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Evaluation of broccoli (*Brassica oleracea* var. italica) crop by-products as sources of bioactive compounds

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ABSTRACT

This study was performed to evaluate different by-products of broccoli (*Brassica oleracea* var. italica) production (leaf, inflorescence and stem) as sources of valuable bioactive compounds, considering different cultivars and states of maturation. The total phenolic and chlorophyll contents as well as the antioxidant, ACE-inhibitory and antimicrobial activities were quantified from the ethanolic extracts of the different broccoli tissues at two different maturation stages from five cultivars ('TSX 007', 'Monaco', 'BRO 2047', 'Parthenon' and 'Summer purple'). The major compounds in ethanolic extracts were identified by HPLC-UV-ESI-MS/MS, whereas chlorophylls were determined by UV–vis spectrometry. The leaf extracts showed the highest antioxidant activities and were the best sources of chlorophylls and phenolic compounds, constituting mainly kaempferol and quercetin glucosides. These compounds were more abundant in extracts from the 'Summer Purple' variety and commercial state. Glucosinolates were more abundant in the inflorescence extracts, principally in the flower bud state. The stem and inflorescence extracts, mainly from the 'TSX 007' variety, showed a strong inhibitory effect on the three bacteria studied (*Bacillus cereus, Staphylococcus aureus* and *Listeria innocua*), which was related to a higher concentration of fatty acid derivates. The findings suggest that broccoli by-products are useful and value-added products as sources of bioactive compounds, providing a sustainable alternative to reduce residues from this crop.

1. Introduction

Broccoli is an increasingly popular vegetable consumed under many different cultivar variations worldwide. In 2019, the combined world production of cauliflower and broccoli reached 26 million tons, 0.46 million tons more than in 2018 and 0.58 million tons more than in 2017 (FAOSTAT, 2021). Currently, the market value of the broccoli-related industry is valued at more than one billion dollars (PMG, 2021). The broccoli produced is not only intended for animal and human consumption; it is also exploited as biofuel, as a biofumigant and in medical applications, among many others (Björkman et al., 2011).

A diet rich in broccoli has numerous health benefits, as it provides essential nutrients (vitamins, minerals and fiber) and phytochemicals such as glucosinolates and phenolic compounds (Björkman et al., 2011; Fernandez-León et al., 2012; Nagraj et al., 2020). Many studies have linked its richness in bioactive substances to the properties associated with broccoli, such as anticancer, antioxidant, antimicrobial (Ares et al., 2013; Jang et al., 2015; Owis, 2015), anti-inflammatory and antihypertensive (Dang et al., 2019; Jeffery and Araya, 2009) activities. Its beneficial composition also allows it to be used to treat other health-related issues, such as hypercholesterolaemia, cardiovascular diseases, diabetes or photosensitivity disorders (Ares et al., 2013; Bahadoran et al., 2012; Porter, 2012).

The content and diversity of these compounds in plants, including broccoli, are influenced by several factors: climatic (light, temperature etc.); biotic (cultivar, exposure to pests or diseases, or weeds) and agronomic (genotype, irrigation, soil type, growing season, fertilizers or pesticides) (Björkman et al., 2011; Pék et al., 2013; Mahn, 2017; Turan 2019 and 2021). Pérez-Balibrea et al. (2011) observed a decrease in the content of phenolic compounds as the plant grew, and Di Gioia et al.

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(2018) studied the influence of irrigation with saline water on the content of glucosinolates in the vegetable. Moreover, compounds may vary not only along the crop cycle but also depending on harvest practices, postharvest storage conditions or food preparation methods (Ferreira et al., 2020; Lafarga et al., 2018; Lv et al., 2017; Martínez et al., 2020;).

In general, the vegetable can be harvested once the inflorescence has reached its maximum size without opening (Gómez-Campo, 1999). A late harvest means an overripe product, which is therefore unfit for sale and subsequent consumption. This implies an increase in by-products, which, together with the rest of the discarded parts, represents a large amount of unused plant material. Of all the biomass generated by broccoli crops, only 30% is used in food (broccoli head); the rest of the parts, including leaves, stems and inflorescences, are considered as by-products that are mostly used for composting or incorporated into the soil (Zhang et al., 2017). This discarding means not only the loss of product by farmers but also an increase in waste, generating environmental problems. Considering the health benefits provided by broccoli beyond essential nutrition, due to its extraordinary richness in phytochemicals, the by-products generated from its cultivation can also be rich in bioactive compounds and present beneficial properties (Ferreira et al., 2018; Shi et al., 2019; Hügel et al., 2018). The utilization of these by-products gives them added value, improving crop profitability and increasing farmers' profits. In addition, it would facilitate the production of high-value functional ingredients.

The main objective of this study was to characterize and quantify the bioactive compounds present in the different parts of broccoli (leaf, inflorescence and stem) and to study the dependence of their variation on the maturation stage (flower buds and commercial) and the cultivar. All this was focused on the possible subsequent use of by-products as new sources of bioactive compounds.

2. Materials and methods

2.1. Plant material

Plant material was obtained from a broccoli variety field experiment from which five cultivars with different plant characteristics were select for the study: 'TSX 007′, 'Monaco', 'BRO 2047′, 'Parthenon' and 'Summer Purple'. The experimental plot is located in the Vegas Bajas del Guadiana (Extremadura) at the La Orden farm of the Center for Scientific and Technological Research of Extremadura (CICYTEX). The soil has a clay-loam texture, with a slightly acid pH and low organic matter content (0.4%). The plants were transplanted on 29 August of 2020, with drip irrigation and appropriate cultivation practices for optimum plant development. The experimental design is a randomized block with three replications (elementary plots).

Each cultivar was harvested at two different growth stages, including flower buds and mature broccoli. At each phenological stage, one representative plant of each cultivar was selected per elementary plot. Broccoli was then divided into three parts, namely: leaves, stems and inflorescences. All samples were cut and dried in a forced-air oven at 45 °C for 48 h.

2.2. Bioactive compound extraction

For the extraction of bioactive compounds (phenolic compounds and glucosinolates) from plant material, the method described by Oniszczuk and Olech (2016) was applied, with some modifications (Casquete et al., 2015). Vegetal material (10 g) was extracted in 60 mL ethanol (80%) using an ultrasound bath for 1 h (45–50 °C). The residues were separated, and the process was repeated twice. Excess ethanol was removed by heating at 37 °C in a rotary evaporator under vacuum. The resultant aqueous extracts were combined and lyophilized (Telstar, LyoBeta).

2.3. Total phenolic content (TPC)

The determination of total phenolic content was performed according to the colorimetric method of Folin-Ciocalteu from 0.01 g of lyophilized broccoli powder (1 mL ethanol, 100%). Total phenolic contents were determined spectrophotometrically at 760 nm in triplicate, and the results were expressed as mg of gallic acid equivalents (mg GAE)/100 g of the extract's dry weight.

