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Review

Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds

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ABSTRACT

Broccoli (*Brassica oleracea* L. var. *Italica*) contains substantial amount of health-promoting compounds such as vitamins, glucosinolates, phenolic compounds, and dietary essential minerals; thus, it benefits health beyond providing just basic nutrition, and consumption of broccoli has been increasing over the years. This review gives an overview on the extraction and separation techniques, as well as the biological activity of some of the above mentioned compounds which have been published in the period January 2008 to January 2013. The work has been distributed according to the different families of health promoting compounds discussing the extraction procedures and the analytical techniques employed for their characterization. Finally, information about the different biological activities of these compounds has been also provided.

Contents

Abbreviations: AA, ascorbic acid or vitamin C; AAS, atomic absorption spectroscopy; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ACN, acetonitrile; AES, atomic emission spectroscopy; $AlCl_3$, aluminium chloride; $C_6H_8O_7$, citric acid; Cl_3CH, chloroform; $C_6H_4(SH)_2$, 1,2-benzenedithiol; DAD, diode array detector; DCM, dichloromethane or methylene chloride; DHAA, dehydroascorbic acid; DTT, dithiothreitol; ECD, electrochemical detector; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; EtOAc, ethyl acetate; EtOH, ethanol; FA, formic acid; FAMEs, fatty acids methyl esters; FCR, folin-ciocalteu reagent; FES, flame emission spectroscopy; FID, flame ionization detector; FLD, fluorescence detector; FMOC-Cl, fluorenylmethyloxycarbonyl chloride; FRAP, ferric reducing antioxidant power; FTIR, Fourier transform infrared spectroscopy; FTICR, Fourier transform ion cyclotron resonance; GC × GC, comprehensive two-dimensional gas chromatography; GLSs, clucosinolates; GRA, glucoraphanin; HAc, acetic acid; H_3BO_3, boric acid; HCl, hydrochloric acid; $HClO_4$, perchloric acid; $H_2C_2O_4$, oxalic acid; HNO_3, nitric acid; H_2O_2, hydrogen peroxide; HSCCC, high speed counter current chromatography; IC, ion chromatography; ICP, inductively coupled plasma; IEC, ion-exchange chromatography; ITCs, isothiocyanates; KCl, potassium chloride; KOH, potassium hydroxide; LC, liquid chromatographic; MAE, microwave assisted extraction; MECK, micellar electrokinetic chromatography; β-MET, mercaptoethanol; MeOH, methanol; MES, 2-(N-morpholino)ethanesulfonic acid); MPA, meta-phosphoric acid; MS/MS, tandem mass spectrometry; NAA, neutron activation analysis; $Na_2B_4O_7$, sodium borate; NADPH, nicotinamide adenine dinucleotide phosphate; NaF, sodium fluoride; NaH_2PO_4, sodium dihydrogen phosphate; NaOH, sodium hydroxide; NMR, nuclear magnetic resonance; $NaC_2H_3O_2$, sodium acetate; $NaNO_2$, sodium nitrite; $NH_4C_2H_3O_2$, ammonium acetate; NS, not specified; OES, optical emission spectroscopy; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffer saline; RPLC, reverse phase liquid chromatography; RT-PCR, reverse transcription polymerase chain reaction; SEC, size exclusion chromatography; SDS, sodium dodecyl sulfate; SF, sulforaphane; SFE, supercritical fluid extraction; SPE, solid phase extraction; TEAC, trolox equivalent antioxidant capacity; TCA, trichloroacetic acid; TCD, thermal conductivity detector; TCEP, tris(2-carboxyethyl) phosphine; THF, tetrahydrofuran; TLC, thin layer chromatography; TOC, tocopherol; Tris–HCl, tris–hydrochloride; UPLC, ultra performance liquid chromatography; USDA, United States Department of Agriculture; XRF, X-ray fluorescence.

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1. Introduction

The genus *Brassica* (Brassicaceae or Cruciferae family) includes a large number of vegetables comprising, amongst others, broccoli, cauliflower, Brussels sprouts, kohlrabi, cabbage, mustard, *etc.* [1]. Those vegetables are a good source of many health promoting compounds and potentially protective phytochemicals including phenolics, carotenoids, selenium, glucosinolates or vitamins [2]. Broccoli (*Brassica oleracea* L. var. *Italica*), which is thought to have originally come from the eastern Mediterranean area and to have been introduced to Europe a long time ago (in mediaeval times) [1,3], is nowadays consumed worldwide and is highly valued by large groups of the population due to its flavour, but also due to some health promoting effects, such as anticancer or antioxidant properties, which have been mainly attributed to glucosinolates and their degradation products as well as phenolic compounds, respectively [1]. Consequently, incorporating some of these broccoli health promoting compounds directly or added to pharmaceutical products (nutraceutical) or other foods (functional foods) once they have been isolated and extracted from this vegetable, is a safe and effective way to guard against many of today's most common diseases [2]. Over the past 5 years, the rising interest in the extraction, isolation, characterization and determination of the biological activity of these beneficial broccoli compounds has been demonstrated by the large number of published research papers dealing with this issue (Fig. 1). As can be observed in Fig. 2, several of such compounds have been investigated in this matrix during this period of time, most of the studies being devoted to the analysis of glucosinolates and related compounds (33% of the publications), while phenolic compounds (28%) have also been widely

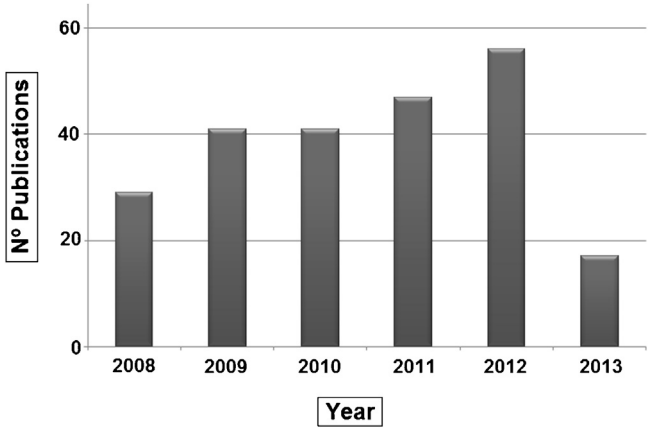

Fig. 1. Evolution of the published works in the last five years related to extraction, chemical characterization and biological activity determination of broccoli health promoting compounds (data up to January 2013). The sources of information were the databases: ISI-Web of Knowledge, Scirus, Scopus and Science Direct. The search has been done using as keywords [(Broccoli) or (*Brassica oleracea* L. var. *Italica*) or (Brassicaceae) or (Brassicaceae or Cruciferae)] and [(phytochemicals) or (glucosinolates) or (lipids) or (vitamins) or (proteins) or (phenolic) or (essential elements) or (amino acids) or (isothiocyanates) or (extraction) or (isolation) or (quantification) or (separation) or (determination) or (chromatography) or (biological activity)] among several others.

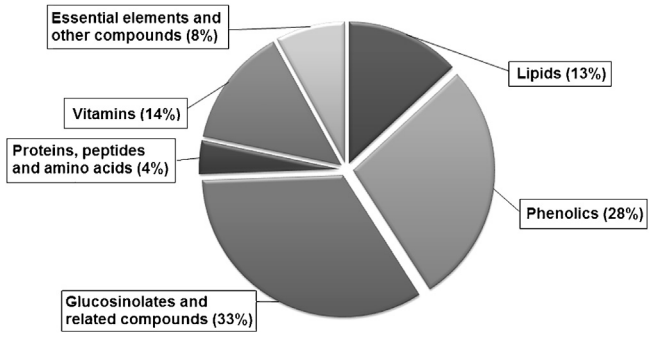

Fig. 2. Summary of the health promoting compounds analyzed of broccoli in the last five years.

studied. It is also interesting to mention that many extraction, separation and determination techniques have been employed to obtain and characterize these compounds from broccoli (see Figs. 3 and 4). As can be seen in Fig. 3, solvent extraction has been predominantly chosen as sample treatment. This finding could be explained because this extraction procedure is usually the cheapest and simplest. However, solid phase extraction (SPE) has been employed in some other cases, especially when analyzing glucosinolates (GLSs), sulforaphane (SF) and vitamins, as it is an effective procedure to obtain cleaner and purer extracts, and at the same time it is possible to concentrate the sample. Hydrolisis has been mainly employed to analyze proteins, GLSs and SF, while an acid digestion was recommended to determine proteins and essential elements. Other sample treatments as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), or Soxhlet extraction have been selected in some specific cases. Meanwhile, liquid chromatography (LC) coupled to several detectors has mainly been used as characterization technique due to the physico-chemical characteristics of the broccoli health promoting compounds, and because it is possible to analyze the content in broccoli of single compounds

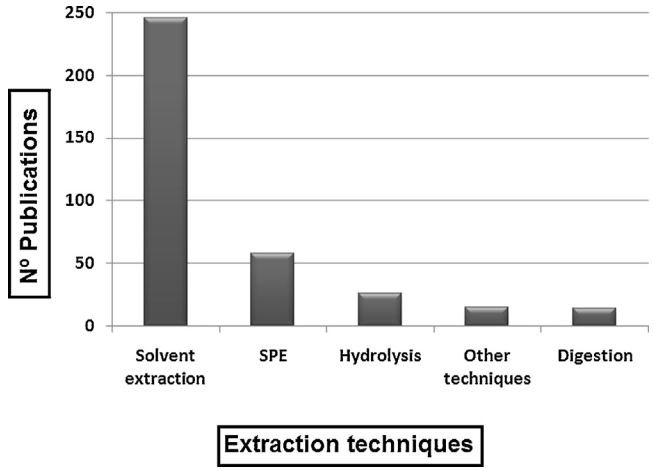

Fig. 3. Summary of the extraction techniques used to analyze health promoting compounds of broccoli in the last five years.

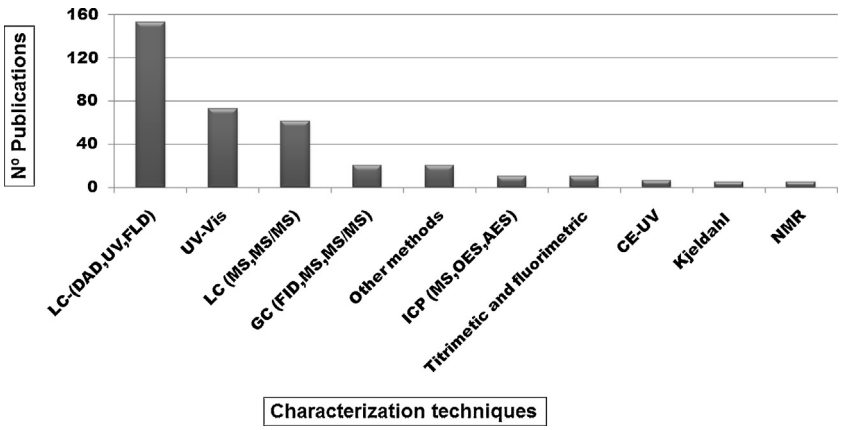

Fig. 4. Summary of the characterization techniques used to analyze health promoting compounds of broccoli in the last five years (AES, atomic emission spectroscopy; DAD, diode array detector; FID, flame ionization detector; FLD, fluorescence detector; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; OES, optical emission spectroscopy).

