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Effect of polyphenol content on the antimicrobial activity of natural extracts from agro-industrial by-products

Einfluss des Polyphenolgehaltes auf die antimikrobielle Wirkung natürlicher Extrakte aus agarindustriellen Nebenprodukten

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Summary

The main objective of the present study was to investigate the effect of the conditions of extraction by Accelerated Solvent Extraction (ASE) technology on the bioactive antimicrobial activity of extracts from by-products of cauliflower, broccoli, orange, and mandarin. The antimicrobial activity of extracts, with concentrated phenol content, was evaluated against four of the most important foodborne pathogens: *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, *Bacillus cereus,* and *Listeria monocytogenes.* The largest phenol content (1252.12 ± 38.29 µg gallic acid/mL) was recovered from cauliflower extract. Cauliflower and mandarin extracts were effective against both Gram-positive and Gram-negative bacteria, showing the highest inhibition zones, 16 ± 1 mm and 17 ± 0.4 mm respectively, against 10^5 cfu/mL *S.* Typhimurium. The antimicrobial effectiveness of the extracts was influenced by the ASE extraction conditions, initial contamination level, and microbial strain.

Keywords: vegetable by-products, Accelerated Solvent Extraction, foodborne pathogens, phenols, natural antimicrobials

Zusammenfassung

Das Hauptziel der vorliegenden Studie war es, die Auswirkungen der Extraktionsbedingungen der Accelerated Solvent Extraction (ASE) Technologie auf die bioaktive antimikrobielle Wirkung von Extrakten aus Nebenprodukten von Blumenkohl, Brokkoli, Orange und Mandarine zu untersuchen. Die antimikrobielle Wirkung der Extrakte mit konzentriertem Phenolgehalt wurde auf vier der wichtigsten lebensmittelbedingten Krankheitserreger untersucht: *Salmonella enterica* Serovar Typhimurium, *Escherichia coli* O157: H7, *Bacillus cereus* und *Listeria monocytogenes*. Der höhste Phenolgehalt (1252,12 ± 38,29 µg Gallussäure/ml) wurde aus dem Blumenkohl-Extrakt gewonnen. Blumenkohl- und Mandarinen-Extrakte waren sowohl gegen Gram-positive als auch Gram-negative Bakterien wirksam. Die größten Hemmzonen (16 ± 1 mm und 17 ± 1 mm) waren gegen *S.* Typhimurium zu beobachten. Die antimikrobielle Wirksamkeit der Extrakte wurde von den ASE-Extraktionsbedingungen, des anfänglichen Kontaminationsgrades und der Bakterienstämme beeinflusst.

Schlüsselwörter: Beschleunigte Lösemittelextraktion, lebensmittelbedingte Krankheitserreger, Phenol, natürliche antimikrobielle Stoffe

1. Introduction

Owing to consumer concerns about synthetic additives, there is growing interest in the use of natural substances obtained from plants as functional food ingredients (Viuda-Martos et al., 2007). At the same time, agro-industrial activities like fruit and vegetable processing result in a huge quantity of waste, which represents an important economic problem for producers and an environmental challenge (O'Shea et al., 2012). However, much of this waste has bioactive compounds that can be recovered and used in other industrial processes. Many studies show that fruit and vegetable by-products and their extracts are significant sources of dietary fiber and bioactive compounds with high nutritional value (Fattouch et al., 2007). These bioactive compounds can be phenolic compounds, essential oils, flavonoids, carotenoids, and vitamin C, whose antioxidant, anticarcinogenic, anti-inflammatory, antiviral, and antimicrobial properties have been reported (Ghafar et al., 2010). By recovering them it is possible to increase the added value of these residues and to some extent mitigate the environmental problem, complying with the European Union's requirement of zero wastes (EUROSTAT, 2010). Therefore approaches involving the use of agri-food waste as by-products to obtain food additives or supplements are now being encouraged. Their antimicrobial effects are of huge interest for the food industry.

New extraction procedures have been proposed with the aim of extracting bioactive compounds from plants, reducing extraction time and solvent consumption and improving analyte recovery (Ballard et al., 2009). They include accelerated solvent extraction (ASE), which maximizes sample throughput, minimizes phytochemical degradation, and is a suitable method that is particularly useful for comparing the phenolic content of fruit, food materials, and by-products (Wibisono et al., 2009).