2.4. Antioxidant activity by free-radical-scavenging ability by the use of a stable DPPH radical and ABTS radical cation

To analyze the antioxidant capacity of broccoli extracts, the 2.2-diphenyl-picryl-hydrazyl (DPPH) and 2.2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays were used by the methods described by Teixeira et al. (2009) and Cano et al. (1998), respectively. Previously, 0.01 g of broccoli sample was dissolved in 1 mL of ethanol (100%) for use in the analysis. All samples were analysed in triplicate. Results were expressed as mg Trolox/100 g dried weight.

2.5. Assessment of ACE-inhibitory activity

ACE-inhibitory activity was measured by fluorescence using the method of Sentrandreu and Toldrá (2006a, 2006b). The extracts were dissolved in 40% methanol (v/v water) at a concentration of 400–100 μg/mL, then 1:2, 1:4 and 1:8 dilutions were made in Milli-Q water. The fluorescence generated was measured every 15 min for an hour using a multicam microplate fluorimeter (FLUOstar optima, BMG Labtech, Offenburg, Germany).

The activity of each sample was tested in triplicate. Inhibitory activity was expressed as the extract concentration required to inhibit the original ACE activity by 50% (IECa50).

2.7. Antimicrobial activity

Antimicrobial susceptibility was tested against foodborne pathogenic bacteria (*Staphylococcus aureus* CECT 976, *Bacillus cereus* CECT 131, and *Listeria innocua* CECT 910). For that, the extracts were diluted in dimethyl sulfoxide (DMSO) (10 mg extract/mL DMSO) and additionally diluted to achieve final concentrations of 80, 60 and 40 ppm. Target cell suspensions were prepared from cultures incubated overnight at 37 °C on brain-heart infusion agar (BHI). After the incubation period, colonies were transferred to a sterile peptone water solution to obtain a turbidity equivalent to 0.5 McFarland standards. The wells of a sterile multiwell plate were then inoculated with 2% (v/v) of each bacterial suspension to which the various concentrations of extract were added. DMSO was used instead of active compounds as a negative control. All experiments were performed in triplicate, and the plates were incubated for 24 h at 37 °C. Microbial growth was detected by optical density (FLUOstaroptima, BMGLabtech, Offenburg, Germany). The results were expressed as% inhibition. This process was repeated twice after the incubation period.

2.8. Identification of bioactive compounds of the extracts by HPLC-UV-ESI-MS/MS

A sample of freeze-dried broccoli (0.08 g) was placed in 2 mL of methanol (HPLC). The prepared samples were analysed by mass-coupled HPLC (Agilent HPLC-QTOF Model G6530, Agilent Technologies, Palo Alto, CA, USA). Detection and identification of bioactive compounds were performed using a quadrupole time-of-flight tandem mass analyser (Q-TOF) with electrospray ionization (ESI). The instrument was operated in negative-ion mode, and the full scan covered the mass range from m/z 100 to 1700. The gas flow was 11 mL/min at 280 °C (nebulizer 35 psi). Gradient elution was carried out with a mixture of 5% hydrocyanic acid in water (solvent A) and 95% hydrocyanic acid in water and 0.1%

formic acid (solvent B), with a flow rate of 0.350 mL/min. The solvent gradient started with 5% solvent B, reaching 90% at 15 and 20 min, and returning to the initial conditions for the last 10 min. Tentative identification of bioactive compounds was elucidated based on the MassBank database and retention time and by comparing the data to published reports (Table 1) (Cartea et al., 2011; Vallejo et al., 2004).

Table 1
Identification of bioactive compounds from dehydrated broccoli byproducts.

Peak	Rt (min)	[M-H]$^-$	MS/MS (*m/z*)	Compound identified
Phenolic components				
1	7.85	**315**	108; 109; 152; 153	Benzoic acid + 2O, O—Hex[a]
2	12.09	**625**	787	Qn-3-diglucoside-7-glucoside[b]
3	12.12	**447**	609	Km-3,7-di-O-glucoside[a]
4	12.24	**1111**	949	Qn-3-caffeoyltriglucoside-7-glucoside[a]
5	12.24	**191**	135; 179; 353	Caffeoyl-quinic acid[c]
6	12.26	**323**		Glabranine/isobavachin[a]
7	12.32	**191**	135; 179; 353; 523	Caffeoyl-quinic acid derivate[d]
8	12.37	**1155**	993	Qn-3-sinapoyltriglucoside-7-glucoside[a]
9	12.37	**1095**	787; 933	Km-3-caffeoyltriglucoside-7-glucoside[d]
10	12.51	**1139**	977	Km-3-sinapoylsophorotrioside-7-glucoside[d]
11	12.52	**993**	787; 831	Qn-3-sinapoylsophorotrioside[a]
12	12.57	**947**	1109	Km-3-feruloylsophorotrioside[b]
13	12.85	**191**	163; 337	Coumaroyl-quinic acid[c]
14	13.12	**609**	285; 447	Km-3-O-diglucoside[c]
15	13.08	**463**	301; 625	Qn-3,4′-O-di-beta-glucoside[a]
16	13.93	**205**	223; 529; 753	1,2-Disinapoyl-gentiobioside[c]
17	14.36	**959**	511; 735	Trisinapoyl-gentionbiose[a]
18	14.46	**929**	705	Feruloyl-disinapoyl-gentionbiose[a]
19	12.55	**771**	933	Km-3-diglucoside-7-diglucoside[d]
20	12.78	**785**	293; 947	Km-3-feruloyldiglucoside-7-glucoside[d]
21	24.48	**577**	578; 579	Km-3,7-O-di-rhamnopyranoside[a]
22	12.25	**609**	771	Km-3-diglucoside-7-glucoside[d]
23	13.41	**609**	173; 284; 285	Km-7-diglucoside[b]
24	13.81	**785**	284; 609	Km-3-O-feruloyldiglucoside-7-O-diglucoside[c]
Glucosinolates and derivatives				
25	3.15	**436**	178; 372	Glucoraphanin isomer 1[e]
26	4.08	**436**	178; 372	Glucoraphanin isomer 2[e]
27	11.90	**463**	169; 267	4-Hydroxiglucobrassicin[e]
28	13.31	**447**	139; 254; 448; 449	Indolymethyl glucosinolate (Glucobrassicin)[a]
29	13.91	**477**	478; 479	Methoxyglucobrassicin 1[a]
30	14.68	**477**	478; 479	Methoxyglucobrassicin 2[a]
Other compounds				
31	2.80	**195**	129; 177	Gluconic acid[a]
32	3.95	**128**	200; 290; 346; 737	Fructosyl-pyroglutamate derivate[a]
33	15.67	**327**	171; 183; 211; 229	FA 18:2 + 3O[a]
34	16.02	**329**	183; 211; 229	FA 18:1 + 3O[a]
35	20.13	**295**	277	9-HODE/13-HODE[a]
36	20.30	**277**	295	9-HODE/13-HODE[a]
37	23.05	**255**		Palmitic acid[a]
38	23.58	**277**	278	Linolenic acid isomer[a]
39	17.08	**121**	185; 211; 235; 289	FA 18:4 + 2O[a]
40	13.28	**655**	259; 461; 624	Unknown 1
41	14.03	**462**	169; 494; 945	Unknown 2

[a] MassBank.
[b] Llorach et al. (2003).
[c] Lin and Harnly (2009).
[d] Ferreira et al. (2020).
[e] Yang and Zhang (2012).

