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Phenolic antioxidants extraction from raspberry wastes assisted by-enzymes

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Abstract The effect of enzymatic hydrolysis on phenolic antioxidant extraction was studied in raspberry solid wastes. This by-product possesses high content of crude fiber (60%) and low values of protein, oil and ash. Raspberry fiber composition suggests that biocatalysts with cellulase, hemicellulase and pectinase activities would be useful for carrying out an enzymatically assisted antioxidant extraction. Hydro-alcoholic extraction was done using different commercial enzymes. Total phenol content and antioxidant activity of enzyme-hydrolyzed residue extracts were measured and compared with those obtained without enzyme application.All biocatalysts evaluated increased soluble solids in comparison to the non-enzymatic control. Among them, Grindamyl and Maxoliva offered the best recovery of polyphenols. Enzymatic assisted extraction with an hydro-ethanolic mixture (75:25, v/v) during 18 hrs at 50°C increased phenolic content up to 35% and antioxidant capacity around 50%, 15% and 30% according to 2,2-diphenyl-1-picrylhydrazyl = 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl) (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) methods, respectively. Use of a higher enzyme concentration significantly improved extraction of phenolic antioxidants.

Keywords: antioxidant activity, enzymatic extraction, phenolic compounds

INTRODUCTION

Antioxidants are substances that, when present at low concentrations with respect to an oxidisable substrate, delay or prevent the oxidation of that substrate. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in the food industry due to their effectiveness and low price. Nevertheless, toxicity of these compounds and the growing interest in safe nutritional additives by consumers has created the necessity to identify natural and safe sources for food antioxidants (Ajila et al. 2007). In this context, solid wastes generated from fruits and vegetables processing are interesting raw materials to obtain phenolic compounds with good antioxidant activity. External fractions of vegetables and other lignocellulosic parts (skins, peels, hulls and seeds) are usually discarded, although they present greater content of phenolic compounds than their corresponding inner fractions (Cruz et al. 2004). Because of the low cost of residues and the possibility to solve environmental problems caused by their disposal, this field of research is highly appealing.

Crude fiber of fruits and vegetables, which includes cellulose, hemicellulose, pectin, β-glucans, gums and lignin, is rich in polyphenols (Sáyago-Ayerdi et al. 2007). Then, high crude fiber content suggests a high antioxidant potential. On the other hand, polyphenols are susceptible to precipitate coupled to proteins, diminishing concentration of free phenolic compounds and avoiding their antioxidant efficacy (Labuckas et al. 2008). Also, minerals and heavy metals contained in ashes can act as pro-oxidants (Maisuthisakul et al. 2006; Maisuthisakul et al. 2008). Due to these facts, some vegetable matrixes have more potential to be a source of antioxidant compounds.

Solid/liquid antioxidant extraction is a very efficient technique, but results depends on several factors such as raw material composition, conditioning, solvent polarity (and phenolic compounds polarity), time, temperature and solid/solvent ratio during the extraction.

Enzymatic treatment of vegetable samples can be a suitable alternative to increase polyphenols recovery. Enzymes commonly used in the food industry catalyze a variety of hydrolytic reactions and a high percentage act on cell wall polymers improving extraction yield of juices, oils and sugars. The main limitation for the application of those enzymes in industrial processes has been their high cost, but with current biotechnology advances it is already possible to obtain enzymatic formulations with lower costs and better quality (Bhat, 2000; Zúñiga et al. 2003).

Few works published have reported application of enzymes in processes that use organic solvents or water to extract phenolic compounds from vegetal samples as a way to increase phenol yields. Moreover, some of them have been developed with the aim to improve the nutritional quality of products obtained from vegetable matrix or increase the yield, as it is the case of oil extraction and juice production processes. Weinberg et al. (1999) studied ensilage assisted by enzymes (cellulases, hemicellulases, and pectinases) on rosemary and sage with the aim of improving their digestibility, observing an increase in phenolic compounds extracted from the herbs. Andreasen et al. (1999) have studied the release of hydroxycinnamic and hydroxybenzoic acids from rye grain with four different commercial enzymatic formulations that degrade vegetable cell walls components with the purpose to improve the nutritional quality of rye bread.