The aim of this study was to test the antimicrobial effectiveness of cauliflower, broccoli, mandarin, and orange by-product extracts against four foodborne pathogens – *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes, Escherichia coli* O157:H7, and *Bacillus cereus* – and determine how their effectiveness is affected by the ASE extraction conditions applied to the type and initial quantity of microbial contamination.

2. Material and Methods

2.1. Bacterial cultures

Glycerinated cryovials of *L. monocytogenes* (CECT 4032), *B. cereus* (CECT 131), *S.* Typhimurium (CECT 443), and *E. coli* O157:H7 (CECT 5947) were obtained from lyophilized cultures provided by the Spanish Type Culture Collection, using the methods described by Belda-Galbis et al. (2013) for *L. monocytogenes* and *E. coli* O157:H7, by Pina-Pérez et al. (2013) for *B. cereus* and by Pina-Pérez et al. (2012) for *S.* Typhimurium.

2.1. Obtainment of natural extracts from vegetable by-products

Dehydrated natural by-products of broccoli, cauliflower, mandarin, and orange were provided dehydrated directly from primary production.

Brassicaceae and *Citrus* extracts were obtained by the ASE technology. Accelerated solvent extraction (ASE) is a sample preparation technique that greatly reduces the amount of time and solvent required to achieve analyte extraction. The rate of extraction is greatly enhanced and the % recovery of analytes consistently increased over traditional techniques such as Soxhlet by using high temperature and pressure to achieve extraction from solid and semi-solid matrices in very short periods (Fig. 1). To perform an extraction, the solid sample is loaded into a sample cell (11 or 22 mL) which is placed on a cell tray and collection vessels are placed ona collection tray. The oven is maintained at the selected operating temperature throughout the extractions. The extraction cell design allows operation of the extractions at high pressures (1600 psi) to maintain the solvents as liquids at temperatures above their boiling points. Once the cell is placed in the oven, the pump immediately begins to deliver the solvent of choice to the sample cell. Single solvents or premixed solvents can be used from a single collection vessel, or any combination of up to three different solvents can be programmed. ASE is attracting interest because it features short extraction times, low solvent use and, high extraction yields, and provides a high level of automation (Hofler, 2002). Carotenes, aflatoxins, glucosinolates and polyphenols have been extracted by using the ASE system (Breithaupt, 2004; Sheibani and Ghaziaskar, 2009; Mohn et al., 2007)

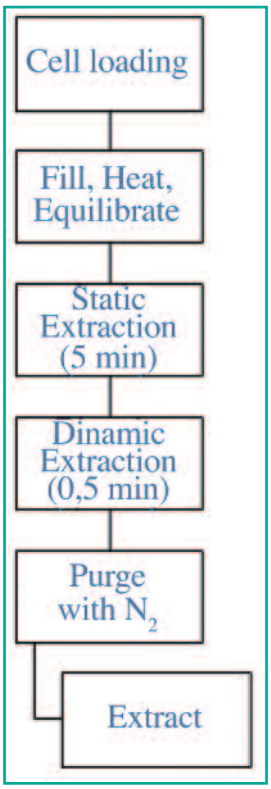

FIGURE 1: *ASE procedure used to obtain the extracts from the by-products of cauliflower, broccoli, mandarin and orange.*

Accelerated Solvent Extraction (ASE 350 by Dionex, Vertex Technics) was employed to obtain the cauliflower, broccoli, mandarin and orange extracts, as shown in Figure 1. The extraction was assumed to be affected by two independent variables: the extraction temperature and the number of extraction cycles.

In this case, a mixture of ethanol and water (20:80) was used as the solvent, the extraction temperature was fixed in the range of 20–120 °C, and the extraction cycles were in the range of one to four. Static extraction time was fixed at 5 minutes and pressure level at 1600 psi (approximately 11 MPa). Each sample was obtained in quadruplicate.