2.9. Chlorophyll content

Chlorophyll extraction was performed according to the spectrophotometric method of García et al. (2005). Dried broccoli samples (4 g) were homogenized with 15 mL of acetone and were centrifuged at 7800 rpm for 15 min at 4 °C. This process was repeated twice. The supernatants were decanted onto glass wool and diluted to 50 mL with acetone. The extracted samples were then analysed using a spectrophotometer (Shimadzu UV Spectrophotometer UV-1800) operating at wavelengths between 350 and 900 nm. Since the ordinary spectral bands of chlorophyll (Chl) a and b strongly overlap in mixtures (Ergun et al., 2004), ^{2}D-spectra were used to determine their concentrations. Chl a and Chl b were determined at 676 nm and 632 nm with a high sensitivity. The results were expressed as mg of chlorophyll/100 g dry weight.

2.10. Statistical analysis

The statistical study was carried out using SPSS Statistics, version 21.0 (IBM Corp., Armonk, Ny, USA). Descriptive statistics of the data were determined, and the differences within and between groups were studied by three-way analysis of variance (ANOVA) and separated by Tukey's honestly significant difference test ($p \leq 0.05$). Principal component analysis (PCA) was performed on the correlation matrix of the variables.

3. Results and discussion

3.1. Extraction yield rates and total phenolic content

The extraction yield rates of bioactive compounds in 80% ethanol from broccoli by-products are shown in Table 2. No significant differences in extraction yields were obtained between varieties, plant parts or ripening stages. Among varieties, the mean extraction yield ranged from 13.4% to 16.3% dry mater. Jaiswal et al. (2011) studied the extraction yield rates of phenolic compounds obtained from different solvent systems (methanol, ethanol and acetone) for different Irish *Brassica* vegetables, observing that methanol was the most efficient solvent for the extraction of polyphenolic compounds from broccoli. However, among the variety of solvents, ethanol is most preferable because it is inexpensive, reusable and nontoxic, and the extracts can be used in the food industry, while methanol generates toxic waste (Oroian and Escriche, 2015).

Unlike the yield, the total phenolic content of the ethanolic extracts obtained from broccoli samples showed significant differences ($p \leq 0.05$) in all the factors studied (Table 2). 'Summer Purple' and 'Parthenon' showed the highest mean values among all varieties studied (1891 and 1518 mg GAE/100 g extract, respectively). This variability associated with the variety factor agrees with the results provided by other studies (Pérez-Balibrea et al. (2011); Borowski et al., 2008; Kaur et al., 2007). Bhandari and Kwak (2015) determined the total phenolic contents of six broccoli and cauliflower cultivars, observing the highest values for purple cauliflower with respect to green varieties, which may be of interest for the selection of cultivars with high contents of these compounds. However, a direct comparison between the values obtained and those reported by these studies is difficult, mainly due to the different extraction methods and solvents applied and the units used for expressing the results.

With respect to the plant part, the highest mean concentration of total phenols was observed in broccoli leaves (2435 mg GAE/100 g of extract), reaching more than twice the values for inflorescences (1074 mg GAE/100 g of extract) and stems (939 mg GAE/100 g of extract). These results are in concordance with those reported by other studies: total phenols in the different parts of broccoli decreased in the order

Table 2
Extraction yield (%), total phenolic compounds (TPC) (mg GAE/ 100 g dry extract), antioxidant activity (mg Trolox/100 g dry extract) by two methods (DPPH and ABTS) and antihypertensive activity (IECa50) of extracts in 80% ethanol from different broccoli varieties, by-products and ripening stages.

Factors	TPC			Extraction yield			DPPH			ABTS			IECa50		
Levels	Mean		SD[1]	Mean		SD	Mean		SD	Mean		SD	Mean		SD
Cultivars (V)															
TSX 007	1392^{bc}	±	555	13.9	±	3.4	385^{bc}	±	155	1062	±	342	410^{b}	±	48
MONACO	1335^{c}	±	753	16.1	±	3.0	402^{ab}	±	202	1084	±	412	239^{c}	±	90
BRO 2047	1279^{c}	±	659	15.9	±	1.4	360^{c}	±	102	843	±	396	430^{b}	±	72
PARTHENON	1518^{b}	±	940	16.3	±	4.0	437^{a}	±	129	1631	±	584	143^{d}	±	32
SUMMER PURPLE	1891^{a}	±	875	13.4	±	2.6	436^{a}	±	201	1218	±	203	1141^{a}	±	1249
Parts (P)															
Stems	939^{c}	±	296	17.2	±	3.3	299^{c}	±	81	882	±	399		–	
Leaves	2435^{a}	±	537	13.5	±	2.7	567^{a}	±	146	1636	±	364	538	±	706
Inflorescences	1074^{b}	±	301	14.7	±	1.9	346^{b}	±	99	985	±	240		–	
Stage of maturity (M)															
Flower buds	1389	±	763	15.8	±	3.4	360	±	135	1206	±	488	764	±	929
Commercial	1577	±	799	14.5	±	2.6	448	±	175	1129	±	467	287	±	122
P-values															
P^{V}		< 0.001			0.788			< 0.001			0.415			< 0.001	
P^{P}		< 0.001			0.430			< 0.001			0.380			–	
P^{M}		< 0.001			0.547			< 0.001			0.317			< 0.001	
P^{M*V}		< 0.001			–			< 0.001			0.413			< 0.001	
P^{M*P}		< 0.001			–			< 0.001			0.380			–	
P^{V*P}		< 0.001			–			< 0.001			0.447			–	

[1] SD: standard deviation; [abc] Values with different superscripts are significantly different between each of the factors (Tukey's test; $p \leq 0.05$).

leaves, florets and stems (Faller and Fialho 2010; Kim et al., 2014; Liu et al., 2018; Thomas et al., 2018). However, the differences in total phenols between the parts of broccoli depend on the variety studied ($P_{V*P} \leq 0.05$), being greater for the varieties `Parthenon' and 'Monaco' (Fig. 1).