(see Fig. 4). It should be also commented that MS detectors have been employed in most cases to confirm the presence of specific compounds when the standards were not available, rather than to quantify those compounds as it was not required a great sensitivity because they are usually found at high concentrations. It should be also commented that spectrophotometric methods (UV–vis) have been preferred to determine the total content or to study the profile of a group of phytochemicals, as they are simpler and cheaper than other characterization techniques. Moreover, gas chromatography (GC) has been chosen to analyze lipids, proteins, SF and some essential elements, although it should be pointed out that its use has been more limited than LC. In relation to other characterization techniques, it should be commented that their use have been focused on specific groups of phytochemicals. For example inductively coupled plasma (ICP) has been mainly selected to analyze essential elements, while titrimetric and fluorimetric methods were predominantly used to determine vitamins.

Several interesting reviews and research studies focusing on the general health benefits of broccoli and its related compounds have been published [4–9]. For example, an in-depth study of the chemical and biological characterization of some phytochemicals from broccoli was published in 2006 [4]. In this work, more than 120 research papers were summarized, and although most of them were related to the isolation and determination of glucosinolates and isothiocyanates, several other compounds such as selenium, organic anions and cations were also investigated. Moreover, the influence of processing on the phytochemical composition of broccoli and the bioavailability of these compounds were also discussed. In a more recent publication, a summary was made of some of the literature on bioactive compounds from edible crucifers, including broccoli, indicating their beneficial health promoting effects and their possible role in building up defence against diseases, especially cancer [2]; however, nothing was said about the isolation and characterization of these compounds. Some other studies focused on phytochemicals [5], health affecting compounds [6], and the dietary constituents [7] of broccoli, among other Brassicaceae vegetables, have been published, but, as previously stated, their attention was not focused on the extraction and characterization of those compounds. Mention should also be made of an interesting review studying the variation in the bioactive components (glucosinolates, vitamins, flavonoids, *etc.*) found in broccoli due to genetics, environment and post-harvest processing [8]. The influence of some of these factors on phytochemicals obtained from Brassicaceae vegetables has been also reported [5]. As has been previously seen, several studies have demonstrated that broccoli might be beneficial for human health by reducing the risk of developing certain types of cancer, or on account of its antioxidant activity. However, recent *in vitro* and experimental animal studies indicate that broccoli, its extracts and glucosinolate-derived degradation products might also have undesirable effects, especially genotoxic activities [1,9], although the relevance of these to human health is as yet unknown. However, in this review attention will be focused on the health promoting effects of bioactive compounds extracted from broccoli. Considering all these aspects, the aim of the study is to present and discuss the main extraction and analytical techniques used to obtain, identify, characterize and/or quantify broccoli health promoting compounds in the period January 2008 to January 2013. The review is structured according to the different families of bioactive compounds (lipids, vitamins, proteins, glycosides, phenolic compounds, *etc.*), whilst the last section of the manuscript discusses the biological activity of broccoli and its related compounds and how this was determined.

2. Health promoting compounds of broccoli

2.1. Lipids

Lipids constitute a group of naturally occurring molecules that include fats, fatty acids, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, phospholipids, carotenoids and others. Some of the biological functions of lipids are related with energy storage, composition of cell membranes, and molecular signalling [10]. Although humans and other mammals use various biosynthetic pathways to both break down and synthesize lipids, some essential lipids cannot be made in this way and must be obtained from diet, and broccoli is a potential source of some of these compounds. They have usually been extracted from broccoli by means of solvent extraction, but in two cases supercritical fluid extraction (SFE) was employed to analyze fatty acids in broccoli leaves (Table 1). Moreover, acetone and mixtures of this solvent with water have been predominantly used to extract lipids from broccoli (see Supplementary information, Fig. S1). It can be also observed in this table that gas chromatography (GC) was the technique of choice when determining fatty acids and sterols, although fatty acids should be converted into their corresponding methyl esters (FAMEs) prior to their detection, as more robust and reproducible chromatographic data are obtained in this way. Finally, spectrophotometric methods and reverse phase liquid chromatography (RPLC) coupled to diode array detector (DAD) were employed to determine the other groups of lipids in broccoli (Table 1). It should be commented that the use of separation techniques like GC and LC is necessary in order to facilitate the individual determination of

Table 1
Applications in the analysis of lipids in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
13 FAMEs [11]; 6 FAMEs and 5 sterols [12]	NS (cv. Grandeur) [11]; broccoli roots [12]	Solvent extraction (toluene/MeOH) and acetylation [12]	GC-FID	[11,12]
15 FAMEs	Flowers and stems	Soxhlet extraction (hexane)	GC-FID	[13]
22 FAMEs	Leaves (cv. Nubia, Naxos, Viola, Parthenon and Marathon)	SFE (using CO_2/15% MeOH) or Soxhlet extraction (hexane)	GC–MS	[14]
45 FAMEs [15]; 47 FAMEs [16]	Leaves (cv. Naxos, Nubia and Viola)	SFE (using CO_2/15% MeOH)	GC × GC-FID	[15,16]
Total carotenoid content	Heads (cv. Lvxiong) [18]; Kailan-hybrid [19]; florets (cv. Green Star) [20]; Edible parts (cv. Sebastian) [27]	Solvent extraction (acetone/petroleum ether) [18]; (hexane/MeOH/acetone) [19]; (water/acetone) [20]; (MeOH) [27]	UV–vis	[18–20,27]
Lutein [21]; Lutein and β-carotene [28]	Kailan-hybrid stems and florets [21]; Edible parts [28]	Solvent extraction (hexane/MeOH/acetone) [21]; (EtOH/DCM/hexane) [28]	RPLC-DAD	[21,28]
Lutein and β-carotene	Heads (cv. Parthenon)	Solvent extraction (acetone)	RPLC-DAD	[22]
Total chlorophyll and carotenoid content	Heads [23]; sprouts [24]	Solvent extraction (acetone) [24]; (acetone/water) [23]	UV–vis	[23,24]
Chlorophyll *a* and *b*, total chlorophyll and carotenoid content	Bimi® broccoli (NS)	Solvent extraction (MeOH/acetone)	UV–vis	[25]
Chlorophyll *a*, *b*; and 6 major carotenoids	Edible parts	Solvent extraction (acetone or MeOH)	RPLC-DAD	[26]
Chlorophyll *a*, *b*, Lutein and β-carotene	Heads (cv. Monaco [33] and Parthenon [29,33]); florets [34]	Solvent extraction (acetone)	RPLC-DAD [29,33,34]; UV–vis [34]	[29,33,34]
Chlorophyll *a*, *b* [31]; total chlorophyll content [30,32,35]	Florets [30,35] (cv. iron [31]); heads (cv. Cicco) [32]	Solvent extraction (acetone) [30]; (acetone/water) [31,32,35]	UV–vis	[30–32,35]
Chlorophyll *a*, *b*, and 6 carotenoids	Heads and florets (cv. VI-158, BNC, Pirate, purple-headed)	Solvent extraction (EtOH)	UPLC-DAD	[37]
Total chlorophyll content	Heads (cv. Chaoda) [36]; leaves and roots [38]	Solvent extraction (EtOH/water) [36]; (MeOH) [38]	UV–vis	[36,38]
Chlorophyll *a*, *b*, Lutein, β-carotene total chlorophyll and carotenoid content	NS	Solvent extraction (THF)	RPLC-DAD	[39]

DAD, diode array detector; DCM, dichloromethane; EtOH, ethanol; FAMEs, fatty acids methyl esters; FID, flame ionization detector; GC × GC, comprehensive two-dimensional gas chromatography; MeOH, methanol; NS, not specified; THF, tetrahydrofuran.

these compounds, but when the main goal of the study is to determine the total content (carotenoid or chlorophyll), it is acceptable to simply use UV–vis.

Fatty acids have been extracted in different ways from broccoli; solvent extraction followed by an acetylation process was successfully employed to extract FAMEs and sterols [11,12], Soxhlet extraction was also selected in two cases [13,14], while SFE was used in other publications [14–16]. It should be added that in one of these publications [14], the results obtained with SFE and Soxhlet were compared, and it was concluded that SFE is a promising alternative to the traditional Soxhlet methods for extracting lipids from broccoli leaves. Moreover, compared with Soxhlet extraction, supercritical fluid extracts presented higher percentages of unsaturated fatty acids, and SFE is also a more environmental friendly extraction technique. As seen previously, GC (non- or low polar columns) coupled to flame ionization (FID) or mass spectrometry (MS) detectors was used in all the studies in which fatty acids were analyzed in broccoli [11–16]. Moreover, in two of these publications [15,16], the usefulness of comprehensive two-dimensional gas chromatography (GC × GC) was demonstrated by separating and determining more than 40 FAMEs in a single chromatographic run. In these studies, they were employed highly polar columns in the first dimension and non-polar columns for the second dimension. Taking into account those publications, the best option to characterize fatty acids is the use of GC. In relation to the extraction methods, it could be said that the simplest and cheapest option is solvent extraction, although SFE has provided also promising results, which were comparable or even better than the obtained using Soxhlet extraction.

Carotenoids, which possess great importance because of their nutritional and physiological activities, cannot be synthesized by animals, so they should be acquired through the diet [17]. Total or individual (lutein, lycopene, β-carotene, *etc.*) carotenoid contents have also been studied in different broccoli parts such as heads, florets and sprouts (Table 1). In all cases, solvent extraction with different solvents or composition has been used to isolate these compounds, and in most cases acetone was predominantly employed as extractant (alone or in mixtures with other solvents) [18–26], while in other cases methanol [27] or mixtures of ethanol with other solvents [28] were also selected. Only spectrophotometric detectors (UV–vis, DAD) were employed to determine the compounds, although in certain cases prior separation was required, which was usually accomplished by RPLC with C_{18} [21,22,28,34,37,39] and C_{30} [26,29,33] stationary phases.

The last group of lipids that have been studied in broccoli were chlorophylls. Individual (chlorophyll *a* and *b*), as well as total chlorophyll content was determined in broccoli heads, florets and sprouts. As with carotenoids, solvent extraction has been employed in all the publications where these compounds were analyzed (Table 1). Acetone [23,24,26,29–35], ethanol [36,37], methanol [26,38], tetrahydrofuran [39] or acetone and methanol mixtures [25] were the solvents employed. Finally, spectrophotometric methods with or without previous separation (RPLC with C_{18} [37,39] and C_{30} [26,29,33] columns), depending on the goal of the study (total or individual content), were chosen to identify and quantify the compounds. So it can be postulated that to analyze carotenoids and chlorophylls in broccoli, acetone and a spectrophotometric detector (coupled to RPLC if it is necessary to

determine single compounds) are recommended as extractant and characterization method, respectively.

2.2. Phenolic compounds

More than 4000 compounds divided into 12 subclasses belong to this group of natural compounds, and several of them can be found in broccoli. 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 and others), and are commonly found conjugated to sugars and organic acids [40]. The main activity reported for phenolic compounds has been as antioxidants, although they are also associated with other health promoting effects such as anti-carcinogenic, anti-inflammatory, anti-ageing, and anti-thrombotic activity [41].