Enzyme incorporation in oil extraction processes produce a high content of antioxidant compounds in olive oil (García et al. 2001), in defatted meal of evening primrose (Collao et al. 2007), and in borage oil and its defatted meal (Soto et al. 2008). Additionally, antioxidant capacities were increased in the same assays. Also, the authors indicated the importance of parameters as type of enzyme and solvent used in extraction by solvents.

Enzyme application in red wine elaboration is usual. In this context, Pardo et al. (1999) evaluated the effect of four commercial enzymatic formulations on anthocyanins and total polyphenol content, proving that all of them increase polyphenols and anthocyanins with respect to non-enzymatic control. A similar behavior was observed by Bautista-Ortín et al. (2005), who reported improvement in chromatic and sensory characteristics of enzyme-treated wine in comparison to control wine. In the case of juice preparation, good results of phenolic compounds and/or antioxidant activities have been observed when enzymes were incorporated during the process, as it is occur in asparagus juice (Sun et al. 2005), bilberry juice (Koponen et al. 2008; Puupponen-Pimia et al. 2008), and blackcurrant juice (Koponen et al. 2008). Those reports show that content of antioxidants compounds in press cake increase o decrease according to the type of enzyme incorporated.

Enzyme application on agroindustrial residues with the aim to increase phenolic compounds also has been studied. Meyer et al. (1998) and Maier et al. (2008) showed an increase of phenolic compounds extracted from grape pomace by means of enzyme incorporation. Also, more pigments (anthocyanins) can be obtained from vinification process residues if enzymes are applied (Muñoz et al. 2004). Tobar et al. (2005) assessed the antioxidant potential of defatted meal of grape seed; the phenolic compounds content of a defatted meal obtained by an enzyme assisted oil extraction process was better those

Table 1. Proximal composition of raspberry residue (% dry matter). Results are expressed as the mean (n = 3) ± SD.

Component	
Protein	1.87 ± 0.05
Oil	1.38 ± 0.04
Ash	5.97 ± 0.05
Crude fiber	59.76 ± 0.02
Nitrogen free extract	31.02 ± 0.13

obtained from control oil extraction process meal, without enzymes. At pilot-plant scale, Kammerer et al. (2005) showed the benefits of enzyme application on polyphenols extraction from grape by-products, demonstrating that conditions such as enzyme type, enzyme-substrate ratio and temperature are the most relevant in the process. Lee and Wrolstad (2004) established that enzymatic treatment had a little effect on total monomeric anthocyanins content and on total phenolics recovery from blueberry processing waste. Kim et al. (2005) evaluated the effect of an enzymatic treatment with a *Thermobifida fusca* cellulase on apple peel finding a good correlation between phenolics released, reducing sugar production, and antioxidant capacity. In the same way, Li et al. (2006) analyzed the enzymatically assisted aqueous extraction from five different citrus peels, determining that the most important parameters in the extraction process are the condition of the peels, temperature of extraction, type of enzymes, enzyme concentration and citrus species. Landbo and Meyer (2001) reported that the incorporation of enzymes into the extraction process from blackcurrant juice residues promotes the polyphenols and anthocyanins recovery. A similar result was observed by Kapasakalidis et al. (2009), using a commercial cellulose enzyme on blackcurrant pomace.

In spite of the abundant literature about the presence of phenolic antioxidants in fruits and vegetables, there is not enough information about the influence of variables on the extraction process. Many conventional extraction systems could diminish the activity of the phenolic antioxidants, which are very sensitive to specific solvents and high temperatures. This has impelled worldwide research for developing process to obtain natural antioxidant extracts under mild conditions. Application of low cost commercial enzymes, used in the food industry, to degrade main components of vegetable cell walls might ameliorate the phenolic compounds extraction without using denaturing conditions and thus preserving their antioxidant activity. Based on before mentioned, the aim of this work is to evaluate the effect of enzymatic technology and solvent type, on recuperation of phenolic compounds as an eco-friendly way of increase the functional value of phenolics in raspberry residues.