Concerning the ripening stage, in general, the total phenolic content was higher in the 'Commercial' than in the 'Flower Bud' stage, indicating an increase in phenols as plant maturity progressed (Table 2). Bhandari et al. (2019), analysed the total phenolic content in broccoli at three different stages of maturity, obtaining a higher content in the marketable stages than in the immature stage in all genotypes studied. Likewise, the intensity of the change in total phenols during maturation depended on the variety ($P_{V*M} \leq 0.05$) and the plant part ($P_{P*M} \leq 0.05$). Concretely, the increase in total phenols during broccoli maturation was more intense for 'TSX 007′ varieties and leaves (Fig. 1).

These results showed that ethanolic extracts differ in total phenolic content dependent on the variety, part and stage of development of the plant and the interaction between these factors. This variability in TPC is responsible for the variation in activities of the studied ethanolic extracts.

3.2. Antioxidant activity

Among the health benefits of broccoli, antioxidant capacity is one of the most important due to the presence of numerous antioxidant compounds. In this study, antioxidant activity was determined by the DPPH and ABTS methods. The data obtained for DPPH and ABTS showed differences depending on the variety, with values ranging from 436 to 360 and from 1631 to 843 mg Trolox/100 g of extract, respectively. The 'Summer Purple' and 'Parthenon' varieties showed the highest antioxidant capacity, in concordance with the values for TPC found for these varieties. This dependence of antioxidant activity on variety was also observed in other studies (Bhandari and Kwak, 2015; Borowski et al., 2008). As in other studies performed in broccoli, the mean values obtained were higher using the ABTS radical than with DPPH, given that the highly pigmented and hydrophilic antioxidants are better reflected by the ABTS than by the DPPH assay (Arnáiz et al., 2016; Floegel et al., 2011; Sun et al., 2007).

Similarly, antioxidant activity showed differences depending on the plant part, with activity decreasing in the following order: leaves, inflorescences and stems (Table 2). In other works, higher antioxidant activity in the leaves of broccoli compared with the stems had been reported using the DPPH method (Dominguez-Perles et al., 2011; Hwang and Lim 2015). In our study, the varieties 'Parthernon' and 'Summer Purple' showed the greatest differences in DPPH values between the studied plant parts ($P_{V*P} \leq 0.05$; Fig. 2). For the ripening stage, the flower bud samples presented lower antioxidant activity than the commercial samples using the DPPH method (360 and 448 mg Trolox/100 g extract, respectively). Other studies also observed this increase in antioxidant activity in broccoli and other Brassica family vegetables during maturation (Bhandari et al., 2019; Soengas et al., 2012).

3.3. ACE-inhibitory activity

The human renin-angiotensin system (RAS), a regulator of blood pressure, is controlled by the protease activities of the angiotensin-converting enzyme (ACE). Thus, *in vitro* enzyme inhibition is used to measure potential antihypertensive effects. The IECa50 inhibition values obtained for the different broccoli samples are shown in Table 2. The values for the different varieties studied ranged from 143 to 1141 ppm, with the 'Parthenon' variety showing the highest antihypertensive capacity. Concerning the plant part, antihypertensive activity was only found in the extracts obtained from broccoli leaves (Table 2). On the other hand, the antihypertensive capacity increased with increasing maturation time of broccoli, since the amount of extract needed to inhibit ACE at 50% was lower in the 'Commercial' than in the 'Flower Bud' samples, according to the increase of TPC values previously found. Other studies have also related the decrease in cardiac pressure with an increase in polyphenols in broccoli and other vegetables, mainly in leaves (Alashi et al., 2018; Hügel et al., 2016).

3.4. Antimicrobial activity

The antibacterial activity of extracts obtained from cruciferous vegetables has been widely described (Hu et al., 2004). The inhibitory effect of the extracts studied on the three bacteria tested showed significant differences associated with the three factors evaluated (variety, part of the plant and maturation) and its concentration ($p \leq 0.05$; Table 3). Concerning the effect of the variety, extracts of 'TSX 007′ and 'Monaco' presented the highest inhibitory effect on the three bacteria studied. The inhibition percentages ranged from 56.3 to 86.7% against