Several sample treatments have been published in order to extract these compounds from broccoli, the majority being solvent extractions or microwave assisted extraction (MAE). As can be observed (see Supplementary information, Fig. S2) methanol a water mixtures have been predominantly used to extract those compounds from broccoli, while methanol, ethanol and water have been also widely employed. Moreover, spectrophotometric techniques have been successfully used to determine the total phenolic content in broccoli (see Table 2). However, in some cases, single phenolic compounds were specifically analyzed by using other techniques like LC, GC and CE. It should also be mentioned that LC with C_{18} analytical columns has currently become the technique of choice to perform this task [25,29,41–47,49–57,60], while GC–MS with a low polar column [48] and capillary electrophoresis (CE, fused silica capillaries) with UV [49] were scarcely employed.

One of the classic methods used to analyze total phenolic content is the Folin Ciocalteu reagent (FCR), in which the measured colour change is associated with the reduction of a molybdate–tungstate reagent induced by the phenols in the sample [41]. This reagent does not only react with phenols, but with any reducing substance of the broccoli sample. As can be observed in Table 2, FCR has been mainly employed to determine the total phenolic content in broccoli. Solvent extraction is the most commonly used sample treatment when analyzing phenolics, with several extractants, such as water [24,61–63], methanol [20,27,41,43,64,65,76], ethanol [30,32,66,67], mixtures of water and methanol [25,34,36,44–46,73,74], water with other solvents [19,22,42–48,68], or mixtures of ethanol with hexane [63], being employed. Accordingly, it can be concluded that a mixture of methanol and water is recommended to perform the solvent extraction in this case. Moreover, the effect of independent variables of MAE (extraction temperature, solvent concentration and extraction time) on total phenolic and flavonoid contents of broccoli extracts was also investigated [74]. After that, the samples were directly measured spectrophotometrically by UV–vis to determine the total phenolic content, as it was the fastest and simplest procedure when it was not required to analyze a specific phenolic compound. All types of broccoli parts are analyzed in these studies (florets, heads, sprouts, *etc.*).

Flavonoids are a family of phenolic compounds that have been investigated in broccoli. Quercetin and kaempferol are the predominant flavonoids in broccoli. In general, their levels depend on several factors like environmental pressures, cultivar, post-harvest transport or genotype [44]. Several research papers have been published in order to determine their total content in broccoli samples [45–47,62,71,74–77]. As with total phenolic content, solvent extraction was mainly used to isolate flavonoids, although an additional step of mixing the broccoli extract with sodium nitrite, aluminium chloride and sodium hydroxide was required [45,47,62,71,74]. However, other extractants have been employed in order to analyze total flavone [75], flavonoid [25] and anthocyanin [47,76–78] content, such as methylene chloride [75], mixtures of methanol and water [25], methanol and hydrochloric acid [76,77] and hydrochloric acid with potassium chloride, ammonium or sodium acetate [47,78]. Spectrophotometric (UV–vis) detection was used in most of the research, and in some of the studies devoted to analyzing anthocyanins [47,78] an extra pH differential method was needed due to the fact that they had different colours depending on the pH value. Some special cases are related to the analysis of acylated anthocyanins in broccoli sprouts [59,60] or in stems and florets [42,43]. It has been necessary to perform chromatographic (RPLC) separation subsequent to solvent extraction of these compounds with water, methanol and formic acid [59,60] or with water and methanol [42,43] mixtures, respectively. Identification and quantification were performed by DAD [42,43] or DAD with electrospray ionization tandem mass spectrometry (ESI-MS/MS) [59,60]. A different group of phenolic compounds, flavonoid glycosides, have been also determined in broccoli. It should be pointed out that most of these compounds have been analyzed in conjunction with total flavonoid or phenolic content, so the preferred sample treatment, as can be expected, is solvent extraction (see Table 2). Similar solvents to those mentioned previously have been employed to analyze flavonoid glycosides. Methanol [79], water [52,58] or solvent mixtures such as ethanol and phosphate buffer (PBS) [80], methanol and water [39,48–51,53–57,59,81,82], or hydrochloric acid with ethanol [29] and methanol [33], have been chosen to perform the extractions in these studies. Meanwhile, it should be added that in one case the use of SPE [49] was required. The separation, identification and quantification of these compounds were largely conducted by RPLC (C_{18} columns) coupled to DAD [25,29,41–44,46,49–59,79,81], UV–vis [82,83], or MS [29,33,50–59] detectors. Moreover, CE with UV–vis detector [49] and GC–MS [48] were also employed to determine flavonoid glycosides. It should be also mentioned that in one case [49], flavonols and phenolic acids were isolated and concentrated by SPE with C_{18} cartridges.

After studying the scientific literature related to the analysis of phenolic compounds in broccoli, it can be recommended a solvent extraction with a mixture of methanol and water as sample treatment, while spectrophotometric detection (coupled to RPLC in order to analyze single or several compounds) is the best choice to characterize those compounds.

2.3. Proteins, peptides and amino acids

The consumption of proteins, peptides and/or amino acids offers several benefits for human health, as they are involved in antibacterial, antioxidant, immuno-stimulating, anti-thrombotic and anti-inflammatory activities, among other positive effects in the organism. Such compounds can be found in broccoli, and their extraction and identification requires the use of different analytical techniques, such as SPE, SFE, acid digestion, hydrolysis or solvent extraction, due to the complexity of these compounds (see Table 3). However, solvent extraction was selected in several studies, because it is usually simpler, faster and cheaper than other alternatives as SPE or SFE. Although with the latter techniques it was possible to obtain cleaner and purer extracts. Moreover, RPLC (C_{18} columns), GC (Zebron^{TM} ZB-AAA column), UV–vis, and amino acid analyzers have been commonly used to study them (Table 3).

Proteins (soluble and total) have been extracted from broccoli florets using different solvents like trichloroacetic acid (TCA) [66] or buffer solutions [30,32,35], but in this case it could not be recommended one specific solvent, as all of them were equally employed (see Supplementary information, Fig. S3). The protein

Table 2
Applications in the analysis of phenolic compounds in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Total phenolic content	NS [61,68]; Kalian-hybrid broccoli [19]; sprouts [24]; edible parts [65,70]; florets (cv. Volta F1) [73]; heads [72] (cv. Parthenon [22], Chaoda [36], Cicco [32]); florets [69] (cv. iron [30], Monaco [64], Cicco [66], Green Star [20], Parthenon [34]); seeds [67]; NS (cv. Sebastian) [84]	Solvent extraction (water) [24,61]; (EtOH/water/HCl) [22]; (acetone/water) [68]; (acetone/FA) [19,69,70,73]; (MeOH/water) [34,36,73]; (EtOH) [30,32,66,67]; (MeOH) [20,27,64,65]; (acetone/water/HAc) [72]	UV–vis (FCR)	[19,20,22,24, 27,30,32,34,36, 61,64–70,72,73]
Total phenolic content[a,b] and flavonoid compounds[b]	Bimi® broccoli (NS) [25]; sprouts [41]	Solvent extraction (MeOH/water) [25]; (MeOH) [41]	UV–vis (FCR)[a]; RPLC-DAD[b]	[25,41]
Phenolic and flavonoid compounds	Heads (cv. Nubia and Naxos [56], Parthenon [29]); heads, stalks and leaves (cv. Marathon) [50]; leaves and stalks (cv. Nubia, Marathon and Viola) [51]; broccoli beverages from leaves and stalks (cv. Nubia) [52]; plants (cv. Parthenon and Naxos) [53]; sprouts [55] (cv. Nubia, Marathon and Viola) [54]; edible sprouts and seeds [57]	Solvent extraction (EtOH/water/HCl) [29]; (MeOH/water) [50,51,53–57]; (water) [52]	RPLC-DAD-ESI-MS/MS	[29,50–57]
Phenolic and flavonoid compounds	Heads (cv. Parthenon and Monaco)	Solvent extraction (MeOH/HCl)	RPLC-ESI-MS/MS	[33]
Phenolics and flavonoids compounds	NS [39] (cv. Sebastian) [79]; sprouts (cv. Cezar) [80]; leaves and florets [82], roots and sprouts (cv. Marathon) [81], brocoletto heads [83]	Hydrolysis enzymatic and solvent extraction (MeOH/water) [39,81–83]; (MeOH) [79]; solvent extraction (EtOH/PBS) [80]	RPLC-DAD [39,79–81]; RPLC-UV [82,83]	[39,79–83]
Total phenolic content[a,b], anthocyanin[b], flavone[b] and flavonoid[b] compounds	Irish florets [42]; stems (cv. Monaco) [43]	Solvent extraction (EtOH or acetone with water) [42]; (MeOH) [43]	UV–vis (FCR)[a]; RPLC-DAD[b]	[42,43]
Total phenolic[a,b] and flavonoid[b] content	Florets	Solvent extraction (acetone/water)[a]; (MeOH/water)[b]	UV–vis (FCR)[a]; RPLC-DAD[a,b]	[44]
Total phenolic[a] and flavonoid[b] content	Heads (cv. Calabrese and Shouthern star) [45]; sprouts (cv. Wiarus) [71]; inflorescences [62]; heads (cv. Green Comet) [63]; NS (cv. Marathon) [74]	Solvent extraction (MeOH/water) [45][a] (water) [62][a]; (hexane/EtOH) [63][a,b]; (acetone/FA) [71][a,b]; ($NaNO_2$/$AlCl_3$/NaOH) [45,62,63,71,74][b]; MAE and solvent extraction (MeOH/water) [74][a,b]	UV–vis (FCR)[a]; UV–vis[b]	[45,62,63,71,74]
Total phenolic[a,b] and flavonoid[b] content	Heads (cv. Monaco)	Solvent extraction (MeOH/water)[a]; (MeOH or acetone)[b]	UV–vis (FCR)[a]; RPLC-DAD[a]; UV–vis[b]	[46]
Phenolic[a], flavonoid[b], and anthocyanin[c] compounds	Florets and stems (green and purple-sprouting)	Solvent extraction (MeOH/water)[a]; ($NaNO_2$/$AlCl_3$/NaOH)[b]; (KCl/HCl/$NaC_2H_3O_2$)[c]	UV–vis (FCR)[a]; UV–vis[b]; UV pH differential method[c]	[47]
Phenolic and flavonoid compounds	Florets	Solvent extraction (MeOH/water)	UV–vis (FCR); GC–MS	[48]
Phenolic and flavonoid compounds	NS	Solvent extraction (MeOH/water) and SPE	CE-UV; RPLC-DAD	[49]
Acylated anthocyanin compounds	Sprouts (cv. Marathon, Nubia, Viola)	Solvent extraction (MeOH/FA/water)	RPLC-DAD-ESI-MS/MS	[60]
Total flavone content	Heads and stalks	Solvent extraction (DCM)	UV–vis	[75]
Total phenolic[a] and anthocyanin[b] content	Florets and leaves (cv. Windsor) [76], sprouts (cv. Youxiu) [77]	Solvent extraction (MeOH) [76][a]; (EtOH/water) [77][a]; (MeOH/HCl) [76,77][b]	UV–vis (FCR)[a]; UV–vis[b]	[76,77]
Anthocyanin compounds	Sprouts (cv. Youxiu)	Solvent extraction (KCl, HCl and $NH_4C_2H_3O_2$)	UV pH differential method	[78]