In order to increase polyphenols recovery and to preserve their functional properties, different parameters such as type of biocatalyst, ethanol concentration in the extraction mixture and concentration of the enzyme were studied and compared with controls without enzyme addition.

MATERIALS AND METHODS

Vegetal solid wastes

Raspberry residues were provided by Bayas Del Sur, S.A. (Chile). Raw material was dried at 60ºC and grounded in a knife mill. Powdered samples were sieved to select particles smaller than 2 mm. Later, they were defatted during 6 hrs with petroleum ether and stored at room temperature in the dark until their use.

Enzymes

Twelve commercial biocatalysts with mainly pectinolitic and cellulotic activities were used: Cellubrix (cellulose and hemicellulases), Olivex (pectinase and hemicellulase), Pectinex Ultra SPL (pectinase), Ultrazym 100G (pectinase) and Viscozym (carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) were from Novozymes A/S; Rohapect 10L (pectinase), Rohapect DA6L (pectinase), Rohapect Max (pectinase) and Rohavin L (pectinase, cellulase) were supplied by AB Enzymes; Granozyme (Xylanase) was from Granotec Inc.; Grindamyl CA 150 (pectinase) was from Danisco; and Maxoliva (pectinase) was supplied by DSM.

Analytical methods

Proximal and fiber composition. Total nitrogen was determined by the micro-Kjeldahl method and protein was calculated as nitrogen x 6.25. Oil was extracted for 6 hrs with petroleum ether in a Soxhlet system. Ash was determined by incineration in a furnace at 550ºC and weighed. Crude fiber was measured as the residue remaining after acid and basic hydrolysis. Dry matters were determined by drying the samples to a constant weight at 105ºC (Figuerola et al. 2005). Detergent fibers were analyzed according to Zuñiga et al. (2003) and pectin was determined as it is mentioned in Soto et al. (2004).

Determination of total polyphenolic compounds. Total phenolics were assayed colorimetrically by the Folin-Ciocalteu method. Briefly, 3.75 ml of water, 0.25 ml two-fold diluted Folin-Ciocalteu reagent, 0.5 ml of phenolic extract and 0.5 ml of 10% sodium carbonate were mixed. The absorbance was measured at 765 nm after 1 hr at room temperature. A mixture of water and reagents was used as a blank. Total phenolic content was expressed as mg gallic acid/g dry sample (mg GAE/g d.s.) (Conde et al. 2009).

Determination of the antioxidant activity. The 2,2-diphenyl-1-picrylhydrazyl = 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl) (DPPH) radical scavenging capacity was measured according to Moure et al. (2000). 2 mL of a 3.6 x 10^{-5} M of methanolic solution of DPPH (Sigma) was added to 50 µL of a methanolic solution of the phenolic extracts. The decrease in absorbance at 515 nm was recorded in a spectrophotometer after 16 min. Results were expressed as mmol 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox)/100 g dry sample (mmol TE/100 g d.s.).