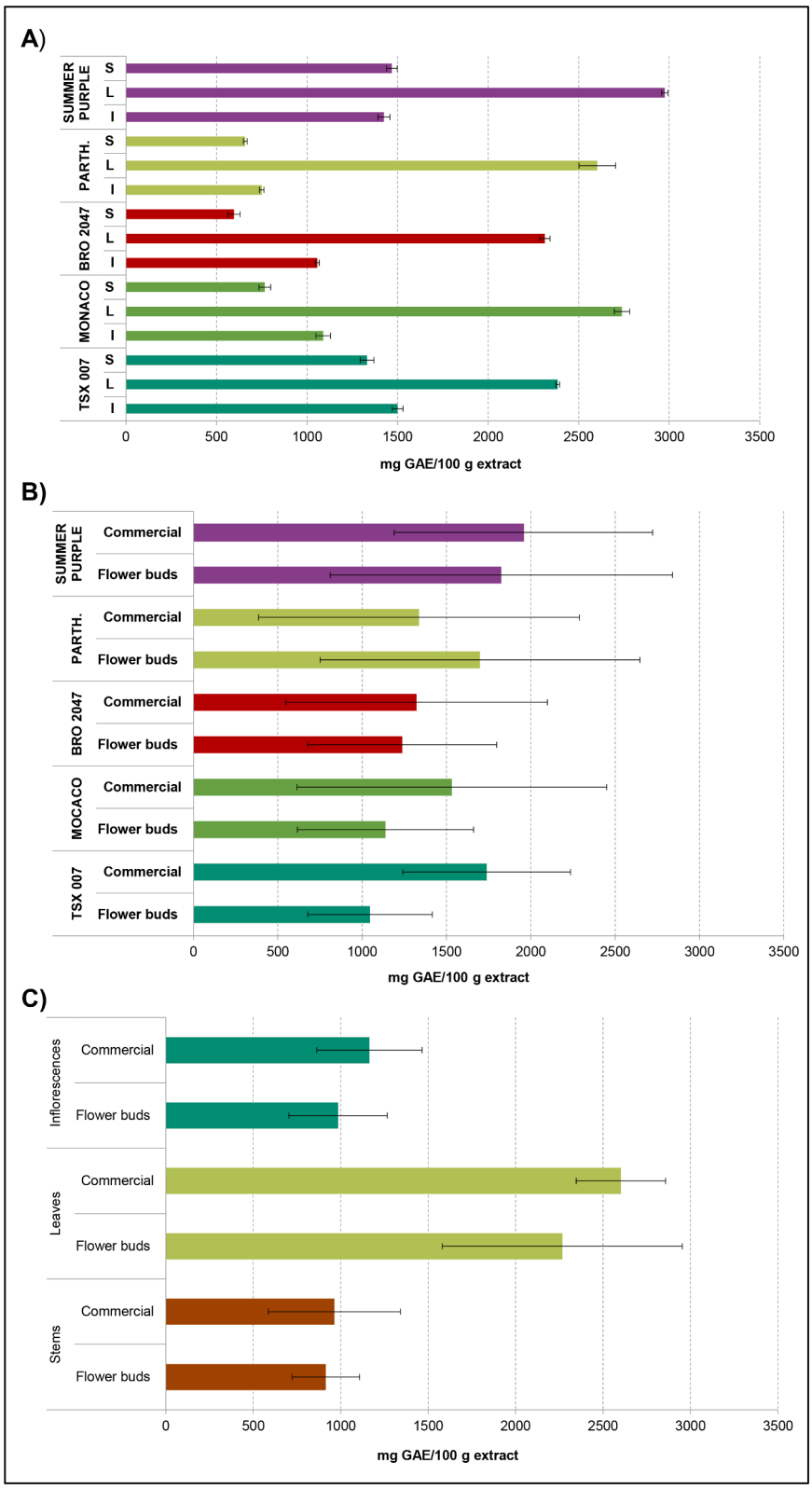

Fig. 1. Interaction effect on the total phenolic compounds; (A) cultivars * part of the plant; (B) cultivars * stage of maturity; (C) part of the plant * stage of maturity. S: stems; L: leaves; I: inflorescences.

B. cereus, 82.0 to 94.2% against *S. aureus*, and 69.3 to 93.1% against L. *innocua*. All broccoli varieties showed a higher inhibitory effect against *S. aureus*, followed by L. *innocua* and, to a lesser extent, *B. cereus*. Vale et al. (2015) also found higher antibacterial activity against *S. aureus* than against *B. cereus* in broccoli sprouts.

The antibacterial activity was also influenced by the part of the plant from which the bioactive compounds were extracted (Table 3). Stem ethanolic extracts showed a higher inhibitory effect against *B. cereus* and *L. innocua*, while *S. aureus* was more affected by inflorescence extracts. These results contrast with those obtained by Pacheco-Cano et al. (2018)

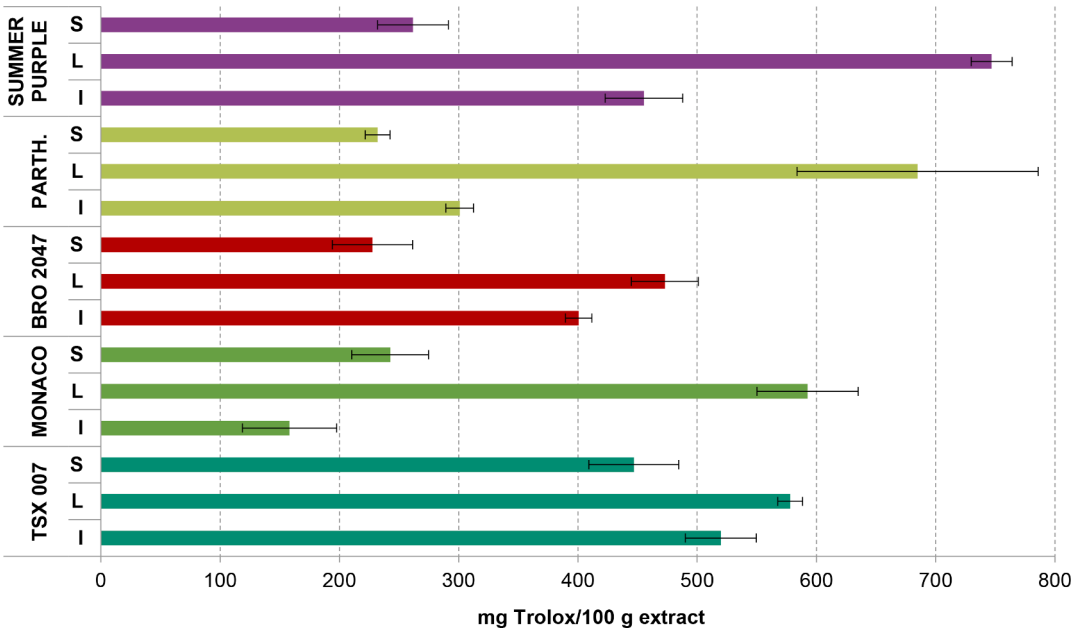

Fig. 2. Interaction effect cultivars * part of the plant on the antioxidant activity (DPPH). S: stems; L: leaves; I: inflorescences.

Table 3
Effect of variety, plant part, concentration and ripening stage on the antibacterial activity (% inhibition) of broccoli extracts.

Factors	***B. cereus***			***S. aureus***			***L. innocua***		
levels	Mean		SD[1]	Mean		SD	Mean		SD
Cultivars (V)									
TSX 007	86.7^{a}	±	22.8	94.2^{a}	±	14.9	93.1^{a}	±	20.7
MONACO	81.8^{b}	±	33.5	87.6^{b}	±	27.3	85.8^{b}	±	31.9
BRO 2047	56.3^{d}	±	32.6	85.4^{c}	±	25.6	69.3^{d}	±	33.1
PARTHENON	63.8^{c}	±	33.7	82.0^{d}	±	27.2	69.2^{cd}	±	36.8
SUMMER PURPLE	64.8^{c}	±	31.2	81.9^{d}	±	29.7	72.2^{c}	±	34.4
Parts (P)									
Stems	81.3^{a}	±	27.1	89.3^{b}	±	22.5	81.9^{a}	±	28.7
Leaves	57.5^{c}	±	33.5	77.8^{c}	±	30.7	69.5^{b}	±	37.9
Inflorescences	73.3^{b}	±	33.5	91.6^{a}	±	20.6	82.7^{a}	±	30.4
Stage of maturity (M)									
Flower buds	66.5	±	33.1	89.0	±	21.6	75.6	±	32.7
Commercial	74.8	±	32.1	83.4	±	28.9	80.4	±	33.2
Concentration (ppm) (C)									
80	84.1^{a}	±	22.0	97.2^{a}	±	9.8	95.2^{a}	±	13.4
60	71.9^{b}	±	31.6	90.1^{b}	±	18.8	82.0^{b}	±	24.2
40	56.0^{c}	±	37.1	71.4^{c}	±	34.3	56.9^{c}	±	41.9
P-values									
P^{V}		< 0.001			< 0.001			< 0.001	
P^{P}		< 0.001			< 0.001			< 0.001	
P^{C}		< 0.001			< 0.001			< 0.001	
P^{M}		< 0.001			< 0.001			< 0.001	
P^{M*C}		< 0.001			< 0.001			< 0.001	
P^{M*V}		< 0.001			< 0.001			< 0.001	
P^{M*P}		< 0.001			< 0.001			< 0.001	
P^{C*V}		< 0.001			< 0.001			< 0.001	
P^{C*P}		< 0.001			< 0.001			< 0.001	
P^{V*P}		< 0.001			< 0.001			< 0.001	

[1] SD: standard deviation; [abc] Values with different superscripts are significantly different between each of the factors (Tukey's test; $p \leq 0.05$).

for crude extracts of broccoli. They found a greater inhibitory effect in florets than in stems, against both gram-negative and gram-positive bacteria; moreover, extracts were more effective against *B. cereus* than against *S. aureus*. However, these authors also indicated that this antibacterial activity was, in part, proteinaceous in nature. Therefore, different antibacterial compounds are unevenly distributed throughout the plant, of interest in the identification of bioactive compounds present in ethanolic extracts.

