania)	i) Solvent extraction (methanol/water)[All] ii) FCM[TPC] iii) AlCl$_3$[TFC]	UV–vis[TPC,TFC] RPLC-DPPH[IP] RPLC-ECD[IP]	[45]
Resveratrol and piceid isomers (4)	NS (Spain)	i) Cleaning (hexane) ii) Solvent extraction (ethanol/water)	RPLC–MS	[47]

AlCl$_3$: aluminium trichloride; **AD:** amperometric detector; **Al(NO$_3$)$_3$:** aluminium nitrate; **BPE:** bound phenolic extract; **DAD**: diode array detector; **DPPH**: 1,1-diphenyl-2-picrylhydrazyl; **ECD**: electrochemical detector; **FCM**: Folin Ciocalteu method; **FPE**: free phenolic extract; **IP**: individual phenolic content; **NaNO$_2$**: sodium nitrite; **NS**: not specified; **PDA**: photodiode array detector; **RPLC**: reverse phase liquid chromatography; **SPE**: solid phase extraction; **TANC**: total anthocyanin content; **TFC**: total flavonoid content; **TFLC**: total flavonols content; **TPC**: total phenolic content.

tain studies [37,39,43–46], several phenolic compounds like rutin, quercetin, caffeic acid, resveratrol or kaempferol were detected in bee pollen samples from different countries. The health promoting effects of these phenolics are quite diverse. For example, rutin and quercetin, which possess very similar chemical structures, are used in many countries as medications for blood vessel protection, while resveratrol and its related compounds have all been positively linked to health benefits, including anticancer, anti-inflammatory, antioxidant, antimicrobial, and antiaging properties [46]. In one of the above-mentioned studies, twelve phenolic compounds, including rutin, quercetin, vanillin, resveratrol, rutin and catechin, were determined by RPLC-PDA in free (FPE) and bound (BPE) phenolic extracts of rape bee pollen [37]. The sample treatment was much more complex, as several additional steps were required (cleaning, a second extraction, hydrolysis, evaporation and reconstitution). In another study [44], in which compounds from phenolic extracts of bee pollen samples were identified by an ultra-high performance liquid chromatography system coupled to PDA and MS detectors, diversity in composition and structure was apparent. Most of the compounds identified were flavonoids, such as quercitrin, kaempferol or isorhamnetin, and were common for all bee pollen samples. According to the publications summarized in Table 2, there has been scant use of MS detectors like those employed in the previous study. These are more selective and sensitive than spectrophotometric detectors, but are also more complex and expensive, which means they are not affordable for

every laboratory. Finally, mention should be made of an interesting study in which the antioxidant activity of bee pollen was evaluated by analyzing the phenolic content determined by spectrophotometric and chromatographic techniques (RPLC coupled with an 1,1-diphenyl-2-picrylhydrazyl (DPPH) reaction detector and electrochemical detector (ECD)) [45]. The on-line RPLC-DPPH method permits specific assessment of which compounds are responsible for the radical scavenging activity in the DPPH assay, as not all compounds are active in the DPPH model system. RPLC-ECD analysis, meanwhile, revealed electrochemically active compounds in the pollen extracts, since polyphenolic compounds are reducing agents and their electrochemical responses are due to the donation of electrons. The latter examination technique showed thirteen electrochemically active phenolic compounds, whilst RPLC-DPPH analysis revealed six compounds responsible for DPPH inhibition. As previously stated, not only chromatographic methods have been employed to analyze phenolic compounds in bee pollen. CE with amperometric detection (AD) has been used to investigate the phenolic profile of several bee pollen samples [40]. Under optimum conditions, 13 such components can be well separated or nearly baseline separated within 29 min. Thus, it has been demonstrated that CE is a promising alternative to LC when determining phenolic compounds in bee pollen; certain advantages of CE over LC are high separation efficiency, low consumption of sample and reagents, and cost.

To conclude, several sample treatments have been published for extracting phenolic compounds from bee pollen. In most cases these involved a solvent extraction with a mixture of an alcohol (methanol or ethanol) and water, together with the FCM and AlCl$_3$ approaches (see Table 2). Spectrophotometric techniques have generally been used to determine total contents in bee pollen, although electrochemical and MS detectors have been selected in some specific studies. Occasionally, however, single phenolic compounds were specifically analyzed mainly by means of RPLC with C$_{18}$ analytical columns. Ultra-high performance liquid chromatography (UHPLC) and CE were rare.

2.3. Proteins, peptides and amino acids

Consumption of proteins and AAs, which form part of the bioactive compounds found in bee pollen, offers several benefits for human health. They are involved in antibacterial, antioxidant, immuno-stimulating, anti-thrombotic and anti-inflammatory activities, amongst other positive effects on the organism [35]. Proteins are one of the major components of bee pollen and, as can be expected, their content is highly variable among plant species (10–40% dry weight) [5,6]. Total protein content (TPROC) has been established in many publications (See Table 3), and in most cases this was the result of nitrogen measurement by a Kjeldahl method [1,2,9,14–18,20,22–25,29,30,42,48,53,55–58]. This method can be broken down into three main steps: digestion, distillation and titration. Briefly, hot digestion is accomplished by boiling a sample in sulfuric acid, together with potassium sulfate and a catalyst, resulting in digestion containing ammonium sulfate. Afterwards, a base (NaOH) is added to convert ammonium sulfate to volatile ammonia, which is determined by titration [59]. In some of the above-mentioned publications, use was made of micro-Kjeldahl extraction equipment, which has been designed for Kjeldahl extraction on a micro scale to determine the protein content of food [2,14,16–18,48,53]. In one study, the bee pollen samples were combusted and the nitrogen content was determined via an elemental analyser [21]. Once total nitrogen content has been established, a conversion factor must be employed to transform the nitrogen into protein content. As can be seen in Table 3, two different conversion factors, 5.60 and 6.25, have been used. This could be