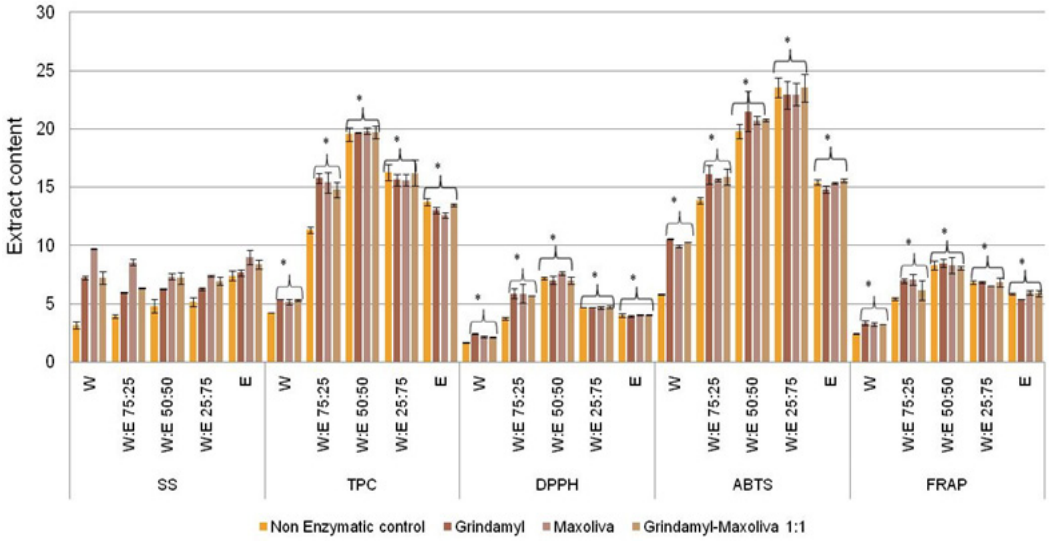

Fig. 1 Ethanol concentration effect on enzymatically assisted extraction of phenolic antioxidants from raspberry wastes. Conditions: 1 g; 50ºC; 18 hrs; 150 rpm; Liquid/Solid ratio 20:1; W: water; E: ethanol. Results are expressed as the mean (n = 3) ± SD. SS: g/g d.w.; TPC: mg; GAE/g d.s.; DPPH: mmol TE/100 g d.s.; ABTS: mmol TE/100 g d.s.; FRAP: mmol AAE/100 g d.s. Values grouped with * means not significant differences (P > 0.05).

For 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay, a stock solution of ABTS + radical cation was prepared by mixing ABTS solution and potassium persulfate solution at 7 mM and 2.45 mM final concentrations, respectively. The mixture was maintained in the dark at room temperature for 12-16 hrs before use. The working solution was diluted to obtain an absorbance of 0.700 (± 0.020) at 734 nm. Phenolic extracts (10 µL) were allowed to react with 1 mL of the diluted ABTS working solution for 10 min in a dark condition. Then the absorbance was taken at 734 nm. Results were expressed as mmol trolox/100g dry sample (mmol TE/100 g d.s.) (Conde et al. 2009).

For ferric reducing antioxidant power (FRAP) assay, the stock solutions included 300 mM acetate buffer pH 3.6, 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM FeCl3 x 6H2O solution. The fresh working solution was prepared in a ratio of 10:1:1 and then warmed at 37ºC before using. Phenolic extracts (0.1 mL) were mixed with 3.0 mL of the FRAP solution for 6 min in dark condition. Then the absorbance was monitored at 593 nm using the spectrophotometer. Results were expressed as mmol ascorbic acid/100g dry sample (mmol AAE/100 g d.s.) (Conde et al. 2009).

Experimental methodology

Prior to antioxidant extraction, raspberry wastes were characterized chemically and the fiber content established, with the aim to approach the real potential to recover compounds with antioxidants and to determine the requirements of enzyme activity to produce the cell wall degradation.

Extraction of phenolic compounds. Samples of 1.0 g of dry, crushed finely and defatted raw material were extracted in batch system with a 1:20 solid/liquid ratio, using magnetic agitation at 150 rpm. Extraction was done at 50ºC, during 18 hrs. Solvents of extraction were ethanol, methanol, distilled/deionized water and hydroalcoholic mixtures of ethanol/water (25/75). Commercial enzymes were applied directly on each crushed and defatted sample, prior to solvent incorporation, in a 10% respect to dry matter. The enzymatic treatment and the antioxidant extraction were done altogether.

The crude extracts were filtered using fiber glass paper and stored at 4ºC for the determination of the antioxidant power and the quantification of total phenolic compounds. All the extracts were prepared in triplicate.

The effect of biocatalysts type, solvent applied and enzyme concentration was evaluated too. In the first, twelve commercial biocatalysts with pectinolotic, cellulolitic and hemicellulolitic activities (as it is reported in materials section) were used, selecting those that promote the best total phenolic compounds recovery with a high antioxidant capacity.