$AlCl_3$, aluminium chloride; DCM, dichloromethane; DAD, diode array detector; ESI-MS/MS, electrospray ionization coupled to tandem mass spectrometry; EtOH, ethanol; FA, formic acid; FCR, Folin-Ciocalteu reagent; HAc, acetic acid; HCl, hydrochloric acid; KCl, potassium chloride; MeOH, methanol; MAE, microwave-assisted extraction; $NaC_2H_3O_2$, sodium acetate; $NaNO_2$, sodium nitrite; $NH_4C_2H_3O_2$, ammonium acetate; NS, not specified; PBS, phosphate buffer saline; SPE, solid phase extraction.
Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

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

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Soluble[a] and total protein[b] content	Florets (cv. iron [30], Cicco [32])	Solvent extraction (buffer solution tris–HCl/β-MET/EDTA)[a]; (NaOH/SDS)[b]	UV–vis (FCR)[b]	[30,32]
Soluble protein content	Florets (variety non-specified)	Solvent extraction (buffer solution tris–HCl/DTT/EDTA)	UV–vis	[35]
Total protein content	Florets (cv. Cicco)	Solvent extraction (TCA)	UV–vis	[66]
Crude protein content[a], glutathione [84][b]	Heads (cv. Monaco [84], Shogun F1 [85], Pirate F1 [85], Sultan F1 [85], Marathon F1 [85])	Digestion (H_2SO_4) with selenium mixture as catalyst [84,85][a]; solvent extraction (HPO_3) [84][b]	Kjeldahl method[a]; RPLC-DAD [84][b]	[84,85]
Crude protein content[a], 15 amino acids[b]	Seeds (cv. Plenk)	Digestion (H_2SO_4) with selenium mixture as catalyst[a]; hydrolysis (HCl)[b]	Kjeldahl method[a]; IEC[b], photometrically[b] (amino acid analyzer)	[86]
Crude protein content[a], 17 amino acids[b]	Florets (cv. Lord F1)	Digestion (H_2SO_4) with selenium mixture as catalyst[a]; hydrolysis (HCl)[b]	Kjeldahl method[a], photometrically[b] (amino acid analyzer)	[87]
Crude protein content[a], 15 amino acids[b]	NS (cv. Grandeur)	Digestion (H_2SO_4) with selenium mixture as catalyst[a]; hydrolysis (HCl) and derivatization (FMOC-Cl)[b]	Kjeldahl method[a]; RPLC-FLD[b]	[88]
Proline	Leaves (cv. Parthenon and Naxos)	SPE	UV–vis	[53]
20 amino acids	Leaves (cv. Nubia, Naxos, Marathon, Parthenon and Viola)	SFE (MeOH as organic modifier) or solvent extraction (MeOH/water or water)	GC–MS	[91]
Cysteine and methionine	Leaves (cv. Monaco)	Solvent extraction (EtOH)	RPLC-DAD-ESI-MS	[92]

DAD, diode array detector; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization coupled to mass spectrometry; EtOH, ethanol; FCR, Folin-Ciocalteu reagent; FLD, fluorescence detector; FMOC-Cl, fluorenylmethyloxycarbonyl chloride; IEC, Ion-exchange chromatography; MeOH, methanol; NS, not specified; SDS, sodium dodecyl sulfate; SPE, solid phase extraction; β-MET, β-mercaptoethanol; TCA, trichloroacetic acid; Tris–HCl, tris–hydrochloride.
Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

content was determined using UV–vis, but in one case the FCR method was employed [30,32]. Meanwhile, crude proteins were obtained from broccoli [84–88] using acid digestion catalyzed with a selenium mixture. Thus, it must be noted that crude protein content was determined in broccoli heads following the procedures of the Association of Official Analytical Chemists [89] or the European recommendations [90], where it was stated that the crude protein content was calculated by multiplying the nitrogen content by 6.25. Total nitrogen was determined by the Kjeldahl method.

Several amino acids have also been found in different broccoli parts such as florets, sprouts and leaves. Acid hydrolysis with hydrochloric acid was selected as the sample treatment in three cases [86–88], while SFE [91], solvent extraction [91,92] and SPE [53] were also successfully employed. These compounds were determined photometrically by means of amino acid analyzers [86,87], an UV–vis spectrophotometer [53], a DAD [92] or a fluorescence detector (FLD), subsequent to conversion of the amino acids with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl), or MS [91,92]. It should be made clear that occasionally the amino acids were previously separated by ion-exchange chromatography [86], GC [91] and RPLC [88,92], in order to perform a single compound determination.

Glutathione has been extracted from broccoli heads by the use of ice-cold meta-phosphoric acid [84]. It was quantified using a homemade electrochemical detector (ECD), and the purity and identification of the peak was confirmed with a DAD. In this case, it was also necessary to perform a previous RPLC separation, in order to facilitate the isolation of glutathione from other matrix compounds.

Finally, it could not be recommended any specific analytical methodology to determine proteins, peptides and amino acids, as they have been employed several and quite different extraction and characterization methods. However, the advantages and disadvantages of some of them have been indicated, which could help potential readers to select the most adequate procedure depending on the analyte, and the equipment of their laboratories.

2.4. Vitamins

Brassica vegetables such as broccoli contain high levels of vitamins, which are organic compounds essential in trace amounts for the normal growth and maintenance of life. These compounds have diverse biochemical roles as regulators of mineral metabolism or of cell and tissue growth and differentiation, antioxidant activity and some are precursors of enzyme cofactors. 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 [4]. Several sample treatments based on solvent extraction have been proposed in order to isolate and obtain ascorbic acid (AA) from broccoli (see Table 4 and Supplementary information, Fig. S4). After examining these data, it can be recommended the use of MPA or a solvent mixture ($C_6H_8O_7$/EDTA/NaF/methanol/water) to perform this task. It should be mentioned that common deficiencies have been found in the existing methods used to determine AA in foods such as lack of specificity, not efficient extraction or stabilization of AA during analysis, and incomplete separation of AA from food-specific interferences in chromatographic analyses [93]. For example, titrimetric and fluorimetric methods are simple and, therefore, popular, but they are not chemically specific for AA. The titrimetric method relies on reduction of the blue dye 2,6-dichloroindophenol by AA to a colourless solution. Meanwhile, the fluorimetric method is based on oxidation of AA to dehydroascorbic acid (DHAA), followed by reaction with o-phenylenediamine to produce a fluorescent quinoxaline derivative [93]. As can be seen in Table 4, RPLC and spectrophotometric methods have additionally been employed to perform the determination of AA in order to solve some of the problems related to the titrimetric and fluorimetric methods, as specificity and separation from matrix compounds.

Use has been made of different solvents to extract AA from broccoli prior to carrying out titrimetic [13,23,24,27,85,94,95] and fluorimetric [69] determination. Methanol [24,27], meta-phosphoric acid (MPA) [13,69], or mixtures of MPA and acetic

Table 4
Applications in the analysis of vitamins in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
AA [13,23,85,94,95] and L-AA [24,27]	Flowers and stems [13]; heads [23]; sprouts [24,27]; heads (cv. Shogun F1, Pirate F1, Marathon F1 and Sultan F1) [85]; florets (cv. Marathon) [94]; florets (cv. Sultan F1, Majestic F1 and Marathon F1) [95]	Solvent extraction (MPA) [13]; (MeOH) [24,27]; (MPA/HAc) [94,23,85,95]	Titrimetric method	[13,23,24,27, 85,94,95]
Total Vitamin C (AA and DHAA) content	Kailan-hybrid florets [19]; florets (cv. Marathon) [34]; Inflorescences (cv. Marathon) [50]; florets [51], leaves [6] and stalks [6] (cv. Marathon, Nubia and Viola) [54]; sprouts [55]; sprouts (cv. Nubia, Marathon and Viola) [54] purple-sprouts [56]; heads (cv. Naxos and Nubia) [59]; sprouts (cv. Marathon) [81]; leaves and florets (cv. Marathon) [82]	Solvent extraction ($C_6H_8O_7$/EDTA/NaF/MeOH/water), SPE and DHAA after pre-column derivatization	RPLC-DAD [19]; RPLC-UV [34,50,51,54–56,59,81,82]	[19,34,50,51, 54–56,59,81,82]
AA	Florets and stems (cv. British green and purple-sprouting)	Solvent extraction (MeOH)	(UV–vis) FCR	[47]
L-AA	Florets (cv. Marathon)	Solvent extraction (MPA)	UV–vis	[62]
AA and DHAA	Florets (cv. Cicco)	Solvent extraction (TCA)	UV–vis	[66]
Total vitamin C (AA and DHAA)	Florets (cv. Volta F1)	Solvent extraction (MPA) and DHAA after pre-column derivatization	FLD	[69]
Total vitamin C (AA and DHAA)[a], L-AA[b]	NS	Solvent extraction (MPA/HAc/EDTA)	UPLC-DAD[a], iodometric titration[b]	[96]
AA	Heads (cv. Parthenon [22,29] and Marathon [33]); stems and leaves [93]	Solvent extraction (MPA/EDTA) [22,29,33]; (MPA/EDTA/TCEP) [93]	RPLC-DAD	[22,29,33,93]
Total vitamin C (L-AA and L-DHAA)	NS [39]; florets [44,97,98]; stalks [97,98]; heads [97,98]	Solvent extraction (MPA) [44]; ($H_2C_2O_4$) [39]; (NaH_2PO_4/EDTA) [98,97] and DHAA after pre-column reduction	RPLC-UV [97]; RPLC-DAD [39,44,98]	[39,44,97,98]
AA	Heads (cv. Lvxiong) [18]; florets (cv. Monaco) [64]; florets and stems (cv. Youxiu [77,99] and Lvling [99])	Solvent extraction ($H_2C_2O_4$) [18,77]; (MPA) [64]; (water) [99]	RPLC-UV [18,99]; RPLC-DAD [64,77]	[18,64,77,99]
Vitamins B (B1, B2, B3, B5, B6, B9, B12) and C	NS	Solvent extraction (water/NaOH/PBS)	RPLC-DAD	[100]
AA[a] and 5-methyl-tetrahydrofolate[b]	Heads (cv. Green Star)	Solvent extraction (MPA)[a]; ($NH_4C_2H_3O_2$/TCA)[b]	RPLC-UV[a], RPLC-FLD[b]	[20]
α,γ-Tocopherol and vitamin A	Edible parts	Solvent extraction (DCM/hexane)	RPLC-DAD	[28]
α,γ-Tocopherol	Florets (cv. VI-158, BNC, Brocolette Neri E. Cespuglio and Violet Queen)	Solvent extraction (MeOH)	UPLC-DAD	[37]
Total tocopherol (α,β,δ,γ-tocopherol)	Leaves	Saponification (KOH)	RPLC-FLD	[38]
Ascorbate and α-tocopherol	Heads (cv. Monaco)	Solvent extraction (MPA)	RPLC-DAD	[84]
5-Methyl-tetrahydrofolate	Florets [101] (cv. Belstar [102])	Solvent extraction (PBS) and chemical derivation	RPLC-FLD	[101,102]