explained by the fact that some authors claim factor 6.25 may overestimate protein content, and recommend the use of factor 5.60 to convert nitrogen into protein content. These authors reported that about 30% of nitrogen content determined in pollen samples may be of non-protein origin, because a large part of it comes from free amino acids (FAAs) [25]. Not only has TPROC been determined by previously establishing the nitrogen content. Some researchers have decided to employ the Bradford assay (BA) to quantify protein levels in bee pollen extracts obtained after diluting with water [26,31] or by means of a buffer composed of tris(hydroxymethyl)aminomethane (Tris) and ethylenediaminetetraacetic acid (EDTA) [51]. The BA involves the binding of a dye (Coomassie Brilliant Blue G-250), dissolved beforehand in ethanol and acidified with phosphoric acid, to protein; this causes a shift in the absorption maximum of the dye, which is monitored via a UV–vis spectrophotometer [60]. This assay is very reproducible and rapid, with the dye binding process virtually complete in approximately 2 min. In addition, there has been a study of the potential of near infra-red spectroscopy (NIRS) technology together with a remote reflectance fibre-optic probe for determining the major components, including proteins, in bee pollen [58]. The results demonstrated that the proposed analytical method, employing the NIRS technique, namely, a fibre-optic probe and measuring of the samples by direct application of the probe, does not require treatment of the samples; moreover, it is rapid, non-polluting, and can be used for determining protein in bee pollen samples, with results comparable to those obtained with classic chemical methods, such as Kjeldahl or BA. Proteins have been also individually determined in bee pollen samples [32]. Extracts were homogenized with acetone containing trichloroacetic acid and 1,4-Dithiothreitol, and proteins were extracted with an isoelectric focusing (IEF) lysis buffer. Bee pollen proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and two-dimensional gel electrophoresis (IEF/SDS). In addition, areas of interest were cut out from the two-dimensional gels and after in-gel tryptic digestion, the tryptic peptides were subsequently determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). It was possible to identify 9 proteins with a molecular weight of about 12, 15, 30, and 50 kDa. To conclude our discussion of the publications in which proteins and peptides were determined, we should refer to a study evaluating the polypeptide content of bee pollen [54]. Here, the authors have developed a new methodology to determine polypeptides which avoids some of the drawbacks of classic methods. The proposed sample treatment comprises several steps: **i)** protein precipitation; **ii)** contaminant removal; **iii)** acetone residual removal; **iv)** extraction of polypeptides with sodium dodecyl sulfate (SDS) and phenol buffers; **v)** precipitation of polypeptides, washing and air-drying. Quantification of total polypeptides was performed by means of the bicinchoninic acid (BCA) method. This combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with a colorimetric detection of the cuprous cation (Cu^{+1}). The chelation of two molecules of BCA with one cuprous ion formed a reaction product that could be spectrophotometrically monitored. In comparison with Kjeldahl, it could be said that this proposal permits the differentiation of polypeptide nitrogen from non-polypeptide nitrogen, whilst being simpler, safer and cheaper. Moreover, combination with the BCA assay makes it possible to quantify the polypeptides independently of their amino acid composition, reducing the protein-to-protein variation encountered with BA. As seen in the Introduction, bee pollen generally contains all the essential AAs needed for the human organism, but amounts may vary among plant species. Indeed, AA composition may define the nutritional value of pollen more accurately than protein content, since the former is reduced when inadequate amounts of the essential AAs are present [21]. According to the related bibliography, there are two

Table 3
Applications in the analysis of proteins, peptides and amino acids in bee pollen samples.