The effect of solvent type on polyphenols recuperation was evaluated using the selected enzymes. Pure ethanol, water and ethanol/water mixtures (75/25, 50/50, 25/75 v/v) were used. Enzyme concentration was studied in the range of 0.75 to 10 g/100 g dry raw material, using the ethanol/water mixture selected in the previous stage and using one biocatalyst, which promote the best results. Other extraction conditions are above mentioned.

RESULTS AND DISCUSSION

Raspberry residues characterization

Proximal free-moisture composition of raspberry wastes is shown in Table 1.

The high crude fiber content of raspberry residues suggests that they possess a great potential as phenolic antioxidant source. Crude fiber, which consists of a heterogeneous mixture of non starch polysaccharides including cellulose, hemicellulose, pectin, β-glucans, gums, and lignin (Figuerola et al. 2005), and contains frequently polyphenols associated with non starch polysaccharides, that could be released from the vegetal matrix conferring antioxidant activity (Sáyago-Ayerdi et al. 2007). Raspberry wastes presented low protein content. Polyphenols can precipitate with proteins through different mechanisms, such as hydrophobic and ionic interactions, hydrogen and covalent bindings; these interactions can diminish free phenolic concentration reducing their antioxidant efficacy (Labuckas et al. 2008). Also, raspberry residues presented low ash content, which could positively influence the antioxidant capacity of raspberry extract, since ashes contain minerals and heavy metals, including iron that can act as pro-oxidants (Maisuthisakul et al. 2006; Maisuthisakul et al. 2008). Furthermore, low

Table 2. Fiber composition of raspberry residue. Results are expressed as the mean (n = 3) ± SD.

Fiber	
Hemicellulose	14.89 ± 0.53
Cellulose	5.36 ± 0.88
Lignin	63.16 ± 1.52
Pectin	15.38 ± 1.37
Others	1.21 ± 0.2

ash content could be beneficial in reducing the risk of enzymatic action inhibition. In the case of lipid content, raspberry residues was low, preventing the rancidity and favoring the stability of this by-product.

Fiber characterization was done with the aim of pre-select the type of biocatalyst, which could have the best degradative action of raspberry cell wall.

Raspberry residues presented a high percentage of lignin as it is observed in Table 2; other important structural components were pectin and hemicellulose, while cellulose content was less relevant. The high content of lignin in the raspberry residues would indicate a greater presence of phenolic compounds. Pectin content (oxalate soluble) of about 15% confirmed the significance of using a pectinase activity for degradation, because its presence in external layers of cell walls limits other internal hydrolysis components.

A commercial enzyme, which contains a combination of cellulase, hemicellulase and pectinase activities, would be useful for carrying out an enzymatically assisted antioxidant extraction, according to fiber composition of raspberry wastes above showed. Ben Shalom (1986) demonstrated that hydrolysis of vegetable cell walls is more effective when using formulations that contain a cocktail of activities, that when applying pure enzymes, since a synergic effect is produced. Degradation of cell wall depends on sequential hydrolysis of the structural polysaccharides; given that pectic substances are covering cellulose microfibers (which are joined to hemicellullolitic polymers) partially preventing their hydrolysis. Addition of pectinases and the consequent degradation of pectic polymers allows greater exposition of cellulose and hemicellulose for the enzymatic action of cellulases and hemicellulases (Massiot et al. 1989; Voragen and Pilnik, 1989).