AA, ascorbic acid; $C_6H_8O_7$, citric acid; DAD, diode array detector; DHAA, dehydroascorbic acid; DCM, dichloromethane; EDTA, ethylenediaminetetraacetate disodium salt; EtOH, ethanol; FCR, Folin-Ciocalteu reagent; FLD, fluorescence detector; HAc, acetic acid; $H_2C_2O_4$, oxalic acid; KOH, potassium hydroxide; MeOH, metanol; MPA, meta-phosphoric acid; NaH_2PO_4, sodium dihydrogen phosphate; NaF, sodium fluoride; NaOH, sodium hydroxide; NS, not specified; PBS, phosphate buffer saline; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl) phosphine.
Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method

acid [23,85,94,95], have been used to extract AA or L-AA from different broccoli parts such as florets, heads or sprouts. Furthermore, the differences between LC and titrimetic methods have been investigated in one study aimed at determining AA, DHAA and L-AA in broccoli extracts [96]. In this research, different experiments were carried out, which involved the use of solvent extractions with mixtures of MPA, acetic acid and ethylenediaminetetraacetic acid (EDTA), and quantification and identification by ultra performance liquid chromatography (UPLC) or iodometric titration. It was concluded that L-AA should be determined by using a titrimetic method, while L-AA, DHAA were determined with UPLC-DAD. Several other studies based on LC

methods with C_{18} based stationary phases to determine simultaneously AA, L-AA or DHAA have been published in the last years (see Table 4). In some of them [19,50,51,54–56,59,81,82] the same sample treatment was employed, involving solvent extraction with mixtures of $C_6H_8O_7$/EDTA/NaF/methanol/water) after which a SPE with C_{18} cartridges was performed. The compounds were determined using a DAD [19] or UV–vis detectors [34,50–54,59,81,82]. It must be specified that in some of the above-mentioned publications, DHAA was converted into a fluorophore [34,50–54,59,81,82] whilst in one conversion occurred by mixing AA and 1,2-phenylenediamine [19]. DHAA can also be determined indirectly in broccoli (sprouts, leaves, stalks, heads or florets) after its conversion to L-AA by RPLC-UV [20,97] and RPLC-DAD [39,44,98]. In two of these analyses [97,98], pre-column conversion of DHAA to L-AA was carried out using tris(2-carboxy-ethyl) phosphine solution in hydrochloric acid following extraction with mixtures of sodium dihydrogenophosphate and EDTA. Meanwhile, DL-1,4-dithiothreitol (DTT) was used in other studies to convert DHAA into L-AA after solvent extraction with oxalic acid [39] or MPA extractions [20,44]. Several publications were focused on determining AA or L-AA individually (see Table 4). Different solvents were employed as extractants such as MPA [64,84], oxalic acid [18,77], water [99] and mixtures [22,33,93,100]. The determination of those phytochemicals in broccoli florets [64], heads [22,29,33], sprouts [77], stems or leaves [93] have been mainly achieved by RPLC (C_{18} columns) coupled to DAD [22,29,33,64,77,84,93,100] or UV–vis [18,99]. Finally, spectrophotometric detection (UV–vis) was employed for the analysis of AA and related compounds after performing a solvent extraction with methanol (florets and stems) [47], MPA (inflorescences) [62] or TCA (florets) [66].

B-group vitamins, which are water-soluble vitamins related to metabolism, were also investigated in broccoli samples [100]. In this research, vitamins B1, B2, B3, B5, B6, B9 and B12 were extracted from broccoli samples using a composite formed by water, sodium hydroxide and PBS, and were determined by RPLC-DAD (C_{18} stationary phases). Folate, also known as vitamin M or vitamin B9, has been associated with neural reduction defects and with certain beneficial effects against some types of cancer and other diseases. RPLC-FLD (C_{18} stationary phases) was the preferred analytical technique to determine folate and related compounds in broccoli florets [101,102] and heads [20]. In two studies, chemical conversion to 5-methyl-tetrahydrofolate was conducted prior to its extraction with PBS [101,102], while solvent extraction with TCA and ammonium acetate was sufficient in the other study [20].

Tocopherols (TOC), which are a class of chemical compounds many of which have vitamin E activity, are involved in the protection of membrane lipids from oxidative damage, acting as anti-inflammatory agents. These compounds have been determined in broccoli by RPLC with C_{18} stationary phases (DAD [28,84]; FLD [38]) or UPLC-DAD [37]. Solvent extraction with MPA (heads) [84], methanol (florets) [37] and a mixture of DCM and hexane (edible parts) [28] was used in some cases, or saponification with potassium hydroxide [38] was employed as the sample treatment.

It can be concluded that a sample treatment based on solvent extraction is required in order to obtain vitamins from broccoli. However, the selection of the solvent should be done accordingly to the vitamin group. For example, MPA or a solvent mixture ($C_6H_8O_7$/EDTA/NaF/methanol/water) is recommended to analyze AA, L-AA or DHAA, while other solvents like PBS, MPA, methanol or different solvent mixtures are usually employed to extract B-group vitamins and tocopherols. Moreover, the use of SPE is necessary in some cases in order to obtain cleaner and eliminate some matrix compounds that could interfere with the determination of vitamins. Finally, RPLC coupled to several detectors (UV, DAD, and FLD) is the best option to determine this group of compounds as it solves most of the problems related to the titrimetric and fluorimetric methods. Although, it should be also mentioned that it is more complicated and expensive than these latter methods.

2.5. Glucosinolates and related compounds

Glucosinolates (GLSs) are thioglucosides containing a cyano group and a sulphate group [50]. Because GLSs coexist with myrosinase in the plant, fresh plant material processing in the presence of water (grinding, cutting) will initiate a rapid hydrolysis of the parent compounds. For this reason, analytical approaches could be divided into methods for total GLSs, individual GLSs and breakdown products (thiocyanates, isothiocyanates (ITCs), and oxazolidine-2-thiones) [103]. GLSs and/or their breakdown products have long been known for their fungicidal, bactericidal, nematocidal and allelopathic properties, and they have recently gained research interest because of their anticancer activity. The potential beneficial effects of GLSs and related compounds, such as ITCs, in relation to several diseases (cancer, cardiovascular and neurological diseases) have been previously discussed in some review articles [104,105]. Meanwhile, another study sought to provide evidence for and against ITCs as chemopreventive agents [106]. In relation to determining GLSs in broccoli, it should be specified that these compounds are usually established according to the presence (intact) or absence (desulfo-derivatives, desulfo-GLSs) of the sulphate group in their structure. A common approach, which is widely used [107], is to convert the intact GLSs into desulfo-derivatives, as they can be more easily determined by RPLC. Identification of the GLSs by means of this method is based solely on comparing retention times and UV spectra with reference standards, but due to the lack of availability of many GLSs standards it is difficult or even impossible to determine unequivocally unknown GLSs. For that reason, the use of MS detectors appears to be a solution for a more trustworthy identification of GLSs.

An on-column enzymatic desulfation treatment has been adopted in order to determine by RPLC desulfo-GLSs in most of the broccoli parts such as seeds, florets, heads, shoots, roots or leaves (see Table 5). These studies largely made use of a preliminary solvent extraction (see Supplementary information, Fig. S5) with heated solvents such as mixtures of methanol and water at different ratios (70:30, v/v) [69,84,108–124], (80:20, v/v) [125,126], (90:10, v/v) [62], although water alone has also been employed [18,45,78,99,127,128] followed in most cases by a SPE desulfation. In one study, however, desulfo-GLSs were extracted using a microwave digestion procedure [100]. It can be concluded after studying the summarized data (Table 5 and Supplementary information, Fig. S5) that the best choice to extract desulfo-GLSs is the use of a mixture of water and methanol (70:30, v/v). Furthermore, RPLC (C_{18} analytical columns) is the technique of choice to analyze these compounds, and this has been coupled to several detectors like spectrophotometric (UV–vis [77,99,108,117,118,122,124,125,127,128] or DAD [78,84,111,112,115,116,119,121,123,126]), and MS detectors such as ESI-MS [18,121,123] or ESI-MS/MS [69,110–113], in order to perform identification and quantification. It should be pointed out that MS detectors have been predominantly used with identification and confirmation purposes, as it is not necessary a great sensitivity to determine those compounds because they are present in high concentrations in broccoli. Several aliphatic, indole and aromatic desulfo-GLSs were analyzed in the above-mentioned publications. Other methodologies have been developed, not based on RPLC, to determine desulfo-GLSs [45,120]. For example, a CE-DAD method was employed to determine several desulfo-GLSs, subsequent to boiling solvent extraction with a mixture of methanol and water (70:30, v/v) [120]. Meanwhile, total desulfo-GLSs content was measured spectrophotometrically (UV–vis) in broccoli heads [45]. In this study, solvent extraction with boiling

Table 5
Applications in the analysis of glucosinolates and related compounds in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Desulfo-GLSs	Heads (cv. Lvxiong) [18]; florets [127,109,79] and stems (cv. Youxiu [127,109,99], Lvling [99] and Ironman [112], Volta F1 [69]; seeds (*cymosa* Duch. Monopoly [125], F1 Hybrid [125], Syngenta Enkhuizen [125], Netherlands [125], Marathon [113]); edible parts [108]; roots and shoots (cv. Marathon) [110]; shoots (38 cultivars) [126]; heads (cv. Youxiu [115], Lvxiong [115], Sijilv [115], Shenglvand [115], Yangguang [115], '1997' [117], Monaco [84]); spouts [116,27] (cv. Youxiu [78]); inflorescences (cv. Marathon [62,118] and Parthenon [118]); leaves (cv. GDDH33) [114]	Solvent extraction (water) [18,78,99,127]; MeOH [39]; (90/10 MeOH/water, v/v) [62]; (80/20 MeOH/water, v/v) [125,126]; (70/30 MeOH/water, v/v) [69,84,108–118] and SPE desulfation	RPLC-MS [18]; RPLC-UV [39,99,108,109,117,118,125,127]; RPLC-DAD [78,84,115,116]; UPLC-DAD [126]; RPLC-DAD-ESI-MS/MS [69,110–113]; NS [62,114]	[18,39,62,69, 78,84,99,108–118,125,126,127]
Total desulfo-GLSs content	Heads (cv. Calabrese and Southern Star)	Boiling solvent extraction (water), SPE desulfation, column isolation and tubes caption	UV–vis	[45]
Desulfo-GLSs[a] and SF[b]	Sprouts (cv. Youxiu [77,128]); boiled broccoli (NS) [119]	Boiling solvent extraction (H_2O) [77,128][a]; (70/30 MeOH/H_2O, v/v) [119][a] and SPE desulfation; solvent incubation (MES buffer solution) and extraction (DCM) [77,128][b]; conjugation with 2-mercaptoethanol [119][b]	RPLC-UV [77,128][a]; RPLC-DAD [119][a,b]; GC-FID [77,128][b]	[77,119,128]
Desulfo-GLSs	Steamed, raw and cooked broccoli (NS)	MAE digestion	RPLC-MS/MS	[100]
Desulfo-GLSs[a] and ITCs[b]	Heads (cv. Monaco) [120]; florets (cv. Monaco), sprouts (cv. Calabrese), Se and indole-GLS enrichment broccoli [124]	Boiling solvent extraction (70/30 MeOH/water, v/v) [120,124][a] and SPE desulfation; GLSs hydrolisis by water and solvent extraction (EtOAc) [120][b]; (DCM) [124][b]	RPLC-UV [124][a,b]; CE-DAD [120][a]; GC–MS [120][b]	[120,124]
Desulfo-GLSs[a] and ITCs[b]	Sprouts (cv. Calabrese [121]); heads [122]; NS (cv. Cezar) [123]	Boiling solvent extraction (70/30 MeOH/water, v/v) and SPE desulfation [121–123][a]; GRA hydrolysis by acidic water and solvent extraction (DCM) [121,122][b]; (EtOAc) [123][b]	RPLC-UV [122][a]; RPLC-DAD-ESI-MS [121,123][a]; RPLC-DAD [123][b]; GC–MS [122][b]; GC-FID [121][b]	[121–123]
Intact-GLSs[a] and SF[b]	Kailan-hybrid stems and florets [21]; Florets (cv. Marathon and Blooster™) [138]	Boiling solvent extraction (water) [138][a]; (70/30 MeOH/water, v/v) [21][a]; GRA hydrolysis by acidic water and solvent extraction (DCM) [21,138][b] and SPE [21][b]	RPLC-ESI-MS/MS [138][a,b]; RPLC-DAD-ESI-MS/MS [21][a]; RPLC-DAD [21][b]	[21,138]
Intact-GLSs	Heads/florets and controlled samples (cv. Parthenon) [22,29,34]; commercial broccoli [39]; leaves [51], stalks [51], sprouts [54] and seeds [54] (cv. Nubia, Viola and Marathon); green-tea infusions of inflorescences (cv. Nubia) [52,68]; inflorescences (cv. Marathon) [50]; shoots (cv. Parthenon and Naxos) [53]; sprouts [55] (cv. Marathon) [81,129]; leaves and florets (cv. Marathon) [82]; heads (cv. Parthenon [33], Monaco [68], Nubia [56] and Naxos [56]); leaves (cv. Monaco) [90]; florets and stems (cv. purple-sprouting) [59]; edible sprouts and seeds [57]; NS [130,92,132]	Boiling solvent extraction (70/30 MeOH/water, v/v) [22,29,33,34,50,51,55–57,59,68,81,92,129,82,130]; (MeOH) [39,52]; (80/20 MeOH/water, v/v) [53,54]; (water) [132]	RPLC-DAD-ESI-MS/MS [33,39,50–57,59,68,81,92,129,82]; RPLC-ESI-MS/MS [22,29,34]; RPLC-ESI-FTICR-MS/MS [131]; UPLC-ESI-MS/MS [132]	[22,29,33,34,39, 50,57,59,68,81, 92,129,82, 130,132]
Total intact-GLSs[a] content and SF[b]	Heads (cv. Chaoda)	Boiling solvent extraction (95/5 EtOH/water, v/v)[a]; (acetonitrile)[b] and myrosinase hydrolysis	Phenol–sulfuric acid method (UV–vis)[a]; RPLC-UV[b]	[36]