2.3. Determination of phenol content

A modified version of the Glories method (Glories, 1979) was used to determine the total phenol content and phenolic composition of ASE extracts.. Samples were diluted 1:10 with 10 % ethanol and then, 0.25 mL of sample or standard was placed in a test tube and 0.25 mL of 0.1 % HCl in 95 % ethanol and 4.55 mL of 2 % HCl were added. The solution was mixed with a vortex (Heidolph) and allowed to incubated for approximately 15 min before reading the absorbance at 272, 323, 368, and 522 nm with a spectro-

Journal of Food Safety and Food Quality 66, Heft 1 (2015), Seiten 1–24

photometer. To estimate total phenolic content, the absorbance (A) at 272 nm was used, and the absorbances at, A323 nm, A368 nm and A522 nm were used to estimate tartaric esters, flavonols and anthocyanins, respectively. Standards used were gallic acid (Sigma Aldrich Co., Madrid, Spain) in 10 % ethanol for total phenolics, caffeic acid (Sigma Aldrich Co., Madrid Spain) in 10 % ethanol for tartaric esters, quercetin (Sigma Aldrich Co., Madrid, Spain) in 95 % ethanol for flavonols, and malvidin-3-glucoside (Extrasynthese) in 10 % ethanol for anthocyanins.

The statistical analysis was performed with STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA). The analysis included average and standard deviation calculations for the three repetitions and an ANOVA analysis to test significant differences depending on extraction conditions.

2.4. Determination of antimicrobial activity

Antimicrobial activity of the natural extracts was measured using the agar diffusion method. One milliliter of stock vials of each microorganism at different concentrations (10^5 cfu/mL and 10^7 cfu/mL) was spread on the surface of Mueller-Hinton agar plates (Scharlau, S.A., Barcelona, Spain). Sterile filter paper discs (7 mm in diameter) were impregnated with 50 µL of the vegetable extracts. The extract was replaced with buffered peptone water (Scharlab, S.A., Barcelona, Spain) as a control sample.

The plates were then kept at ambient temperature for 30 min to allow diffusion of the extracts prior to incubation at 30 °C for 48 hours for *B. cereus,* at 37 °C for 48 hours for *L. monocytogenes,* and at 37 °C for 24 hours for *S.* Typhimurium and *E. coli* O157:H7.

Finally, the inhibition diameter of each disc was measured with a slide gauge. Studies were carried out in triplicate and the average and standard deviation of three values were calculated using STATGRAPHICS Centurion XV (version 15.1.03; STATGRAPHICS, Warrenton, VA).

3. Results and Discussion

3.1. Phenol content in broccoli, cauliflower, mandarin, and orange ASE extracts

Vegetable ASE extracts were characterized in terms of their total phenolic content expressed in mg gallic acid/L, and then various phenolic families which are present in a wide variety of fruits and vegetables, such as flavonols, tartaric esters and anthocyanins, were characterized from the total phenolic content. Table 1 presents the total phenol content of extracts for the various extraction conditions (temperature and extraction cycles) and their polyphenolic characterization, expressed in percentages.

Independently of extraction conditions, the maximum phenol content (1252.12 ± 38.29 mg gallic acid/L) was obtained from cauliflower leaf by-product, followed by mandarin, broccoli, and orange, with 893.67 ± 105.84, 748.15 ± 86.70, and 570.48 ± 26.55 mg gallic acid/L, respectively.

With regard to the effect of temperature on the amount of total phenolic compounds extracted, Wibisono et al. (2009) reported that the total phenol content of turnip leaf, Red Delicious apple puree, and elderberry extracts was slightly greater when the samples were extracted at a higher temperature (100 °C) than when they were extracted at a lower temperature (40 °C). However, no significant effect ($p \leq 0.05$) on the phenol content of the cauliflower extract was found when the extraction was carried out at 80 °C as compared with the extracts obtained at 100 °C (as can be seen in Table 1). Similar results were observed with the mandarin and broccoli extracts, where no significant differences ($p \leq 0.05$) were found between the extraction temperatures, 100 and 120 °C (mandarin) or 20 and 120 ºC (broccoli), regardless of the number of cycles. In the case of orange extract, the total phenol content at an extraction temperature of 120 ºC with 4 cycles was higher than at an extraction temperature of 80 ºC with 2 cycles

With regard to the number of cycles for the same by-product and extraction temperature, it appears that this did not affect the total phenol content of the extracts. The total polyphenol contents of the mandarin extracts obtained with 2 and 4 cycles at 120 °C were not significantly different ($p \leq 0.05$).

A comparison of the total polyphenol content in the mandarin and orange extracts, extracted under the same conditions, 4 cycles at 120 °C, shows that mandarin extract had more total phenol content than orange extract ($p \leq 0.05$). A comparison of the total polyphenol content of the orange and cauliflower extracts, extracted with 2 cycles at 80 °C, indicates that the total phenol content expressed as gallic acid/L was higher in cauliflower than in orange ($p \leq 0.05$). According to these results it appears that orange by-products have the lowest total phenol content, and accordingly this by-product is the least attractive for the valorization industry.