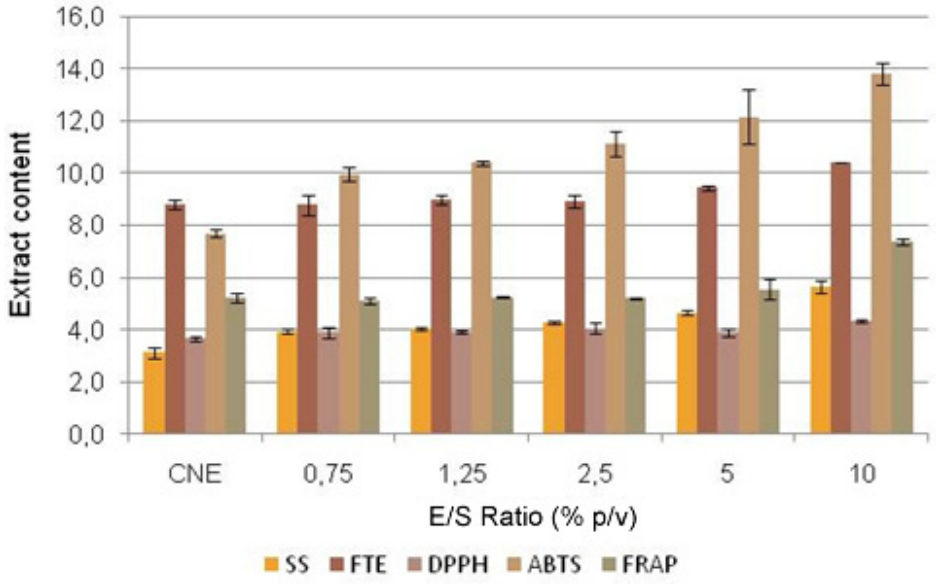

Fig. 2 Enzyme concentration effect on hydroethanolic (75/25%) enzymatically assisted extraction of phenolic antioxidants from raspberry wastes. Conditions: 1 g; 50ºC; 18 hrs; 150 rpm; Liquid/Solid ratio 20:1. Results are expressed as the mean (*n* = 3) ± SD. SS: g/g d.w.; TPC: mg gallic acid/g d.s.; DPPH: mmol TE/100 g d.s.; ABTS: mmol TE/100 g d.s.; FRAP: mmol AAE/100 g d.s.

Enzymatically-aided antioxidant extraction process

Enzymatic mediated extraction of phenolic antioxidants from the vegetable matrix may occur via hydrolytic degradation of the cell wall polysaccharides, which can retain phenolics in the polysaccharide-lignin network by hydrogen or hydrophobic bonding. Another mechanism may be the direct enzyme catalyzed breakage of the ether and/or ester linkages between the phenols and the plant cell wall polymers, as it is mentioned by Pinelo et al. (2008).

Table 3 shows results obtained using twelve biocatalysts. As it is possible to observe, all the biocatalysts evaluated increased yields of soluble solids extraction with respect to non-enzymatic control, except for Rohavin enzyme, which do not show significant difference ($P > 0.05$). This increment can be explained in terms of the hydrolytic capacity of enzymes to degrade the main components of the cell wall, releasing sugars from macro-molecules like cellulose, hemicellulose and pectin. A similar behavior has been referenced previously by Barzana et al. (2002) for the carotenoids extraction from marigold flower flowers.

Under the experimental conditions applied in this study, commercial enzymes such as Cellubrix (cellulase and xilanase), Viscozym (pectinase), Rohapect 10 L, Rohapect DA6L, Ultrazym 100 G and Granozyme (xilanase) diminished strongly the total phenolic compounds content from raspberry residues (significant differences $P < 0.01$), possibly due to the presence of secondary activities like β-glucosidases or β-galactosidases. The later enzymes can cause hydrolysis of anthocyanins as was demonstrated by Versari et al. (1997). These authors determined that the use of commercial pectinases modifies the phenolic composition of berry juices, dismissing the content of anthocyanins in a 20% when Pectinex BE, Rohapect B1L, Rohament MAX, and Pectinex 3XL were used during 6 hrs. In the cases of Rohavin, Pectinex Ultra SPL and Olivex did not exhibit comparative advantages in this study with respect to the non-enzymatic control ($P > 0.05$). These results do not agree with those determined by García et al. (2001), who using Olivex during the olives malaxation, observed an increase in phenolic compounds (vainillinic acid, vainillin, pinoresinol, and luteoline).

Considering the high percentage of lignin in residues of raspberry (63%), one of the factors that could explain the low effectiveness of some of the biocatalyst previously mentioned on phenolic antioxidant release from raspberry wastes is nonproductive absorption of enzymes to lignin or other polysaccharides in the cell wall matrix, as it is reported by Meyer et al. (1998).