Table 5 (*Continued*)

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Intact-GLSs	Leaves	Boiling solvent extraction (20/80, 0.1%FA/MeOH v/v) and SPE	RPLC-ESI-MS	[131]
Novel intact-GLSs	Florets	Boiling solvent extraction (water) and SPE	RPLC-UV-ESI-MS/MS [134,135]; NMR [134,135]; FTIR [135]	[134,135]
Intact-GLSs	NS (cv. Rubra)	Cold maceration (80/20 MeOH/water, v/v) and SPE	TLC, paper chromatography and RPLC-UV	[140]
GRA	Florets	Boiling solvent extraction (water)	MECK-DAD, RPLC-DAD	[133]
GRA[a] and SF[b]	Seeds and sprouts (ZS ZaoSheng, XB XueBai, RF RuiFan N732, YX YinXing 100, TY TaiYou, LLX LuLingxiang and XMYH XiaMenYinhua) [136]; seeds (cv. Calabrese, DeCiccoand Romanesco) [137]	Boiling solvent extraction (water) [136,137][a] and SPE [136][a], GRA hydrolysis by acidic water and solvent extraction (EtAcO) [136][b]; (DCM) [137][b]	RPLC-UV [136][a,b]; RPLC-UV-MS [137][a]; GC-FID [137][b]	[136,137]
Intact-GRA[a] and SF[b]	Sprouts	Boiling solvent extraction (water or solvent)	HILIC-DAD[a]; RPLC-DAD[b]	[139]
GRA configuration	Seeds	NS	RPLC-UV-MS/MS and NMR	[141]
SF and related compounds	Heads and stalks [75]; sprouts [103,151]	Hydrolysis with water and solvent extraction (Cl_3CH) [151]; (DCM) [75,103]	Titrimetric method [75]; GC-FID–MS [103,151]	[75,103,151]
SF	NS [142,143]; heads (cv. Grandeur) [144]	GRA hydrolysis with acidic water and solvent extraction (DCM) and SPE	RPLC-UV	[142,143,144]
SF[a] and total ITCs[b]	NS	Solvent extraction (DCM) and SPE[a]; incubation with $Na_2B_4O_7$ and $C_6H_4(SH)_2$ and $C_6H_4(SH)_2$ following solvent extraction (hexane)[b]	RPLC-DAD	[149]
SF	Seeds	GRA hydrolysis by water, solvent extraction (EtAcO), SPE	HSCCC-UV, MS, NMR	[150]
Se-GLS[a] and Se-ITCs[b]	Florets	Boiling solvent extraction (80/20 EtOH/water, v/v)[a]; (50/50 diethyl ether/pentane, v/v)[b]	RPLC-MS/MS[a]; GC–MS/MS[b]	[152]
SF	Seeds	Boiling water, hydrolysis by acidic water (HCl)	RPLC-ESI-MS and NMR	[153]

Cl_3CH, chloroform; $C_6H_4(SH)_2$, 1,2-benzenedithiol; DAD, diode array detector; DCM, dichloromethane; ESI-MS/MS, electrospray ionization coupled to tandem mass spectrometry; EtOAc, ethyl acetate; EtOH, ethanol; FA, formic acid; FID, flame ionization detector; FTIR, Fourier transform infrared spectroscopy; FTICR, Fourier transform ion cyclotron resonance; GLSs, glucosinolates; GRA, glucoraphanin; HSCCC, high speed counter current chromatography; ITCs, isothiocyanates; MECK, micellar electrokinetic chromatography; MeOH, methanol; MES, 2-(N-morpholino)ethanesulfonic acid); MAE, microwave-assisted extraction; $Na_2B_4O_7$, sodium borate; NS, not specified; NMR, nuclear magnetic resonance; SF, sulforaphane; SPE, solid phase extraction.

Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

water was performed, desulfation being carried out in a SPE cartridge, and finally the desulfo-GLSs were isolated and purified on small ion-exchange columns.

Otherwise, most of the treatments proposed to analyze intact-GLSs included a heating process, as it is necessary to deactivate the enzyme myrosinase in order to achieve a better extraction of the compounds. As can be seen (Table 5 and Supplementary information, Fig. S5), several studies have been published dealing with this issue, and the preferred choice to extract intact-GLSs from broccoli extracts was the use of heated mixtures of methanol and water at different ratios (70:30, v/v) [21,22,29,33,34,50,51,55–57,59,68,81,92,129,82,130] (80:20, v/v) [54], although different mixtures, for instance, methanol and formic acid 1% in water (80:20, v/v) [131] or ethanol and water (95:5, v/v) [36] have been also employed. Moreover, other proposals exist in which heated methanol [39,52] and water [132–139] were used individually to carry out extraction. Prior to identifying and quantifying the intact GLSs, it was necessary to include a cold maceration with methanol and water [140] or SPE procedures [131,133–136] in order to obtain a better purification of the extracts. As occurred when analyzing desulfo-GLSs, several analytical techniques have been chosen to determine the intact-GLSs in broccoli extracts, which were obtained from all its parts, and RPLC (C_{18} columns) coupled to several detectors (UV–vis [36,134–136,140], DAD [21,33,39,50–57,59,68,81,92,129,82,139], MS [131,137] and MS/MS [21,33,34,39,50–57,59,68,81,92,129,82,132,134,135,138,140]) was selected in most of the studies (see Table 5). It must be pointed out that micellar electrokinetic chromatography [133] and hydrophilic interaction liquid chromatography [139] were also chosen to study intact glucoraphanin (GRA) in broccoli florets [133] and sprouts [139]. Meanwhile, GLSs fractions were also separated using thin layer chromatography or paper chromatography. Taking into these data, it is recommended the use of RPLC with UV or DAD detectors when it is possible to obtain the intact-GLSs standards, while MS detectors should be employed if they are not available.

Not only chromatographic methods have been employed to analyze intact GLSs. Fourier transform ion cyclotron resonance MS was chosen to identify 24-intact GLSs identified in broccoli [130], while nuclear magnetic resonance (NMR) has been also selected to carry out other studies in which some new GLSs were investigated [134,135] or the GRA configuration was specified [141]. Moreover, Fourier transform infrared spectroscopy (FTIR) was also used in one study to determine novel intact GLSs in broccoli florets [135]. The quality and quantity of data obtained with these latter analytical techniques are really high, but it should be pointed out that they are more complex and expensive than the LC or spectrophotometric methods.

After examining the scientific literature related to the analysis of GLSs in broccoli, it can be recommended a solvent extraction with a heated mixture of methanol and water, which is followed by a SPE desulfation when analyzing desulfo-GLSs, as sample treatment. Meanwhile, RPLC with UV or DAD detectors is the best choice to determine those compounds when it is possible to obtain individual standards, and MS detectors are recommended if they are not available.

The other huge sulphur-containing phytochemical group that can be found in broccoli are ITCs. Their separation and identification have been typically accomplished by RPLC. However, the application of this technique to investigate certain glucosinolate breakdown products may be limited due to the volatility of many compounds. For this reason, some studies opted for using GC. One of the most extensively studied ITCs is sulforaphane (SF), because of its potential health benefits (see Section 3). Most of the studies in which this compound was analyzed were based on the hydrolysis of its precursor (GRA) by using acidic water at different pHs, and a further extraction step with an organic solvent such as DCM [21,75,77,103,121,122,124,128,137,138,142–147,149], ethyl acetate [120,136,150], or chloroform [146,151] (see Supplementary information, Fig. S5). In some cases, instead of acidic water, SF was incubated with 2-(N-morpholino) ethanesulfonic acid [77,128], or it was conjugated with 2-mercaptoethanol [119]. It should also be mentioned that in some studies SPE with silica cartridges was performed to purify the broccoli extracts [21,142–144,149,150]. Meanwhile, in other study, the myrosinase enzyme was used to perform SF hydrolysis before extracting with acetonitrile [36]. Most of the above-mentioned research studies employed RPLC with C_{18} based stationary phases (DAD or UV–vis [21,36,119,133,139,142–145,149], MS [138]), and GC with non- or low polar capillary columns (FID [77,103,121,128,137,147,148,151] and MS [103,120,122,146,151]). However, it has been postulated that in some cases SF was thermally degraded in the injection ports of GC equipment [143], and usually the GC analysis times were longer than those of LC. As happened with GLSs, MS was not usually employed because of the high SF content in broccoli. It must be added that in one study [150] separation was carried out by high-speed counter-current chromatography, while SF presence was confirmed by NMR and MS. NMR has also served to analyze SF in broccoli seeds [153]. Moreover, a titrimetic method was applied to determine SF and related compounds in broccoli heads and stems by using hexahydropyridine as a reagent [75]. So, in order to determine SF in broccoli, it is recommended to carry out a hydrolysis with acidic water, a further solvent extraction with DCM, and a SPE procedure with silica cartridges before injecting the extracts in a RPLC-UV or DAD system.