Also, the polyphenol characterization of each extract,, enables us to identify the main polyphenol families that are present in each ASE extract. The cauliflower and broccoli extracts have a similar polyphenol pattern, with tartaric esters as the main polyphenol family, followed by flavonols and anthocyanins. Cartea et al. (2011) also found a similar polyphenolic composition of *Brassicaceae* vegetables. In

TABLE 1: *Total phenol content and phenolic characterization of by-product extracts.*

Extract	Temperature (°C)	Cycles	Total phenol content (mg gallic acid/L)	Flavonols	Tartaric Esters	Anthocyanins	Other
Broccoli	20	3	734.60 ± 82.90[a]	22 %	36 %	20 %	22 %
	120	1	748.15 ± 86.70[a]	20 %	35 %	19 %	26 %
Cauliflower	80	2	1252.12 ± 38.29[b]	21 %	33 %	20 %	26 %
	100	2	1071.87 ± 108.04[b]	22 %	34 %	21 %	23 %
Orange	80	2	294.34 ± 19.20[c]	13 %	49 %	7 %	31 %
	120	4	570.48 ± 26.55[d]	13 %	43 %	9 %	35 %
Mandarin	100	4	836.24 ± 107.62[e]	19 %	50 %	14 %	17 %
	120	2	893.67 ± 105.84[e]	22 %	50 %	21 %	7 %
	120	4	810.40 ± 68.36[e]	12 %	51 %	14 %	23 %

[a-e]: superscript letters indicate significant ($p \leq 0.05$) differences between rows.

TABLE 2: *Total phenol content and antimicrobial effect of vegetable extracts (50 µL), tested by disc diffusion method, against L. monocytogenes, B. cereus, S. Typhimurium, and E. coli O157:H7 (10^5 cfu/mL)*.*

Extract	Extraction conditions		Inhibition halo (mm)			
	Polyphenol content (mg gallic acid/L)	T °C/Cycles	*L. monocytogenes*	*B. cereus*	*S.* Typhimurium	*E. coli* O157:H7
Broccoli	734.60 ± 82.90[a]	20/3	NI	NI	NI	11 ± 1.4
Broccoli	748.15 ± 86.70[a]	120/1	NI	NI	NI	NI
Cauliflower	1252.12 ± 38.29[b]	80/2	13 ± 1.4	12 ± 0.5	16 ± 1	9 ± 0.2
Cauliflower	1071.87 ± 108.04[b]	100/2	14 ± 1.4	15 ± 2	15 ± 1	NI
Mandarin	893.67 ± 105.84[e]	120/2	NI	NI	14 ± 0.6	8 ± 0.1
Mandarin	836.24 ± 107.62[e]	100/4	8 ± 0.1	NI	17 ± 0.4	10 ± 0.1
Mandarin	810.40 ± 68.36[e]	120/4	11 ± 0.6	13 ± 0.1	15 ± 0.1	8 ± 0.8
Orange	294.34 ± 19.20[c]	80/2	NI	NI	11 ± 0.1	NI
Orange	570.48 ± 26.55[d]	120/4	NI	NI	10 ± 0.1	NI

[a-e]: superscript letters indicate significant ($p \leq 0.05$) differences between rows. *: Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 7 mm. NI: No inhibition.

TABLE 3: *Total phenol content and antimicrobial effect of vegetable extracts (50 µL), tested by disc diffusion method, against L. monocytogenes, B. cereus, S. Typhimurium, and E. coli O157:H7 (10^7 cfu/mL)*.*