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
TPROC	Different botanical origins (NS [6,14,20,25,30],3 [1],4 [42],5 [16],9 [17,29], 10 [7,18],11 [15,22],15 [24], 21 [48],30 [23],32 [2], 36 [56],42 [53], 46 [57]) (Italy [1,56], Brazil [2,14,17,18,22,25,30], Spain[6,15], Portugal [7,15,29], Saudi Arabia [16],Colombia [20],Francia[23],Serbia [24], Chile [42],Argentina [48,53], Greece [57])	i) Digestion ii) Distillation iii) Titration	Kjeldahl [1,6,7,15,20,22–25,29,30,42,56,57] Micro-Kjeldahl [2,14,16–18,48,53] (TN × 6.25) [2,7,14–18,20,23,30,48,53,56,57] (TN × 5.60) [6,16,22,29,57] NS [1,24,25,42]	[1,2,7,14–18,20,22 –25,29,30,42,48,53,56,57]
TPROC	Corn (Zea mays L.) (Thailand)	NS	NS	[4]
TPROC, TAAs and FAAs (18)	12 different botanical origins (China)	i) Digestion[TPR∘C] ii) Distillation[TPR∘C] iii) Titration[TPR∘C] iv) Solvent extraction (SSA)[FAA] v) Acid hydrolysis[TAA] vi) Basic hydrolysis[TRP] vii) Evaporation[TAA,TRP] viii) Dilution (citrate buffer)[TAA,TRP]	Kjeldahl[TPR∘C] (TN × 6.25) AAA[FAA,TAA,TRP]	[9]
TPROC and FAAs (18)	Aloe greatheadii var. davyana (South Africa)	i) Combustion[TPR∘C] ii) Acid hydrolysis[FAA] iii) Pre-column derivatization (PITC)[FAA]	EA[TPR∘C] (TN × 6.25) RPLC-UV [FAA]	[21]
TPROC	Different botanical origins (NS[31],12[26],18[51]) (Chile[26], Israel[31], Spain[51])	i) Dilution (water)[26,31] ii) Solvent extraction (Tris/EDTA buffer)[51] iii) BA[All]	UV–vis	[26,31,51]
IPRO (9)	Red clover (Trifolium pratense L.) (Lithuania)	i) Acetone/TCA/DTT ii) Solvent extraction (IEF lysis buffer)	SDS/PAGE IEF/SDS MALDI-TOF	[32]
TAAs and FAAs	Chesnut (Castanea sativa) (Italy)	i) Solvent extraction (ethanol/water)[FAA] ii) Evaporation[FAA] iii) Dilution (water)[FAA] iv) Acid hydrolysis[TAA] v) Acidic ninhydrin, water and toluene[proline] vi) Ninhydrin/ethylene glycol and ispropanol[FAA,TAA]	UV–vis	[43]
FAAs (22) and TAAs	NS (Spain)	i) Solvent extraction (ethanol/water)[FAA] ii) Evaporation[FAA] iii) Dilution (water)[FAA] iv) Acid hydrolysis[TAA] v) Pre-column derivatization (OPA/MEC)[FAA,TAA] vi) Acidic ninhydrin and water[proline]	RPLC-FLD[FAA,TAA] UV–vis[proline]	[49]
AAs (18)	Rape (Brassica napus L.) (China)	i) Acid hydrolysis ii) Evaporation iii) Dilution (borate buffer) iv) Pre-column derivatization (BCEC-Cl)	RPLC-FLD	[50]
TTRP and FTRP	NS (China)	i) Basic hydrolysis[TTRP] ii) Washing (HCl)[TTRPC] iii) Dilution (water)[TTRPC] iv) Solvent extraction (water/acetonitrile)[FTRPC]	RPLC-FLD	[52]

Table 3 (Continued)

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
Polypeptides	15 different botanical origins (France)	i) Protein precipitation ii) Contamination removal (TCA/methanol, methanol and acetone) iii) Solvent extraction (SDS and phenol buffers) iv) Precipitation of polypeptides, washing and drying v) Dilution (guanidine/HCl)	BCA method (UV–vis)	[54]
TPROC and FTRPC	8 different botanical origins (Slovenia)	i) Digestion^{TPR-C} ii) Distillation^{TPR-C} iii) Titration^{TPR-C} iv) Solvent extraction (water/methanol and sodium phosphate)^{FTRPC}	Kjeldahl^{TPR-C} (TN × 6.25) RPLC-ECD^{FTRPC}	[55]
TPROC	NS (Spain)	i) Digestion^{K} ii) Distillation^{K} iii) Titration^{K}	Kjeldahl^{K} (TN × 6.25) NIRS	[58]

AAA: amino acid analyzer; **BA:** Bradford assay; **BCA:** bicinchoninic acid; **BCEC-Cl:** 2-(11Hbenzo[α]-carbazol-11-yl) ethyl chloroformate; **DTT:** 1,4-dithiothreitol; **EA:** elemental analyzer; **ECD:** electrochemical detector; **EDTA:** ethylenediaminetetraacetic acid; **FAA:** free amino acid; **FLD:** fluorescence detector; **FTRPC:** free tryptophan content; **IEF:** isoelectric focusing; **IPRO:** individual proteins; **K:** Kjeldahl; **MALDI:** matrix-assisted laser desorption/ionization; **NIRS:** near infra-red spectroscopy; **NS:** not specified; **OCC:** open column chromatography; **OPA:** o-phthaldialdehyde; **PAGE:** polyacrylamide gel electrophoresis; **PDA:** photodiode array detector; **PITC:** phenylisothiocyanate; **RPLC:** reverse-phase liquid chromatography; **SDS:** sodium dodecyl sulfate; **SSA:** 3,5-sulfosalicylic acid; **TAA:** total amino acids; **TCA:** trichloroacetic acid; **TN:** total nitrogen; **TOF:** time-of-flight; **TPROC:** total protein; **Tris:** tris(hydroxymethyl)aminomethane; **TRP:** tryptophan; **TRPC:** total tryptophan content.