Grindamyl pectinase was shown to be efficient in releasing free and esterified ferulic acid from sugarbeet pulp (Micard et al. 1994). It also significantly enhanced the amount of phenols recovery from

Table 3. Soluble solids yields polyphenols content and antioxidant power of raspberry phenolic extracts obtained with several enzymatic formulations. Extraction conditions: 50ºC, 18 hrs; enzyme concentration 10% (g/100 g raw material); solvent: ethanol/water 25/75; solid/liquid ratio 1/20.

Enzymatic Formulation	Soluble Solids (SS) g extract/ 100 g d.s.	Phenolic compounds (TPC) mg GAE/g d.s.	Antioxidant Activity (DPPH) mmol TE/100 g d.s.	Antioxidant Activity (ABTS) mmol TE/100 g d.s.	Antioxidant Activity (FRAP) mmol AAE/ 100 g d.s.
Non enzymatic control	3.86 ± 0.14	11.35 ± 0.27	3.76 ± 0.11	13.73 ± 0.27	5.31 ± 0.23
Cellubrix	9.47 ± 0.70	9.75 ± 0.39	3.34 ± 0.03	13.02 ± 1.12	4.34 ± 0.20
Granozyme	5.41 ± 0.24	9.59 ± 0.16	3.48 ± 0.02	12.14 ± 0.51	4.89 ± 045
Grindamyl CA 150	5.97± 0.05	15.79 ± 0.43	5.88 ± 0.39	15.95 ± 081	6.96 ± 0.16
Maxoliva	8.56 ± 0.24	15.40 ± 0.91	5.70 ± 0.47	15.02 ± 1.77	7.06 ± 0.48
Olivex	7.81 ± 0.51	11.10 ± 0.36	3.74 ± 012	13.86 ± 0.61	5.10 ± 0.22
Pectinex Ultra SPL	7.89 ± 0.11	11.09 ± 0.6	3.71 ± 0.25	13.63 ± 0.09	5.09 ± 0.03
Rohapect 10L	5.44 ± 0.24	10.48 ± 0.84	4.07 ± 0.19	13.28 ± 041	5.41 ± 0.27
Rohapect DA6L	6.09 ± 0.50	10.70 ± 0.29	3.74 ± 0.10	12.79 ± 79	4.91 ± 0.30
Rohapect Max	5.58 ± 0.32	12.17 ± 0.31	4.02 ± 0.11	14.36 ± 0.14	5.36 ± 0.04
Rohavin	4.17 ± 0.32	11.06 ± 0.09	3.67 ± 0.02	13.81 ± 0.20	5.35 ± 0.43
Ultrazym 100G	12.19 ± 0.60	10.21 ± 0.19	3.79 ± 0.14	13.76 ± 0.24	5.40 ± 028
Viscozym	7.7 ± 0.17	10.11 ± 0.18	3.44 ± 0.12	13.73 ± 0.47	4.79 ± 0.19

grape pomace (Meyer et al. 1998) and in black currant juice press residues (Lanbdo and Meyer, 2001). According to its supplier, Grindamyl contains pectinolytic activities, but it also exhibits cellulolytic and hemicellulolytic activities, a suitable combination to extract polyphenols from raspberry residues.

Table 3 shows that only Grindamyl CA 150 and Maxoliva produced a significative increment ($P < 0.05$) of antioxidant capacity measured by the methods of the DPPH, ABTS and FRAP. Therefore, both enzymes were selected for the subsequent study of the ethanol concentration effect used in the enzymatically assisted extraction of phenolic antioxidants from raspberry wastes.

Effect of extracting solvent on phenolic recovery

Both commercial biocatalysts tested as well as its mixtures in equal proportion increased soluble solids content compared to non-enzymatic controls. This increase could be explained in term of the hydrolytic capacity of enzymes able to degrade the main cell wall components, releasing sugars from macromolecules such as cellulose, hemicellulose and lignin (Meyer et al. 1998).