Extract	Extraction conditions		Inhibition halo (mm)			
	Polyphenol content (mg gallic acid/L)	T °C/Cycles	*L. monocytogenes*	*B. cereus*	*S.* Typhimurium	*E. coli* O157:H7
Broccoli	734.60 ± 82.90[a]	20/3	NI	NI	NI	10.5 ± 2.38 mm
Broccoli	748.15 ± 86.70[a]	120/1	NI	NI	NI	NI
Cauliflower	1252.12 ± 38.29[b]	80/2	11.5 ± 0.7 mm	15 ± 0.5 mm	NI	9 ± 0.2 mm
Cauliflower	1071.87 ± 108.04[b]	100/2	11 ± 0.0 mm	11 ± 1 mm	NI	NI
Mandarin	893.67 ± 105.84[e]	120/2	NI	NI	12 ± 0.2 mm	10 ± 0.1 mm
Mandarin	836.24 ± 107.62[e]	100/4	NI	NI	12 ± 0.5 mm	9 ± 1 mm
Mandarin	810.40 ± 68.36[e]	120/4	NI	9 ± 1 mm	NI	9 ± 0.5 mm
Orange	294.34 ± 19.20[c]	80/2	NI	NI	11 ± 1 mm	NI
Orange	570.48 ± 26.55[d]	120/4	NI	NI	NI	8 ± 0.5 mm

[a-e]: superscript letters indicate significant ($p \leq 0.05$) differences between rows. *: Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 7 mm. NI: No inhibition.

the case of Citrus by-products, tartaric esters are also the main polyphenol group, with percentage values higher than in *Brassicaceae* extracts, followed by flavonols and anthocyanins. The percentages of the three polyphenol families analyzed are higher in mandarin than in orange extracts, being, in contrast, the percentage of "other polyphenols" is higher in orange than in mandarin extracts (Table 1). These differences might be due to hydroxycinnamic acids and flavanones such as naringin, which can be found in higher amounts in orange than in mandarin extracts (Abad-García et al., 2014; Khan et al., 2014).

With regard to the effect of extraction conditions on the concentrations of different families of polyphenols (Table 1), the higher the number of extraction cycles, the lower the flavonol and anthocyanin concentrations for mandarin extracts, as occurred with the flavanols of grape skin in the study carried out by Mané et al. (2007). Those authors suggested two possible explanations: exposure to organic solvents leads to reduction of extractability of cellular components in subsequent extraction procedures and enzymatic oxidation could cause degradation of polyphenolic compounds. Also, it can be seen in mandarin extract that, when the temperature was higher, the flavonol content was lower, probably because high temperature increased hydrolyzation and oxidation of phenolic compounds, as indicated by Dai et al. (2010).

3.2. Antimicrobial activity of broccoli, cauliflower, mandarin, and orange extracts

The ASE extracts were then tested for antimicrobial activity against four foodborne pathogens: *S.* Typhimurium, *B. cereus, E. coli* O157:H7, and *L. monocytogenes.*

Tables 2 and 3 show the inhibition halo for each plant by-product and the total phenol content of each extract. Comparing the results, it could be said that, in general, the larger the inoculum concentration, the smaller the inhibition halo, with no inhibition halo in many cases. This was especially relevant for *S.* Typhimurium. All the extracts except broccoli exerted some inhibition against this microorganism when the initial microbial concentration was 10^5, but a non-inhibition halo or a decrease in the halo diameter was observed for almost all the extracts when the initial microbial concentration was 10^7. The effect of inoculum concentration on the efficiency of antimicrobial substances was also observed by Silva-Angulo et al. (2014). Those authors indicated that inoculum size affected the antibacterial effect of carvacrol on *Listeria innocua* and *L. monocytogenes* and this effect should be taken into account in growth ki-

Journal of Food Safety and Food Quality 66, Heft 1 (2015), Seiten 1–24

netic studies. In our study, similar results were obtained in practically all the combinations studied.

As shown on Table 2, cauliflower extract followed by mandarin extract presented the highest antimicrobial activity against the microorganisms tested. Both extracts were effective against Gram (+) and Gram (–) bacteria, with *S.* Typhimurium being the most sensitive microorganism of microorganism tested. Cauliflower showed its highest antimicrobial effect with the 80 °C, 2 cycle extract (polyphenol content of 1252.12 ± 38.29 mg gallic acid/L), with a maximum inhibition zone of 16 ± 1 mm against the 10^5 cfu/mL inoculum concentration of *S.* Typhimurium while the mandarin extract obtained with 4 cycles at 100 °C (polyphenol content of 836.24 ± 107.62 mg gallic acid/L) achieved the greatest inhibition zone, 17 ± 0.4 mm, against *S.* Typhimurium at an inoculum concentration of 10^5 cfu/mL.