principal approaches to establishing AA content: total (TAAs) and free (FAAs). TAAs include free AAs and protein-bound AAs, and this is the method of choice either when AA composition of a protein or peptide is the focus, or when the total protein concentration of a sample is required. On the other hand, FAAs is usually determined when the AAs bound within proteins or peptides are not being considered but rather it is the concentration of the individual unbound AAs which is required [54]. The most representative components of the FAAs fraction are proline and glutamic acid, the ratio of which (proline/glutamic acid) is used as an indicator of the conservation status and manipulation of pollen [12]. In addition, essential AAs such as leucine, isoleucine, phenylalanine, valine, threonine, tryptophan (TRP) and methionine are usually found in this fraction. Some differences were observed in existing sample treatments in terms of the establishing AA content (See Table 3). FAAs have largely been extracted by water mixtures with different organic solvents (ethanol [43,49]; acetonitrile [52]; methanol [55]), although 5-sulfosalicylic acid (SSA) has been also employed in one study [9]. Nevertheless, there are some studies in which a hydrolysis (acid [21] or basic [9,52]) was performed instead of solvent extraction when measuring FAAs. In contrast, acid hydrolysis is the method of choice when determining TAAs [9,43,49,50]. When assessing AA content in bee pollen samples, it should be highlighted that most AAs from the latter show neither natural absorption in the UV region nor natural fluorescence; however, easily detectable AAs derivatives can be obtained through labeling reactions. Generally, derivative separation depends on the use of chromatographic techniques, usually LC, although it is also possible to perform the direct determination of AAs with AA analyzers (AAA) [9] or ECD [55]. Various derivatization reagents have been employed in the publications reviewed. For example, phenylisothiocyanate (PITC) was employed to produce phenyltiocarbamyl AAs, which were further separated and quantified by RPLC-UV–vis [21]. In this study, it was used a column that was specially designed for the reverse phase separation of AAs (Pico.Tag®). This reagent has some advantages and disadvantages. PTIC reacts with primary and secondary amines, yielding stable and UV-absorbing derivatives, without the interference of by-products; nevertheless, successive drying steps are required, which makes it a time-consuming method. Thus, the use of o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (MEC) has been proposed to obtain the corresponding derivatives that could be analyzed by RPLC-FLD [49]. This derivatization procedure is quick, simple and sensitive, but with the limitation that secondary amino groups, such as those of proline or cystine, do not react, while some derivatives are unstable. Consequently, the authors finally decided to determine FAAs by RPLC-FLD with a C_{18} column following a pre-column derivatization with OPA/MEC; proline, meanwhile, was quantified with a procedure based on the colorimetric measurement (UV–vis) of the reaction between an aqueous bee pollen extract with acidic ninhydrin. Ninhydrin was also employed in a study in which TAAs and FAAs, including proline, were determined [43]. As in the latter research, proline was individually measured, but in this case toluene was used to extract the chromophore formed by the union of proline and ninhydrin, prior to the monitoring of its absorbance. Meanwhile, the AAs from the FAAs and TAAs were established by means of a slightly different methodology. Briefly, a citrate buffer and a ninhydrin solution in ethylene glycol were added to corresponding extracts of FAAs and TAAs. Next, the mixture was cooled, and isopropanol was added, and absorbance of the chromophores was also measured by using a UV–vis spectrophotometer. As previously mentioned, conventional derivatization reagents have both advantages and disadvantages; therefore, new compounds were synthesized/tested in order to overcome some of the existing drawbacks. A photochromic molecule 2-(11H-benzo[α]-carbazol-11-yl) ethyl chloroformate (BCEC—Cl) was employed as a labeling reagent

to evaluate the chemical compositions of AAs, including cystine and TRP, in bee pollen by using RPLC-FLD [50]. The results showed that BCEC—Cl exhibited excellent fluorescence sensitivity, especially when determining cystine and TRP, among other interesting qualities like rapid and convenient derivatization or good stability and derivatization yields. Finally, it is interesting to mention two studies in which TRP content in bee pollen was individually investigated [52,55]. This AA possesses an important role in protein biosynthesis, and also functions as a biochemical precursor for some important physiological activity substances, such as serotonin and niacin [52]. In the first of these studies [52], total and free content of this AA were established by employing conventional sample treatments (hydrolysis, total content; solvent extraction-free content) and determination techniques (RPLC-FLD system with a C$_{18}$ column). The authors carried out a basic hydrolysis, in line with other researchers [9], since TRP is more stable than in the case of acid conditions. Moreover, this method has the advantage of being a simple extraction process without the need for derivatization when using FLD.

After an examination of the scientific literature regarding the analysis of proteins, peptides and AAs in bee pollen, different methodologies may be recommended depending on the target compound. A Kjeldahl approach is the method of choice when determining TPROC, although BA, BCA or NIRS could be more rapid alternatives; a solvent extraction followed by an electrophoretic separation has been used to determine proteins individually. In relation to AAs analysis, a solvent extraction with a mixture of water and an organic solvent (FAAs) or an acid hydrolysis (TAAs), a pre-column derivatization and a further RPLC-FLD (C$_{18}$ analytical column) is the most suitable methodology to determine such compounds. In certain cases the derivatization procedure can be omitted, for instance, when alternative sample treatments and different detectors are used.

2.4. Vitamins

Vitamins comprise a diverse group of organic compounds, with varying biochemical roles as regulators of mineral metabolism or cell and tissue growth and differentiation; they possess antioxidant activity and some are precursors of enzyme cofactors. According to the existing literature, bee pollen contains vitamins of B complex, vitamins C, D, E and carotenoids such as β-carotene, which can be pro-vitamin A (See subsection 2.1) [27,28]. As occurred with all the bioactive compounds, vitamins were present in bee pollen in varying amounts depending on the plant source, and some samples were considered dietary sources of antioxidant vitamins in a daily serving (25 g). However, it has also been reported that certain compounds (vitamins A and C) could be lost in the thermal processing of bee pollen, as such compounds are unstable at high temperatures [2,18,28]. Reducing equivalents for biochemical reactions is one of the most important physiological functions of ascorbic acid (vitamin C), and in some vegetables it is responsible for 35–95% of antioxidant capacity [35]. Two different sample treatments based on titrimetric [2,27,28] or fluorimetric (FLM) [18] methods have been proposed in order to isolate and obtain vitamin C from bee pollen (See Table 4). Both procedures display certain advantages and drawbacks. For example, titrimetric and fluorimetric methods are simple and, therefore, popular; yet they are not chemically specific for ascorbic acid, as are, for example, the LC methods. The titrimetric method relies on reduction of the blue dye 2,6-dichloroindophenol by ascorbic acid, previously extracted with a mixture of metaphosphoric and acetic acids, to a colorless solution. Meanwhile, the fluorimetric method is based on oxidation of ascorbic acid by means of active carbon (Norit) to dehydroascorbic acid, followed by reaction with o-phenylenediamine to produce a fluorescent quinoxaline derivative that can be measured by FLD