On the other hand, broccoli samples showed a greater inhibitory effect against *B. cereus* and *L. innocua* as the ripening stage increased, reaching 74.8% and 80.4%, respectively. In the case of *S. aureus*, the less mature samples showed greater antibacterial activity (89.0%). In general, mean inhibition percentages of 84.1%, 97.2% and 95.2% were observed against *B. cereus, S. aureus* and *L. innocua*, respectively, with a concentration of 80 ppm in TPC in the applied extracts (Table 3). As expected, the percentages decreased with decreasing extract concentration. Jaiswal et al. (2011) also observed this relationship between concentration and inhibition of bacteria (L. *monocytogenes* and *S. abony*),

with the percent inhibition decreasing from 69% and 57% to 45% and 39%, respectively, as the extract concentration was serially diluted (2.8% to 1.4%).

3.5. Bioactive compounds of broccoli extracts

Among the phenolic compounds identified in the ethanolic extracts studied, hydroxycinnamic acid esters of kaempferol (Km) and quercetin (Qn) glucosides are highlighted (Table 4). Vallejo et al. (2004) have reported that complex acylated tri- or tetra-glycosides of Km and Qn are the major flavonoids in broccoli inflorescence samples following alkaline hydrolysis. Fernández-León et al. (2012) determined the bioactive compounds in fresh broccoli heads of the varieties 'Monaco' and 'Parthenon', obtaining a higher concentration of quercetin derivatives than of kaempferol after hydrolysis to aglycons of individual phenolic compounds. In the broccoli material used in this study, 11 Km glycosides and only 5 Qn glycosides, mostly in their acylated forms, were tentatively identified as major phenolic compounds in the different cultivars, in concordance with the results obtained by Wu et al. (2019). However, the factor 'plant part' showed the greatest influence on flavonoid concentrations, producing the highest values in leaf extracts (Table 4), which were corroborated by their antioxidant activity.

With respect to the glucosinolates, these were the most abundant bioactive compounds found in ethanolic extracts of broccoli. Glucobrassicin, glucoraphanin and several derivatives were identified in all samples studied, with higher concentrations of glucobrassicin and methoxyglucobrassicin. The concentrations of these compounds varied significantly between plant parts, most of them being more abundant in the inflorescences (Table 5). Other studies also indicate a higher concentration of these compounds in flowers and inflorescences than in stems and leaves (Li et al., 2021; Yang and Zhang, 2012). On the other hand, glucosinolates tend to decrease in concentration with increasing plant maturity. Bhandari et al. (2019) determined glucosinolates at three different stages of inflorescence development, obtaining similar results to this study for glucobrassicin, an inverse relationship between accumulation and maturity. However, the glucoraphanin concentration did increase with plant development. This variation with the present study may be due to difference in varieties, growth conditions or methods of extraction of the compounds. Glucosinolates, mainly glucobrassicin, glucoerucinin and glucoraphanin, exhibit a high antioxidant capacity, albeit lower than that associated with phenolic flavonoid compounds (Bhandari and Kwak, 2015). This relationship can be observed in the data obtained as a function of the broccoli variety but not as a function of the plant part. Despite the higher concentration of glucosinolates in the inflorescence, the leaves showed higher antioxidant activity, possibly due to the higher concentration of total phenols in this part.

In addition to the aforementioned compounds, others were also identified in the ethanolic extracts, such as palmitic and linoleic fatty acids, also found in broccoli extracts (Arnáiz et al., 2011), and more

Table 4
Identification and quantification of phenolic compounds from dehydrated broccoli byproducts analysed by HPLC-UV-ESI-MS/MS.

Factors	*Cultivars (V)*					*Parts (P)*			*Stage of maturity (M)*		*P-values*		
Levels	**TSX 007**	**MONACO**	**BRO 2047**	**PARTH.**	**S. PURPLE**	**Stems**	**Leaves**	**Inflorecences**	**Flower buds**	**Commercial**	**P^V**	**P^P**	**P^M**
Phenolic Components													
1	85.68	76.00	94.12	59.96	57.58	37.62^b	130.69^a	55.69^b	82.80	66.54	0.743	< 0.001	0.415
2	13.24	14.84	15.93	9.31	39.33	2.22^b	43.60^a	9.77^b	27.51	95.49	0.593	0.010	0.150
3	45.57	37.70	55.16	46.36	40.70	4.80^b	114.30^a	16.20^b	49.25	40.95	0.989	< 0.001	0.692
4	2.02	2.05	2.92	0.10	1.14	0.80^b	3.32^a	0.83^b	2.28	1.01	0.236	0.009	0.119
5	0.00	2.22	0.53	0.04	0.48	1.96	0.00	0.00	0.08	1.23	0.547	0.122	0.208
6	119.56	92.31	78.00	95.29	109.86	25.04^c	166.03^a	105.94^b	107.83	90.18	0.866	< 0.001	0.503
7	61.05	51.82	66.23	26.24	51.83	26.78^b	41.53a^b	85.99^a	70.73	32.14	0.755	0.027	0.043
8	4.36	5.02	8.32	1.73	10.97	4.21	6.33	7.71	7.71	4.46	0.079	0.466	0.157
9	21.09	9.67	20.72	8.15	6.87	1.91^b	36.22^a	1.77^b	17.60	9.00	0.722	< 0.001	0.315
10	17.95	10.38	31.52	23.39	19.29	5.73^b	49.77^a	6.02^b	27.93	13.08	0.806	< 0.001	0.160
11	9.46	8.64	13.19	4.12	44.27	7.37	35.59	4.85	23.30	8.57	0.380	0.133	0.297
12	13.83	9.87	10.47	3.46	16.47	1.77^b	23.60^a	7.08^b	9.25	12.38	0.744	0.005	0.614
13	41.99	38.91	36.68	15.09	101.17	14.65	57.10	68.57	62.34	31.20	0.082	0.072	0.130
14	4.78	32.33	37.45	36.56	52.13	2.30^b	67.55^a	28.09^b	32.49	32.81	0.261	< 0.001	0.981
15	2.28	5.28	6.79	3.51	10.46	0.79^b	13.20^a	3.01^b	5.90	5.43	0.617	0.003	0.894
16	24.77	15.27	32.84	24.20	16.09	9.91^b	16.12^b	41.87^a	29.59	15.67	0.614	< 0.001	0.068
17	18.47	4.75	13.32	3.48	9.44	5.10^b	5.26a^b	19.32^a	8.19	11.60	0.342	0.029	0.518
18	5.65	2.98	13.04	7.68	7.30	0.91^b	7.89a^b	13.20^a	5.43	9.23	0.584	0.024	0.327
19	232.05	187.98	293.65	288.42	382.14	14.84^b	791.62^a	24.08^b	326.49	227.20	0.950	< 0.001	0.511
20	191.49	174.11	217.14	206.10	259.22	14.77^b	609.55^a	4.52^b	236.86	182.37	0.993	< 0.001	0.624
21	378.32^a	45.75^b	18.22^b	42.18^b	58.49^b	92.48	108.91	124.38	67.72	149.46	< 0.001	0.936	0.243
22	1.80	1.25	0.41	0.42	1.24	0.00^b	2.88^a	0.19^b	0.83	1.22	0.731	< 0.001	0.600
23	72.13	93.47	45.67	78.31	170.31	8.42^b	226.25^a	41.27^b	104.05	79.90	0.716	< 0.001	0.674
24	94.26	132.26	65.84	86.43	185.44	8.34^b	328.02^a	2.18^b	135.11	90.58	0.861	< 0.001	0.539