Regardless of the extraction conditions, the orange extracts were only effective against *S.* Typhimurium. According to these results, orange extracts seem to have some specificity against *S.* Typhimurium, with a similar inhibition halo both at 120 °C with 4 cycles and at 80 ºC with 2 cycles against an initial cell population of 10^5, although the first of these extracts had a greater total phenol content. The cauliflower and mandarin extracts were also effective against *L. monocytogenes.* Similar inhibition halos were obtained for different cauliflower extracts, which was to be expected because there were no significant differences ($p > 0.05$) in the phenol contents of the cauliflower extracts obtained at different temperatures with the same number of cycles. Mandarin extracts also produced an inhibitory halo in *L. monocytogenes,* although the diameter depended on the extraction conditions because no significant differences ($p > 0.05$) were found among the phenol contents of the mandarin extracts.

B. cereus vegetative cells were also inhibited by the cauliflower and mandarin extracts. The biggest inhibition halo (15 ± 2 mm) was achieved with cauliflower extracts obtained with 2 cycles at 100 °C.

With regard to *E. coli* O157:H7, the broccoli extract was only effective against this microorganism (11 ± 1.4 mm halo), and only with extracts obtained with 3 cycles at 20 °C. There was no significant ($p \leq 0.05$) difference between the phenol contents of the two extracts (3 cycles at 20 °C and 1 cycle at 120 °C).

Generally, considering the effect of the extracts on the various microorganisms, it appears that the extraction conditions are the parameters that have most influence on the activity of the extracts. Therefore, although there were no significant ($p \leq 0.05$) differences in total phenol content among extracts of the same plant genus, these extraction conditions may influence the concentration of the various polyphenol families in the same plant genus (Table 1). Previous studies by Wibisono et al. (2009) confirmed that for the ASE technique 3 cycles (10 min at 40 °C; 2 min at 100 °C) provided optimal conditions for maximizing phenol extraction from apple pomace. Temperature and extraction cycles were also determined as influential when applied to *Cynara* spp. biomass and bioactive compound extraction by ASE (Ciancolini, 2012).

The total phenol content value, which was obtained for each of the extracts under study, appears to exert an influence on their antimicrobial potential. As can be seen in the results obtained, the extracts whose phenol content was higher, were the extracts withgreater antimicrobial activity. In fact, cauliflower *(Brassicaceae)* and mandarin *(Citrus),* were the ASE extracts with the highest antimicrobial effect against most of the microorganisms studied, and they were the extracts with the highest phenol contents.

For the effect of the extracts the type of microorganism is also important. In general, the Gram-negative bacteria showed higher sensitivity to exposure to *Brassicaceae* and *Citrus* extracts than the Gram-positive bacteria. According to Martin-Luengo et al. (2007), bergamot minimum inhibitory concentrations (MIC, µg/mL) range between 400 and 800 µg/mL against *S.* Typhimurium and *E. coli* K-12, whereas 1000 µg/mL was necessary to inhibit *B. cereus,* and no antimicrobial effect was exerted against *L. monocytogenes.* Similarly, the results of Hu et al. (2004) also demonstrated that cabbage extrats had a higher antimicrobial effect against Gram-negative bacteria than against Gram-positive bacteria.

With regard to the extraction conditions of the various ASE extracts (temperature and cycles), for broccoli and orange there were no significant ($p \leq 0.05$) differences between the antimicrobial activity of the same extract obtained at different temperatures or with a different number of cycles. However, for cauliflower and mandarin a rise of temperature (from 80 to 100 °C and from 100 to 120 °C) caused the disappearance of antimicrobial activity against *E. coli* O157:H7 and *S.* Typhimurium, respectively. This behavior could be attributable to the phenolic profile, which might be dependent on the combination of temperature and extraction process cycles, as occurred with the lower flavonol content at higher temperatures in the mandarin extracts.

4. Conclusions

The results showed above clearly suggest that vegetable by-products are a promising potential, economical source of phenolic compounds with a high antimicrobial effect. However, it is important to achieve optimization of the extraction process because of the effect of those conditions on the antimicrobial activity. The present study provides useful information about the value of vegetable by-products for food safety improvement and potential new alternatives for food functional supplementation, and extraction conditions for the ASE technique. Some microbial specificity was found for orange and broccoli extracts. Probably the extraction conditions affect the phenol profile. Studying the phenol profile of each extract could help in understanding the differences observed in the inhibitory capability of extracts from the same plant genus despite the fact that there were no significant differences in the polyphenol contents of the extracts of each genus.

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