[35]. Water-soluble vitamins, like the B complex type, are not normally stored in significant amounts in the body, which entails the need for a daily supply of these vitamins; one of the potential sources could be bee pollen. This group of vitamins plays a key role in the production of cellular energy (B1, B2), whilst facilitating amino acid metabolism (B6) and helping the body to convert carbohydrates into glucose (B3 or PP). As can be seen in Table 4, these compounds have been analyzed with the same methodology in bee pollen samples [10,17,58]. The simultaneous extraction of B vitamins (B1, B2, B3 and B6) was performed in various steps. Firstly, acid hydrolysis followed by enzymatic treatment with fungal diastase was carried out. After enzymatic treatment and cooling to room temperature, the solutions were diluted with water prior to being analyzed by RPLC-FLD. The chromatographic conditions varied for each vitamin. B1 and B2 vitamins were analyzed in the same conditions, whilst B3 and B6 vitamins were quantified differently. Finally, a pre (vitamin B1) and post (vitamin B3) column reaction was required prior to FLD measurement. Tocopherols, a class of chemical compounds many of which have vitamin E activity, are involved in protecting membrane lipids from oxidative damage, acting as anti-inflammatory agents. These compounds have generally been determined by normal-phase LC (NPLC) with the same silica column [2,17,27,28], although in one case an RPLC approach with a C18 column has also been proposed [3]. In all cases determination was carried out with FLD. Certain similarities and differences have been observed between the two methodologies. For example, in both approaches a solvent extraction with acetone [2,17,27,28] or a chloroform and methanol mixture [3] was the first step of the sample treatment; similarly, dilution with a hexane and isopropanol mixture was conducted in both cases. The main difference between the treatments concerns the fact that a partition with a further evaporation step [2,17,27,28] or a saponification of the extracts has been performed prior to determining vitamin E content by LC-FLD.

As a result, it can be deduced by analysing the existing literature that a sample treatment based on solvent extraction is required in order to obtain vitamins from bee pollen, although selection of the solvent should be made in accordance with the vitamin group. As for the determination method, it could be said that simple, economic and not totally specific methods (titrimetric or fluorimetric) have been employed to establish vitamin C, whereas more selective, specific, complex and expensive LC methods have been selected in the case of vitamins B and E.

2.5. Carbohydrates

Bee pollen contains between 15% and 60% carbohydrates (dry weight), and these represent the largest fraction of the substance [6,54]. The floral species from which it derives has an influence on carbohydrate content, as do also the conditions in which processing is carried out and the pollen is collected. Carbohydrates perform numerous essential roles in living beings. Monosaccharides are the major source of energy for the metabolism, while polysaccharides store energy and can act as structural components [34]. Moreover, reducing sugar (RS) is predominant over non-RS, a characteristic that makes it different from the pollen which is isolated directly from the plant [6]. The most abundant reducing sugars in bee pollen are glucose and fructose, while other carbohydrates such as disaccharides (sucrose), polysaccharides, oligosaccharides and dietary fiber can also be found [8]. Total carbohydrate content (TCHC) has been calculated in three different ways in the related publications (See Table 5). In the first method, TCHC was calculated by difference, rather than being analysed directly. With this approach, the other constituents in food (protein, fat, water, alcohol, ash) are determined individually, and then added together and subtracted from the total weight (carbohydrates = 100 − (g fat + g

Table 4
Applications in the analysis of vitamins in bee pollen samples.

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
Vitamin C	Different botanical origins (NS [27], 20 [28],32[2]) (Brazil)	Solvent extraction (MPA/acetic acid)	TTM	[2,27,28]
Vitamin E (α- [2,17,27,28], β- [2], γ- [2], δ- [2] tocopherol)	Different botanical origins (NS [27],9 [17], 20 [28],32 [2]) (Brazil)	i) Solvent extraction (acetone) ii) Partition (petroleum ether) iii) Washing (water) and evaporation iv) Dilution (hexane/isopropanol)	NPLC-FLD	[2,17,27,28]
Vitamin E (α-, γ-, δ-tocopherol)	3 different botanical origins (Italy)	i) Solvent extraction (chloroform/methanol) ii) Dilution (hexane/isopropanol) iii) Saponification	RPLC-FLD	[3]
Vitamins B (B$_1$, B$_2$, B$_3$ (PP), B$_5$, B$_6$, B$_{12}$) and E (α-tocopherol)	Corn (*Zea mays* L.) (Thailand)	NS	NS	[4]
B complex vitamins (B$_1$, B$_2$, B$_3$ (PP), B$_6$)	Different botanical origins (NS [10,61],9 [17]) (Brazil)	i) Acid hydrolysis[All] ii) Enzymatic treatment[All] iii) Dilution (water)[ALL] iv) Pre[B1]-or post[B3]-column reaction	RPLC-FLD	[10,17,61]
Vitamin C (ascorbic acid)	10 different botanical origins (Brazil)	Oxidation (active carbon)	FLM	[18]

FLD: fluorescence detector; **FLM:** fluorimetric method; **MPA:** metaphosphoric acid; **NPLC:** normal-phase liquid chromatography; **NS**: not specified; **PDA**: photodiode array detector; **RPLC**: reverse-phase liquid chromatography; **TTM**: titrimetric method.

protein + g ash) [7,9,15,29]. Obviously, TCHC calculated in this way includes fibre, as well as certain components that strictly speaking are not carbohydrates, like organic acids. Another option was to employ the so-called anthrone method, which is applicable to every kind of foodstuff [6]. Basically, following digestion of the sample with perchloric acid (HClO$_4$), the hydrolysed starch, together with the soluble sugar, is spectrophotometrically assessed (UV–vis) and monitoring takes place of the absorbance provided by the chro-

mophore formed when anthrone and sulphuric acid are added. The third approach was the phenol-sulfuric acid method, which is a simple and rapid colorimetric technique to determine total carbohydrates in a particular sample [42]. Although this method detects almost all carbohydrates, the absorptive aspect of the different carbohydrates varies. Thus, unless a sample is known to contain only one carbohydrate, the results must be expressed arbitrarily in terms of one carbohydrate [62]. Firstly, the sample must be hydrolyzed

Table 5
Applications in the analysis of carbohydrates in bee pollen samples.