As it can be appreciated in Figure 1, solvent type changed phenolic content and antioxidant activity, enhancing with the increase of ethanol concentration in the solution, until the concentration reached 50%. These results agree with those reported by Li et al. (2006) for citruses residues. At equal proportion of water and ethanol in the extractor system, it is not possible to observe the enzymatic treatment effect on the phenolic antioxidant recovery, after which, the yields are reduced. The most effective mixture for conventional solid/liquid extraction from raspberry wastes, water/ethanol (50:50, v/v), resulted in antioxidant activity values of 7.2, 19.8 and 8.3, for DPPH, ABTS and FRAP, respectively and exhibited the highest phenolic content (21.6 mg GAE/g d.s). Soluble solids, phenolic content and antioxidant activity varied significantly with the solvent/mixtures used, but extractions with water and ethanol resulted in lower yields than extracts obtained from hydro-ethanolic mixtures.

Only enzymatic treatment with water and water: ethanol (75:25 v/v) enhanced the phenolic content and antioxidant activity of obtained extracts with respect to non-enzymatic controls ($P < 0.001$). An increase in phenolics content around 25%, (from 4.2 to 5.4) was achieved with water as extractor system under enzymic action. Antioxidant capacity of aqueous extracts from enzymatically treated samples was raised around 40% (from 1.7 to 2.4), around 80% (from 5.8 to 10.5) and around 35% (from 5.4 to 7.0) for the DPPH, ABTS and FRAP methods, respectively. Whereas the hydro-ethanolic mixture (25:75 v/v) enhanced the phenolic content up to 35% and the antioxidant capacity around 50%, 15% and 30% for the DPPH, ABTS and FRAP methods, respectively. The three biocatalyst evaluated did not present significant differences ($P > 0.05$) in their action with all the analyzed solvents/mixtures.

Raspberry ethanolic extracts can inhibit the hydrolytic action of the applied biocatalyst, according to Zabala et al. (2008), because ethanol is a competitive inhibitor which blocks all enzyme active sites, avoiding the enzymatic activity. Similar behavior is reported by Bezerra and Dias (2005), who indicated that when ethanol concentration is increased, the enzyme is denatured. Ethanol also interferes with enzyme (mainly cellobiohydrolases) adsorption to cellulose and modifies the cooperative effect between cellobiohydrolases and endoglucanases, explaining the behavior observed in the Figure 1.

Effect of enzyme concentration on polyphenols extraction

The effect of different enzyme concentrations (Grindamyl CA 150) on hydroethanolic (75/25) phenolic antioxidants extraction from raspberry residues is shown in Figure 2.

All enzyme concentrations increased soluble solids from 26% to 81% with respect to the non-enzymatic control when the enzyme/substrate ratio ranged between 0.75 and 10. Total phenolic content was modified just at high enzyme concentrations, with an increase of 7% and 18%, for 5 and 10% E/S, respectively (from 8.8 to 9.5 and 10.5 g GAE/100 g d.s.); however only 10% E/S show a real difference ($P < 0.001$) with respect to control assay. Also, all enzyme concentrations improved antioxidant activity evaluated by ABTS method with respect to the non-enzymatic control from 30% to 80% when the enzyme/substrate ratio was varied between 0.75 and 10. Enzyme concentrations of 10% resulted in a significant increase ($P < 0.001$) on antioxidant activity evaluated by FRAP method of 42% (from 5.2 to 7.4 mmol AAE/100 g d.s.). In the case of DPPH method, enzyme concentrations of

10% resulted in a slight increase on antioxidant activity with a 4.3 mmol TE/100 g d.s. In general, use of higher enzyme concentration improved greatly the recovery of phenolic antioxidants.

CONCLUDING REMARKS

Summing up, the most significant contributions accomplished in this paper are: Chemical characterization of raspberry residues in a detailed form was realized. This by-product presented considerably content of crude fibre and low values of oil, protein and ash. Also, it possesses a high contents of lignin and pectin; reason why biocatalysts which possess a combination of cellulolytic, hemicellulolytic and pectinolitic activities are adequate for carrying out the enzymatically assisted phenolic antioxidant extraction. Both, concentration of ethanol in the extractor system and concentration of enzyme, influence the polyphenols recoverey mediated by enzymes. Due to the great radical scavenging and iron reducing ability of extracts obtained from raspberry wastes, they could be used for preserving food, cosmetic and pharmaceutical products.

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