[abc] *Values with different superscripts are significantly different between each of the factors (Tukey's test; $p \leq 0.05$). The numerical code corresponds to the compounds listed in Table 1.*

Table 5
Identification and quantification of glucosinolates and other compounds from broccoli by-products analysed by HPLC-UV-ESI-MS/MS.

Factors	*Cultivars (V)*					*Parts (P)*			*Stage of maturity (M)*		*P-values*		
levels	TSX 007	MONACO	BRO 2047	PARTH.	S. PURPLE	Stems	Leaves	Inflorecences	Flower buds	Commercial	P^V	P^P	P^M
Glucosinolates and derivatives													
25	191.42	301.63	479.68	335.71	155.76	135.94^b	59.52^b	683.06^a	413.59	172.09	0.712	< 0.001	0.113
26	148.37	256.53	415.30	286.46	168.41	93.70^b	51.86^b	619.49^a	333.51	176.52	0.758	< 0.001	0.249
27	2.93	7.23	35.15	7.32	73.13	2.04	14.39	59.02	26.14	24.16	0.141	0.051	0.925
28	507.07	989.02	878.15	1290.74	2329.14	139.27^b	307.29^b	3149.91^a	2020.06	377.59	0.754	0.003	0.056
29	232.77	247.43	253.56	259.60	337.89	130.94^b	50.72^b	617.09^a	364.99	167.51	0.991	< 0.001	0.137
30	446.93	1007.53	1337.13	1533.62	699.86	202.39^b	199.97^b	2612.68^a	1779.97	230.05	0.896	0.004	0.031
Other compounds													
31	211.23	184.23	254.75	244.04	183.29	84.22^b	141.67^b	420.64^a	219.48	211.54	0.937	< 0.001	0.902
32	127.64	103.83	79.13	116.40	55.11	156.24^a	43.5^b	89.52a^b	96.59	96.42	0.709	0.022	0.992
33	630.69	544.99	256.44	428.18	302.67	373.11^b	165.43^b	759.24^a	280.67	584.51	0.493	0.002	0.040
34	315.49	203.53	80.24	179.69	102.94	163.45^{ab}	50.70^b	314.98^a	126.62	226.13	0.198	0.003	0.147
35	27.35	31.33	51.41	35.78	38.44	12.72^b	14.08^b	83.79^a	52.17	21.56	0.943	< 0.001	0.086
36	3.78	1.40	7.75	7.67	3.89	3.12^b	1.14^b	10.44^a	5.94	3.86	0.502	0.006	0.439
37	798.61	475.28	48.01	46.66	65.87	159.23	68.38	633.04	355.52	218.26	0.437	0.280	0.662
38	144.48	112.19	102.28	161.61	136.48	19.40^b	40.39^b	334.43^a	228.98	33.84	0.992	< 0.001	0.012
39	15.99	15.55	15.37	18.81	23.60	20.17	11.87	21.54	20.64	15.08	0.781	0.174	0.228
40	168.34	137.12	103.98	92.07	69.54	99.32^{ab}	66.17^b	177.13^a	81.21	147.21	0.350	0.012	0.041
41	19.25	10.32	104.75	12.61	35.58	7.98	13.29	88.24	23.92	49.09	0.329	0.073	0.447
Chlorophyll*													
a	43.97	8.54	25.02	15.12	70.52	10.95^b	56.38^a	27.93^{ab}	31.60	32.89	0.125	0.048	0.941
b	11.24	2.32	7.60	5.78	26.56	2.93^b	20.04^a	7.57^{ab}	10.16	10.89	0.097	0.032	0.908
Total	55.21	10.86	32.62	20.90	97.08	13.88^b	76.42^a	35.50^{ab}	41.76	43.78	0.117	0.042	0.932

^{abc} Values with different superscripts are significantly different between each of the factors (Tukey's test; $p \leq 0.05$). The numerical code corresponds to the compounds listed in Table 1. * Content of chlorophyll a and b and total chlorophyll (mg/100 g dried weight) in broccoli.

abundant in the inflorescence. In the commercial maturation stage, linoleic acid decreased significantly, due to the transformation into derivatives such as hydroxy-oxylipins FA 18:2 + 3O and FA 18:1 + 3O, whose presence increased. Other compounds identified were gluconic acid and fructosyl-pyroglutamate derivate.

3.6. Chlorophyll content

The total chlorophyll content in the varieties ranged from 10.86 to 97.08, distinguishing 'Summer Purple' and 'TSX 007′ from the rest of the cultivars. The different cultivars from this study contained about three to four times more chlorophyll a than chlorophyll *b*, with a greater difference in 'TSX 007′. The chlorophyll a content has been related to antioxidant activity because it is a more effective radical quencher than chlorophyll b (Fernández-León et al., 2012). Regarding the plant part, the mean concentrations of chlorophyll a in stems, leaves and inflorescences were 10.95, 5638 and 27.93 mg/100 g DW, whereas the concentration of chlorophyll *b* was 2.93, 20.04 and 7.57 mg/100 g DW, respectively (Table 5). In this case, the chlorophyll content in different parts of the plant presented significant differences ($p \leq 0.05$), with a higher concentration in leaves, as obtained by Liu et al. (2018). The chlorophyll content was not affected by the stage of maturity studied. These values were similar to the chlorophyll concentrations determined by Guzman et al. (2012) in ethanolic extracts of four different cultivars of broccoli. Nevertheless, Kaur et al. (2007) determined lower chlorophyll concentrations in heptane/ethanol (3:1) extracts. The discordant results may reflect differences between cultivars, as well as the different plant part analysed, or the extraction methods used.