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
RS (GLU and FRU)	Different botanical origins (32 [2],11 [17]) (Brazil)	NS	NPLC-FLD	[2,17]
TCHC, RS (GLU and FRU) and sugars (3)	Corn (*Zea mays* L.) (Thailand)	NS	NS	[4]
TCHC, RS (NS) and sucrose	NS (Spain)	i) Acid digestion[TCHC] ii) Anthrone and sulphuric acid[TCHC] iii) LEM[RS,Sucrose]	UV–vis[TCHC] TTM[RS,Sucrose]	[6]
TCHC	Different botanical origins (2 [9],9 [29], 10 [7],11 [15]) (Portugal [7,15,29], China [9], Spain [15])	NA	BD	[7,9,15,29]
RS (NS)	Different botanical origins (10[7],11[15],30[23]) (Portugal[7,15], Spain[15], France[23])	i) Acid hydrolysis[15] ii) Neutralization[15] iii) Deproteinization[23] iv) Water[All] v) DNS[All]	UV–vis	[7,15,23]
RS (GLU and FRU) and oligosaccharides[56]	Different botanical origins (15[16],36[56]) (Saudi Arabia[16], Italy[56])	i) Water and methanol	NPLC-RID	[16,56]
RS (GLU and FRU) and sucrose[20]	Different botanical origins (NS[20],11[22]) (Colombia[20], Brazil[22])	i) Solvent extraction (water)[All] ii) Zinc acetate and potassium ferricyanide [22]	HPLC-RID[20] NPLC-RID[22]	[20,22]
TCHC	4 different botanical origins (Chile)	i) Acid hydrolysis ii) Neutralization iii) Water iv) Phenol solution v) H$_2$SO$_4$	UV–vis	[42]
RS (NS)	NS (Spain)	LEM	TTM[LEM] NIRS	[58]

BD: by difference; **DNS:** 3,5-dinitrosalicylic acid; **FRU:** fructose; **GLU:** glucose; **IS:** individual sugars; **LEM:** Lane-Eynon method; **NA:** not applied; **NIRS:** near infra-red spectroscopy; **NPLC:** normal-phase liquid chromatography; **NS:** not specified; **RID:** refractive index detector; **RS:** reducing sugars; **TCHC:** total carbohydrate content; **TS:** total sugars.; **TTM:** titrimetric method.

Table 6
Applications in the analysis of minerals, glucosinolates and related compounds in bee pollen samples.

Compound (number)	Plant source (Country)	Sample treatment	Determination method	Refs.
IM (6 [17,20],8 [13],20 [14])	Different botanical origins (NS [13,20],11 [17],12 [9]) (China [9], Brazil[13,17],Colombia [20])	i) Wet acid digestion[9,13,17] (HNO_3 and H_2O_2) [9,13] (HNO_3 and $HClO_4$) [17] ii) (NS) [20]	ICP-AES [9,13,17] AAS [20]	[9,13,17,20]
GLS (9)	NS (Spain)	i) Water ii) SPE (NH_2 cartridges) iii) Evaporation iv) Dilution (water)	RPLC–MS/MS	[63]
SFN	NS (Spain)	i) Solvent extraction (methanol) ii) Evaporation iii) Dilution (methanol)	RPLC–MS/MS	[64]

AAS: atomic absorption spectroscopy; **AES:** atomic emission spectrometry; **GLS**: glucosinolates; **ICP:** inductively coupled plasma; **IM:** individual minerals; **NA**: not applied; **NS**: not specified; **MS/MS**: tandem mass spectrometry; **RPLC**: reverse phase liquid chromatography; **SFN**: sulforaphane.