Finally, research has been published in which ITCs other than SF were analyzed [120,123,124,149,152]. As can be seen in Table 5, the extraction and characterization methodologies employed in those cases were quite similar to the ones selected when analyzing SF.

2.6. Essential elements and other compounds

Broccoli is a good vegetable source of essential elements like Se, minerals for human nutrition (Ca and Mg), main (Na, K, Cl and P) or trace (Fe, Zn, Cu, Mn, I, F, Se, Cr, Mo, Co, and Ni) elements and sugars. Selenium is an essential nutritional element that has attracted interest due to its potential anticancer activity [154]. Moreover, it is known that Broccoli has the ability to accumulate high levels of Se with most of the seleno-amino acids in the form of Se-methylselenocysteine and seleniomethionine. Several papers have been published in last 5 years where selenium was investigated in this matrix (see Table 6 and Supplementary information, Fig. S6). For example, the selenium profile in florets and sprouts was directly determined by means of neutron activation analysis (NAA) [124], whilst total selenium content in florets, leaves and stems was calculated using inductively coupled plasma with mass spectrometry (ICP-MS) [38,112] or FLD [145], although in one case [38] it was necessary to perform acid digestion as a sample treatment. The Se speciation was subjected to study in leaves, roots and sprouts [38,145] by ICP-MS. In both cases it was necessary to conduct a sample treatment (acid digestion [38] and sonication [145]) and a previous separation with ion [38] or size exclusion [145] chromatography in order to obtain satisfactory results. Se-methylselenocysteine and seleniomethionine were also determined in broccoli florets and leaves, after performing acid digestion [73] or solvent extraction [126] and a further liquid chromatographic separation and detection by FLD [73] and DAD [126]. Finally, it should be said that other selenium compounds (organoselenides and volatiles) were identified by GC–MS in broccoli sprouts after performing solvent extraction [152]. As can be seen, different analytical techniques have been employed to determine Se and related compounds, some of them allowed the separation and identification of single analytes (LC and GC), while the other

Table 6
Applications in the analysis of essential elements and other compounds in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Total Se and Cd content[a] and Se speciation[b]	Leaves and roots	Digestion (HNO_3/H_2O_2)	ICP-MS[a]; IC and ICP-MS[b]	[38]
Se-methylselenocysteine	Florets	Solvent extraction (HCl)	RPLC-FLD	[73]
Total Se content	Florets (cv. Ironman)	NS	ICP-MS	[112]
Se profile	Florets (cv. Monaco), sprouts (cv. Calabrese)	NS	NAA	[124]
Cations (Fe, Zn, Cu, and Mn)[a], Total selenium content[b], Se-methylselenocysteine[b] and selenomethionine[b]	Florets and leaves (38 varieties)	Digestion ($HNO_3/HClO_4$)[a]; Solvent extraction (HCl)[b]	ICP-MS[a]; UPLC-DAD[b]	[126]
Total Se content[a] and speciation[b]	Sprouts	NS[a]; Sonication (deionized water)[b]	FLD[a]; SEC and ICP-MS[b]	[145]
Organoselenides and Se volatiles	Florets (cv. Triathlon)	Solvent extraction (pentane/diethyl ether)	GC–MS	[152]
Anions (Cl and P) and cations (Na, K, Ca, Mg, and Fe)	Stem and flower ashes	Solvent extraction (H_3BO_3)	XRF	[13]
(P, Ca, Mg, K, Na, Fe, Mn, and Zn)[a] and total (C and N)[b] content	Leaves and stalks (cv. Nubia, Marathon and Viola)	Digestion ($HNO_3/HClO_4$)	ICP-OES[a]; TCD[b]	[51]
Anions (Cl^-, NO_3^-, SO_4^{2-}, and PO_4^{3-})[a], cations (Ca^{2+}, K^+, Mg^{2+}, and Na^+)[b] and soluble sugar content[c]	Shoots, roots[a,b] and leaves[c] (cv. Parthenon and Naxos)	Digestion (HNO_3/H_2O_2)[a]; SPE[b]	IC-ECD[a]; ICP-AES[b]; UV–vis[c]	[53]
Anions (P, S, and B) and cations (Na, K, Ca, Mg, B, Cu, Fe, Mn and Zn)	Heads (cv. Nubia and Naxos)	Digestion (HNO_3/H_2O_2)	ICP-OES	[56]
Anions (Cl^-, NO^{3-}, PO_4^{3-}, and SO_4^{2-})[a] and cations (Na^+, Ca^{2+}, K^+, and Mg^{2+})[b]	Leaves and florets (cv. Marathon)	NS	IC-UV–vis[a]; ICP-OES[b]	[82]
Anions (N^a and P^b) and cations (K^c, Ca^c, Mg^d, Fe^d, Mn^d, Cu^d, and Zn^d)	Heads (cv. Shogun F1, Sultan F1, Marathon F1 and Pirate F1)	NS	Kjeldahl method[a]; Vanadat-Molibdat method[b]; FES[c], AAS[d]	[85]
Anions (N^a, $NO_3^{-\,b}$) and cations (K^c and Ca^d)	Inflorescences (cv. Parthenon and Marathon)	NS[a,b]; Digestion (HNO_3)[c,d]	Dry combustion[a]; IC-ECD[b]; FES[c]; AAS[d]	[118]
Anions (Cl^{-a}, SO_4^{2-b}, and B^b), cations (Ca, Mg, Na, and K)[c], total S and P content[b]	Leaves, stems and heads (cv. Seminis PX511018)	Digestion ($HNO_3/HClO_4$)	Coulometric–amperometric titration NS[b]; ICP-OES[c]	[156,157]
Anions (Cl^- and NO_3^-)[a] and cation (Na^+)[b]	Leaves (cv. Lord)	NS	CE-UV[a]; ICP-OES[b]	[158]
K	NS	Digestion (HNO_3/H_2O_2)	FES	[160]
Heavy metals (Cd, Pb, Ni, Cu)	Roots	NS	Phytotoxicity scales (agar and filter paper)	[161]
Total [32] and reducing sugar content	Florets (cv. iron)	Solvent extraction (EtOH)	UV–vis	[30–32]
8 sugars	Roots and shoots (cv. Marathon)	Solvent extraction (MeOH)	LC-ECD	[110]
3 soluble sugars	Leaves and heads (cv. Marathon)	Reduction (NADPH)	UV–vis	[155]

AAS, atomic absorption spectroscopy; AES, atomic emission spectroscopy; ECD, electrochemical detector; EtOH, ethanol; FES, flame emission spectroscopy; HNO_3, nitric acid; H_3BO_3, boric acid; H_2O_2, hydrogen peroxide; HCl, hydrochloric acid; $HClO_4$, perchloric acid; ICP, inductively coupled plasma; MeOH, methanol; NAA, neutron activation analysis; NADPH, nicotinamide adenine dinucleotide phosphate; NS, not specified; OES, optical emission spectroscopy; TCD, thermal conductivity detector; IC, ion chromatography; SEC, size exclusion chromatography; SPE, solid phase extraction; UPLC, ultraperformance liquid chromatography; XRF, X-ray fluorescence spectroscopy. Superscript letters (a,b,c,d) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

techniques (NAA, ICP, and FLD) are more recommended to investigate the analyte profiles and total content.

Moreover, some studies have been published in relation to the argument that broccoli could be an alternative source of Ca or Mg in sectors of the population that consume limited amounts of dairy products [155]. As can be seen in Table 6, they were determined in almost all broccoli parts such as leaves, stalks, stems, heads, roots and inflorescences. Most of the proposed sample treatments consisted of acid digestion with nitric acid [118] or mixtures of nitric acid with perchloric acid [51,156,157] or hydrogen peroxide [53,56], yet in one case a solvent extraction with boric acid was used [13]. The characterization mode of choice adopted for simultaneously determining these compounds was ICP coupled to either optical emission spectroscopy (OES) [51,56,156–158] and atomic emission spectroscopy (AES) [53], although X-ray fluorescence (XRF) [13] and flame emission spectroscopy (FES) [118] have been also employed. These compounds were detected in a different way in one publication referring to broccoli heads [85]. In this case Ca was quantified by FES, while Mg was measured by atomic absorption spectroscopy (AAS). Taking into account those findings, it can be recommended an analytical methodology to determine Ca and Mg in which an acid digestion of the broccoli sample should be done prior to analyze the extract by ICP-OES.

In relation to main and trace elements, it should be stated that they have varied functions with regard to humans, as they act as electrolytes, enzyme constituents, or building materials (*e.g.*, in

bones and teeth). However, not all their potential health effects are positive, as is the case with their nitrate content. Human dietary nitrate and nitrite exposure should be controlled as they may be considered a health risk factor. This could be explained by the conversion of nitrates, which are relatively harmless to humans, to nitrites or other N-nitrose compounds perhaps producing toxic products [159]. The composition of these main and trace elements in broccoli has not been examined in depth, and as a consequence not many studies have been published relating to this topic. Different sample treatments have been proposed regarding their determination in several broccoli parts such as florets, leaves, roots, stems, heads and inflorescences (see Table 6). As occurred with Ca and Mg, acid digestion with HNO_3 or mixtures with other compounds [51,53,56,118,156,157] and ICP coupled to OES or AES [51,53,56,156–158] are recommended as sample treatment and determination method, respectively. Other methodologies have been less often employed to perform this task. For example, several of these compounds were extracted with boric acid and measured by XRF in broccoli stems and flower ashes [13], while in another publication [118], different determination methods were proposed according to the compound studied: (i) a dry combustion method was used to determine total nitrogen content; (ii) a digestion method with concentrated nitric acid followed by FES was employed to measure potassium; (iii) ion chromatography separation with ECD was applied to quantify nitrates. Potassium and nitrate have been determined in a similar way in other researches [160] and [82], respectively, but to measure nitrates it was employed UV–vis. Moreover, nitrate and chloride were analyzed in a different study by using CE-UV [158]. Total C and N contents have also been determined in broccoli leaves and stalks with a thermal conductivity detector (TCD) following acid digestion [51]. It is of interest to note that, as special cases, a Kjeldahl method was also employed to determine total N content and a colorimetric method was used to measure total P content, both of them in broccoli heads [85]. Moreover, chloride was determined in several broccoli parts by coulometric–amperometric titration [156,157]. Not only beneficial elements were found in broccoli. In one analysis [161] broccoli roots were used to test the toxicity of four heavy metals (Cd, Pb, Ni and Cu).

Several studies related to investigating sugars in broccoli have been published in the last years (see Table 6). Total and reducing sugar content have been determined in florets. It was necessary to perform a solvent extraction with ethanol prior to quantifying them with a UV–vis spectrophotometer [30–32]. The same detection method was used to measure the content of several soluble sugars in leaves and heads [159] or in shoots, roots and leaves [53], but in these cases the sample treatment consisted of a reduction with NADPH [159] or a SPE procedure [53]. Finally, a solvent extraction method with methanol, followed by LC-ECD with an anion-exchange column, has been proposed in order to determine eight sugars in broccoli roots and shoots [110].