3.7. Multivariate analysis

PCA was carried out for the whole data set to obtain an interpretable overview of the main information. Fig. 3 shows the two-way loadings and score plots, where PC2 was plotted against PC1, explaining more than 60% of the total variance. Antioxidant activities were clearly correlated with higher values of TPC, chlorophylls and the majority of phenolic compounds, as explained by the positive axis of PC1. These compounds and activities were related to leaf samples and, to a lesser extent, samples of the 'Summer Purple' cultivar, which is located in the first quadrant. By contrast, high values for most of the glucosinolates were mainly located on the second quadrant and associated with inflorescences. In the case of 4-hydroxy glucobrassicin (27) and glucobrassicin (28), they were explained by the positive axis of PC2 and were also related to flower bud samples and to samples of the 'Summer Purple' and 'BRO 2047′ cultivars. Antimicrobial and antihypertensive (the lowest values of ICEa50) activities were located in the third quadrant, correlated with higher values of palmitic acid (37) and some hydroxy-fatty acids (32–34). Antibacterial activity towards gram-positive and gram-negative bacteria has been described for several saturated fatty acids, including palmitic acid. In the same way, hydroxy fatty acids have been widely reported as antimicrobial agents (Casillas-Vargas et al., 2021; Shin et al., 2004).

4. Conclusions

In this study, significant differences in ethanol extracts of broccoli by-products were found in terms of activities and the profile of bioactive compounds, depending on the factors considered (variety and ripening stage). Leaf extracts, especially those of the variety 'Summer Purple', showed the highest antioxidant activity related to the highest content of phenolic compounds, constituted mainly by kaempferol and quercetin glucosides. Glucosinolates were more abundant in the inflorescence extracts, whereas antimicrobial and antihypertensive activities were related to the higher concentration of fatty acid derivates found in ethanolic extracts obtained mainly from inflorescences and stems. The high content of phenolic compounds in leaves is relevant to the use of

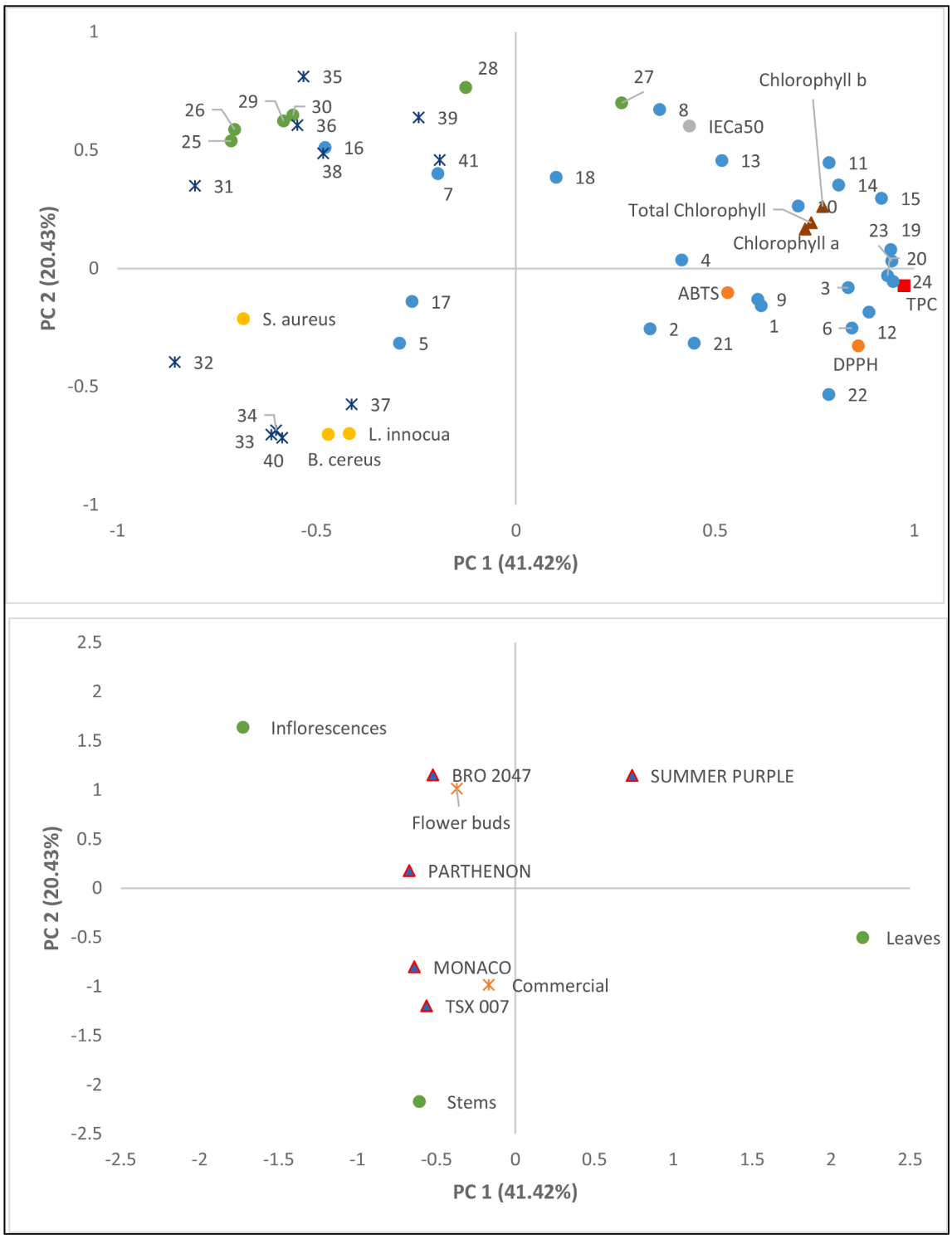

Fig. 3. Principal component analysis of the parameters and compounds analysed (TPC: total phenolic compounds; antioxidant activity: DPPH and ABTS; IECa50: antihypertensive activity; antimicrobial activity against *B. cereus,* L. *innocua* and *S. aureus*. The numerical code corresponds to the compounds listed in Table 1.

broccoli crop residues as raw materials for obtaining these bioactive compounds. In addition, stems and inflorescences that have lost commercial value are an interesting potential source of other bioactive compounds, such as glucosinolates and hydroxy-oxylipins.

CRediT authorship contribution statement

I. Gudiño: Formal analysis, Investigation, Resources, Writing – original draft. **A. Martín:** Methodology, Writing – review & editing, Visualization. **R. Casquete:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization. **M.H. Prieto:** Conceptualization, Funding acquisition. **M.C. Ayuso:** Resources. **M.G. Córdoba:** Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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