and neutralized. Next, water, phenol solution and sulfuric acid are added to the extract, and after a certain period of time the color is measured (UV–vis). As with several other compounds, reducing sugars have been determined as the total or individual (glucose and fructose) content. They are usually analyzed by volumetric (Lane-Eynon method, LEM) [6,58], colorimetric (3,5-dinitrosalicylic acid (DNS) method) [7,15,23], or by LC subsequent to a solvent extraction with water [20,22] or water and methanol mixtures [16,56]. LEM is an example of a titration method for determining the concentration of RS. A Fehlingís solution (containing copper sulfate) and methylene blue (indicator) are prepared and the aqueous bee pollen extract is added to this mixture with a burette. The reducing sugars in the bee pollen extract react with the copper sulfate, and once all the copper sulfate has reacted, any further addition of reducing sugars causes the indicator to change from blue to white. Therefore, the volume of bee pollen extract required to reach the end point is related to RS content. Although this method is simple, it has some disadvantages, such as the lack of differentiation between types of RS, or the fact that it is susceptible to interference from other types of molecules that act as reducing agents. In addition, sucrose content can also be measured by LEM, determining the RS, expressed as inverted sugar, before and after the bee pollen solution is subjected to acid hydrolysis [6]. Finally, RS content obtained with LEM were compared with those resulting from the NIRS methodology, and the results were quite similar in both cases [58]. Thus, as occurred when determining TPROC, the NIRS technique was seen to be a valid alternative for quantifying RS in bee pollen. The DNS method was based on the reaction between the aldehyde group of RS with DNS, which produced its reduced form (3-amino-5-nitrosalicylic acid). It caused a change in the amount of light absorbed that can be monitored by using a UV–vis spectrophotometer. In some cases, it was necessary to perform a previous hydrolysis and neutralization of the extracts [15] or to remove the proteins [23] in order to avoid potential interferences. Due to the physicochemical characteristic of RS, individual content (glucose and fructose) has been mostly determined by NPLC coupled to a refractive index detector (RID) [16,22,56] or FLD [2,17]. In these studies, RS and oligosaccharides [56], were extracted by using water or methanol and water mixtures, although in one case the bee pollen extracts were clarified by the action of zinc acetate and potassium ferricyanide [22]. Separation was performed with an analytical column and a polar phase (NH_2) in all cases. Individual RS, however, has not been determined solely by NPLC. For example, an analytical column (MetaCarb Ca Plus) specifically designed for the separation of carbohydrates was successfully employed to determine RS and sucrose [20]. On the recommendation of the manufacturer, the mobile phase must be composed only

of water, and subsequently the separation mode is not NPLC but steric exclusion.

As can be seen, quite different analytical techniques have been employed to determine carbohydrates in bee pollen. Conventional and classical methods have been predominantly chosen for determining total protein content (by difference; anthrone method; phenol-sulfuric method) and RS (LEM, DNS); meanwhile, a more advanced and recent analytical tool, such as NPLC coupled to RID or FLD, is required to establish the individual content of sugars.

2.6. Other bioactive compounds

Bee pollen is a rich source of essential minerals such as potassium, phosphorus, magnesium, calcium, sodium, sulfur, iron, copper, manganese, zinc, chromium, nickel, and selenium [11–13]. Minerals are essential for proper regulation of metabolic pathways and physiological processes. Their adequate intake is fundamental for maintaining homeostasis, cell protection, functionality, and health, while their deficiency can trigger specific illnesses [13]. Indeed, the presence of zinc, copper and iron, as well as the potassium/sodium ratio makes bee pollen a significant food for balanced diets as regards the mineral content [12]. However, this substance not only contains essential minerals with positive effects for the organism. Detrimental elements, also called contaminants, like barium, cadmium, lithium, lead, mercury and vanadium can also be found in bee pollen [9,13]. Contamination with these elements, which may affect the metabolism of essential trace elements, such as copper, zinc, iron, manganese and selenium, by competing with them for binding in the biological system, usually results from the use of pesticides, fertilizers and other chemicals in agriculture [13]. As can be seen in Table 6, several papers have been published in the last few years in which essential minerals were investigated in this matrix [9,13,17,20]. The proposed sample treatments consisted of wet acid digestion with mixtures of nitric acid (HNO_3) with hydrogen peroxide (H_2O_2) [9,13] and $HClO_4$. The determination mode of choice adopted for simultaneously establishing these compounds was inductively coupled plasma atomic emission spectrometry (ICP-AES), also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES) [9,13,17]. It must be specified that the names (OES and AES) have been used interchangeably over the decades. In fact, OES is a part of AES, and all the significant techniques could be termed AES, as it has been done in Table 6. In one study, however, essential minerals were measured by atomic absorption spectroscopy (AAS) [20]. Both determination methods (ICP and AAS) have their advantages and disadvantages. For example, ICP systems are predominantly simultaneous measuring systems, with the result that analysis times are independent of
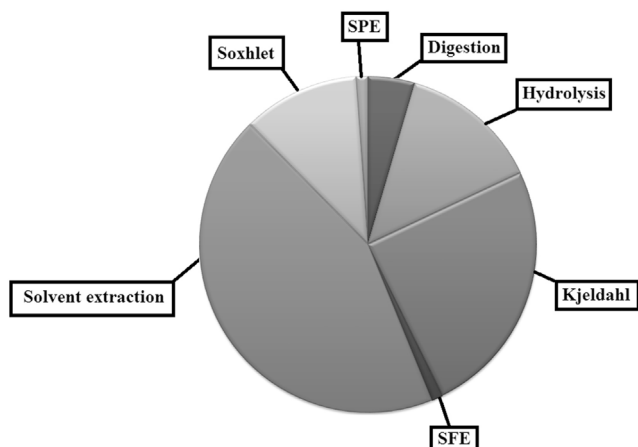
Fig. 4. Summary of the extraction techniques used to isolate the bioactive compounds from bee pollen (**SFE:** supercritical fluid extraction; **SPE:** solid-phase extraction).

the number of elements to be analyzed, which represents a significant advantage if many elements need to be analyzed. AAS is purely sequential (element by element analysis), but at the same time faster and cheaper. Moreover, it provides enhanced sensitivity for group I alkali elements, while ICP generally affords a higher degree of sensitivity for metals whilst allowing for measurement of non-metals.