3. Biological activity determination

As can be deduced from the previous sections of this manuscript, broccoli is an excellent dietary source of phytochemicals, including lipids, phenolic compounds, proteins, peptides, amino acids, vitamins, glucosinolates and their breakdown products, and certain minerals and essential elements. The potential health promoting roles of these compounds have been extensively studied in the last years. Beneficial effects such as antioxidant, anticancer, antimicrobial, amongst several others (Fig. 5), have been reported in many publications. Most of the research that focussed attention on the biological activity of broccoli was related to the antioxidant and anticancer properties of this vegetable due to the action of several of the above-mentioned phytochemicals. In this section the different biological activities of broccoli will be discussed, including many of the recent publications related to this issue, and how these beneficial effects were determined in some specific cases.

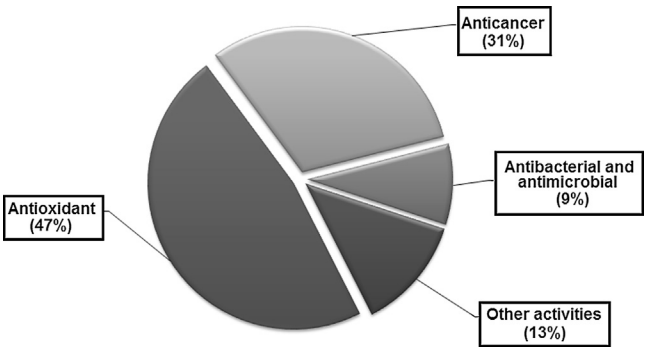

Fig. 5. Summary of the different biological activities attributed to the health promoting compounds of broccoli in the last five years.

3.1. Antioxidant

Broccoli is renowned for its vast range of non-enzymatic bioactive compounds, being rich in both nutritional antioxidants like vitamins, and non-nutritional antioxidants as are carotenoids, and phenolic compounds, particularly flavonoids [47]. It should be added that the antioxidant properties of glucosinolates and related compounds has been also reported [103].

The antioxidant properties of broccoli extracts were studied in many of the publications by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [13,19,20,25,31,32,34,36,42,43,45–48,51,52,54,56,57,61,62,64–67,69,72,73,75,76,83,126,146,162–164]. DPPH is a dark crystalline powder composed of stable free-radical molecules, which is used to monitor chemical reactions involving radicals, and most notably it is a common antioxidant assay. Moreover, in some cases this was undertaken in combination with other antioxidant assays with the aim of obtaining a more detailed understanding of the antioxidant properties of the samples. Thus, DPPH was used together with several other assays as ferric reducing antioxidant power (FRAP) [42,43,46,56,57,61,83,126], ferrous ion chelating capacity [13,45,46], hydroxyl radical-scavenging activity [69,75,163], oxygen radical absorbance capacity (ORAC) [72], 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical method or Trolox equivalent antioxidant capacity (TEAC) [54,56,64]. Finally, it should also be mentioned that the majority of these research studies were performed *in vitro* [31,32,34,47,51,52,54,56,75,76].

A different way of studying this positive health effect is by using the FRAP assay. In this procedure, antioxidants are used as reducing agents in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. These assays have usually been carried out in combination with the DPPH method to determine the antioxidant properties of broccoli extracts [42,43,46,56,57,61,83,126]. In one interesting publication, there was a comparison of three different assays (FRAP, TEAC and total radical-trapping antioxidant parameter (TRAP)) to study the antioxidant characteristics of raw and frozen broccoli florets and stems [39]. Meanwhile, FRAP was exclusively used in one study, in which it was concluded that microwave cooking and boiling cause losses of antioxidants and phenolics in vegetables as broccoli [165].

ABTS assays were also employed to check the antioxidant properties of different broccoli extracts (heads, sprouts and seeds) [22,27,29,33,41,54,56,80,163]. In this procedure, ABTS is converted to its radical cation by the addition of sodium persulfate. This radical cation is reactive towards most antioxidants including phenolics, thiols and vitamins. This assay is often referred to as the TEAC assay.

The ORAC assay measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators, which produce a radical that damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are considered to protect the fluorescent molecule from oxidative degeneration. However, it must be added that in 2012 this was considered biologically invalid by the United States Department of Agriculture (USDA) [166], because no physiological proof *in vivo* existed in support of the free-radical theory. Therefore, this assay was no longer deemed relevant to human diets or biology by the USDA. However, different broccoli extracts have been tested using this method in the last years [63,68,70,72,167,168].

Finally, it should be said that several other assays have also been employed to analyze the antioxidant characteristics of different broccoli extracts, such as ferrous ion chelating capacity [13,45,46], hydroxyl radical-scavenging activity [69,75,163,169], TRAP [39,70] and different methods based on reactive oxygen species, for instance, superoxide anion, peroxyl, and alkoxyl radicals [42,46,63,71,75,163].

3.2. Anticancer

In the last few years, cancer prevention by natural products has received considerable attention. The potentially protective role of cruciferous vegetables, including broccoli, and active components present in these vegetables, such as GLSs and related products (ITCs, especially SF), has been extensively studied in experimental *in vitro* and *in vivo* carcinogenesis models [103]. Recent studies have demonstrated that in humans SF is rapidly absorbed following consumption of liquidized broccoli, but repeated intake of the vegetable does not lead to higher plasma levels, and subsequently to an accumulation in the organism [170]. Several review articles have been published [171,172] which explain and discuss some of the experimental, clinical and epidemiological evidence of anticancer activity among other potential health benefits of SF. This compound has shown itself to be useful as a chemopreventive agent in colon cancer with inactivated or lost p53 [173], as an inhibitor of pancreatic cancer cell growth *in vitro* and *in vivo* tumour suppressor in mouse models [174], and has also shown some beneficial effects in relation to prostate [175] and breast [176] cancer. For all these reasons, it is not surprising that most of the publications devoted to investigating the anticancer activity of broccoli and derived products were related to SF [145,146,149,170,177–188]. In one of these studies [177], it has been demonstrated that there is a synergy between SF and gemcitabine which may enhance the therapeutic index of prevention and/or treatment of cervical cancer [177]. In this *in vitro* study, a colorimetric assay was used for detection of apoptosis in treatment, as well as a reverse transcription polymerase chain reaction (RT-PCR) for RNA isolation and expression analysis. Meanwhile, in order to obtain a better understanding of the temporal effects of SF and broccoli sprouts on gene expression in prostate cells, a comprehensive transcriptome analysis was conducted using cDNA microarrays [181].

Phenolic compounds have also been investigated for their potential anticancer activity [58,80,168,189]. For example, an analysis was undertaken of the effect of the bio-accessibility of phenolic compounds on the *in vitro* anticancer activity of broccoli sprouts [80]. Meanwhile, the compounds present in new broccoli-enriched green tea drinks and their potential antitumor activity *in vitro* were evaluated in a different study [58]. After performing RPLC-PAD-ESI-MS analyses, it was found that the compounds were mainly phenolics and glucosinolates.

Finally, it should be mentioned that one study has been published suggesting that the intake of cruciferous vegetables is inversely associated with lung cancer risk [190], while two different studies argued that is not sufficient to eat broccoli frequently to prevent the occurrence of cancer [191], and that no protective role had been found in a high intake of fruits or vegetables regarding the risk of endometrial cancer in older women [192]. Consequently, after a meticulous revision of existing literature related to this issue, it can be concluded that the consumption of broccoli provides the organism with several phytochemicals which have been shown to possess anticancer activity. However, it cannot be postulated that a high intake of broccoli could increase the above-mentioned anti-carcinogenic effects.

3.3. Other effects

The antibacterial and antimicrobial activities of broccoli extracts have been the subject of study in several publications [19,42,46,151,193–196]. In some of this research [42,46], a comparative study has been conducted of the antibacterial activities of broccoli (florets and stalks), Brussels sprouts, white and York (only in [46]) cabbage extracts against several bacteria. It was observed that broccoli [42] and York cabbage [46] displayed the highest rate of antibacterial activity against most of the tested bacteria in comparison with the other vegetables tested.

Broccoli extracts have also been investigated for their potentially beneficial effects for patients with diabetes, cholesterol, cardiovascular diseases and asthma [139,197–203]. Broccoli sprout powder was used as a supplementary treatment in type 2 diabetic patients [197,199]. It was observed that using broccoli sprout powder as a supplementary treatment in type 2 diabetes could have favourable effects on lipid profiles as risk factors for cardiovascular disease. Although, on the other hand, it has been also postulated that green leafy vegetables such as broccoli may inhibit warfarin, which is nowadays used for many patients on therapy for various cardiovascular diseases, due to the high content of vitamin K [202]. It should be also commented that several of the above-mentioned publications were related to broccoli affecting cholesterol metabolism in rodents [139,200,201]. Moreover, broccoli sprout homogenates with high SF content have been successfully employed to induce phase II enzyme expression in the human airway [203].

Broccoli has shown beneficial effects not only for humans but similarly for insects and fishes [204–207]. For example, the effect of broccoli in the diet on the enzyme activities of tilapia fish during pollutant exposure has been studied in two of these publications [206,207]. Several procedures were applied in order to check different enzyme activities, which included the use of HPLC-DAD and UV–vis spectrophotometers. The results showed that diets containing broccoli induce beneficial changes in the enzymatic systems involved in the detoxification metabolism of fish.

Finally, it is appropriate to mention two research studies referring to particular biological activities observed in plants [208] and eggs [209]. The first article contained a description of the isolation of a broccoli defensive gene and its effect on downy mildew resistance [208]. Meanwhile in the latter study an analysis (HPLC-DAD and GC-FID) was made of the effects of broccoli stem and leaf meal on the production performance and egg quality of laying hens [209].

4. Conclusions

In this paper we have presented an overview of broccoli health promoting compounds over the period January 2008 to January 2013, discussing the different bioactive compounds (lipids, vitamins, proteins, glucosinolates, phenolic compounds, *etc.*), the analytical techniques mainly employed for their extraction, as well as their characterization and determination of biological activity. Scientific interest is demonstrated by the number of research

papers (>200) published on this topic during the period reviewed. It may be concluded that the main group of phytochemicals analyzed in broccoli have been glucosinolates and related compounds, although the study of phenolic compounds in broccoli have also attracted the attention of many researchers. Solvent extraction has been the treatment of choice to isolate broccoli bioactive compounds, as this has been predominantly used for all of them. Moreover, SPE has also been widely employed, especially in the analysis of glucosinolates and related compounds. In relation to characterization techniques, liquid chromatography has mainly been employed, probably due to its versatility, generalized availability and simplicity. Other techniques such as UV–vis, GC, CE, MS, ICP, NMR, UV–vis, or FTIR have also given good results, although their use is not as widespread as LC. The health promoting compounds in broccoli possess a large variety of biological activities, as has been demonstrated by the large number of publications related to this issue. The antioxidant properties of broccoli extracts determined with different assays (DPPH, FRAP, ORAC, ABTS, *etc.*) was a predominant feature in these research papers, although the study of the potential anticancer activities of several broccoli compounds using several methodologies (RT-PCR, arrays, *etc.*), and especially that of sulforaphane, is currently gaining attention. Moreover, some research has also been published focussing on several other biological activities, such as antimicrobial and antibacterial, or the potentially beneficial effects of broccoli for patients with diabetes, cholesterol, cardiovascular diseases and asthma. All the information summarized in this manuscript should facilitate the identification of health promoting compounds in broccoli, their extraction and chemical characterization, as well as making the study of the different biological activities related to these compounds easier.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.07.051.

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