GLSs are secondary plant metabolites that are present in members of the Brassicales order, such as cabbage, cauliflower, or broccoli. Interest in these compounds has been mainly focused on several observations, such as certain compounds appearing to be protective against chemical carcinogens. Additionally, they coexist with myrosinase in plants, especially in *Brassica* vegetables, and in the presence of water are rapidly hydrolyzed by an enzyme (myrosinase) into their breakdown products (isothiocyanates, thiocyanates, nitriles or indoles) [35,63]. One such product is sulforaphane (SFN), which is an isothiocyanate formed by the hydrolysis of a specific GLS, glucoraphanin. It is of special interest, since nowadays there is a large body of research into functional foods in particular, but also into chronic and degenerative diseases [64]. As we can observe in Table 6, only two publications have been devoted to determining GLSs [63] and SFN [64] in bee

pollen. In the first of these studies [63], a new analytical method to determine GLSs in bee pollen was developed. It was based on a solvent extraction with water followed by an SPE procedure with weak anion sorbents (NH$_2$), with determination by RPLC (C$_{18}$ column) using a tandem mass spectrometry detector (MS/MS). Nine of the GLSs examined were identified in certain bee pollen samples, which was an interesting and unreported finding, and, as can be expected, significant differences in GLS content were observed in the samples analyzed. Furthermore, the potential presence of SFN residues in bee pollen has been also investigated [64]. The proposed sample treatment consisted mainly of a solvent extraction with methanol; the determination technique was again RPLC–MS/MS, but in this case a core–shell technology based column (Kinetex C$_{18}$) was selected. SFN residues were detected in most of the bee pollen samples analyzed at low concentration levels. As with the previous study, the relevance of this research resides in the fact that it was the first time this compound had been investigated/detected in bee pollen.

To sum up, the recommended methodology for establishing essential minerals in bee pollen should consist of a wet acid digestion followed by ICP determination if there are a large number of compounds and samples to be analysed. Meanwhile, AAS would be more appropriate if the minerals belong to group one, or if there are few compounds under analysis. Regarding GLSs and SFN, it is not possible to make a comparison as so far there is only one published method for each.

3. Conclusions

This study presents an updated overview of the publications dealing with the extraction and determination of the different bioactive compounds (lipids; phenolic compounds; proteins, peptides and amino acids; vitamins; carbohydrates; other bioactive compounds: minerals, glucosinolates and related compounds) present in bee pollen. Scientific interest is demonstrated by the number of research papers (>50) published on this topic in many different countries around the world during the period reviewed. Indeed, the number of applications has increased sharply in the last two years, mainly due to the current demand for healthy and natural foods, as is bee pollen. In this review, special attention has been focused on the specific analytical techniques employed for extracting and determining each group of bioactive compounds in bee pollen. Moreover, at the end of each subsection a brief comment has
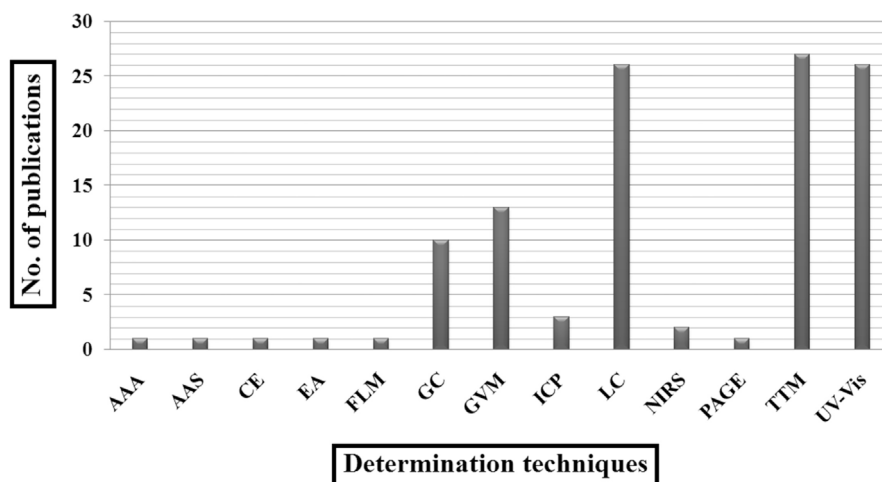

Fig. 5. Summary of the determination techniques used to analyze bioactive compounds of bee pollen in the last years (**AAA:** amino acid analyzer; **AAS:** atomic absorption spectroscopy; **CE:** capillary electrophoresis; **EA:** elemental analyzer; **FLM:** fluorimetric method; **GC:** gas chromatography; **GVM:** gravimetric method; **ICP:** inductively coupled plasma; **LC:** liquid chromatography; **NIRS:** near infra-red spectroscopy; **PAGE:** polyacrylamide gel electrophoresis; **TTM:** titrimetric method).

been included regarding the most suitable methodology for analyzing a specific group of bioactive compounds. It may be concluded that the main group of bioactive compounds analysed in bee pollen have been proteins, although the study of lipids has also attracted the attention of many researchers. Solvent extraction has been the treatment of choice to isolate bioactive compounds from bee pollen, as this has been predominantly used for all of them (See Fig. 4). It has likewise been shown that classic approaches such as Kjeldahl, Soxhlet, FCM or LEM, are today being still used by researchers. In terms of determination techniques, UV–vis has mainly been employed when determining total contents, whereas LC (NP or RP) coupled to several detectors (DAD, PDA, UV–vis, RID, MS, MS/MS) is the technique of choice when determining individual compounds (See Fig. 5). Other techniques such as GC, CE, ICP, AAS, FLD or NIRS have also given good results for determining some specific compounds, although their use is not as widespread as that of LC. To conclude, all the data and information summarized in this manuscript should facilitate the extraction and determination of bioactive compounds in bee pollen, as well as attracting even more attention to this food with its numerous but as yet not widely known health promoting effects.

Conflict of interest statement

The authors of this manuscript declare no conflict of interest

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