Simulation of Multistage Extraction of Antioxidants from Chilean Hazelnut (*Gevuina avellana*) Hulls

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ABSTRACT: The objective of this study was to analyze the factors affecting the ethanol extraction of Chilean hazelnut (*Gevuina avellana*) hulls to obtain antioxidant compounds. The effects of temperature on the kinetics of polyphenolics extraction and on the antioxidant activities of the extracts were assessed. The radical-scavenging activities of the extracts were comparable with that of BHA when used at the same concentration. The optimal temperature for the antioxidant activities of the extracts was 40°C. A four-stage cross-flow extraction was carried out and a four-stage countercurrent extraction was simulated, where each stage lasted 30 min. Best results were obtained with countercurrent extraction, which produced an extract that showed 94.4% α,α-diphenyl-β-picrylhydazyl radical inhibition, compared with 86.2% obtained in the first stage of cross-flow extraction and 92.62% in a batch extraction that lasted 100 h. UV-vis and NIR spectra of extracts from cross-flow and from the simulated countercurrent extraction revealed that the composition of extracts varied along the stages and was affected by the operational strategy.

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KEY WORDS: Antioxidants, countercurrent extraction, cross-flow extraction, ethanol, *Gevuina avellana*, hulls.

Chilean hazelnuts (*Gevuina avellana*) are edible seeds that are native to South America. The oil is composed of 6% saturated, 6% polyunsaturated, and more than 85% monounsaturated FA, the latter showing unusual positional isomers (1). The protein content of the defatted meal is between 18 and 22%, and its composition and digestibility make it valuable for supplementing both feed and food products. *Gevuina avellana* is a promising agricultural resource in the Andean region, mainly if it is processed into oil and protein by means of simple, economic, and versatile technologies. The hulls account for almost 70% (dry basis) of the whole seed dry weight and must be removed before extraction of oil. Owing to both their lignocellulosic nature and heat of combustion, they usually have been destined for burning. The hulls are also a source of antioxidants. The antioxidant activities of extracts from *G. avellana* hulls in organic solvents (e.g., ethanol), aqueous media, and in emulsions are comparable to those of synthetic antioxidants (2).

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Ethanol extracts from these hulls are active radical scavengers and protect against both the oxidation of β-carotene in emulsion and the accelerated oxidation of soybean oil at 70°C (2). Ethanol is also a suitable solvent for the extraction of oil from either *G. avellana* full-fat seeds or from press cakes and is an alternative to hexane (3). Therefore, ethanol may be used in the same facility to extract oil from the seeds and antioxidants from the hulls.

Countercurrent extraction can be simulated on a laboratory scale by sequential batch-contacting stages (4). On this scale, four to six sequential extraction stages with enriched miscella are frequently used. The contact system must be selected according to the characteristics of the solid material. For example, percolation beds are unsuitable for extracting finely ground particles, and a solid–liquid separation stage must be performed. In contrast, with porous materials, percolation beds can be used because these materials do not compact and they do not form preferential paths or dead areas.

The goals of this work were to develop an efficient and economically viable process for extracting antioxidants from *G. avellana* hulls with ethanol and to evaluate their recovery in a multistage countercurrent extraction process. Operational conditions for extraction were selected to maximize both phenolic yields and antioxidant activities of the extracts. Cross-flow extraction and simulated countercurrent extraction in four stages were evaluated.

MATERIALS AND METHODS

Materials. Gevuina avellana seeds were obtained in January 2000 from Chilean local markets. After manual dehulling and air-drying, the hulls were ground in a coffee grinder, sieved in order to select particles smaller than 0.5 mm, and stored at 4°C until use. Moisture content was 14.9 ± 0.1%.

Solvent extraction. The hulls were extracted with hexane at room temperature in capped flasks for 6 h, using a shaker bath at 120 rpm, to remove any trace of oil and other nonpolar compounds. Ethanol extraction of these defatted hulls was performed using 96% ethanol at a liquid/solid ratio of 20 g/g (dry defatted basis). The effect of the extraction temperature was evaluated from 25 to 50°C. Solids were recovered by filtration through filter paper Whatman no. 1 and subjected further to two additional extraction steps under the same conditions.

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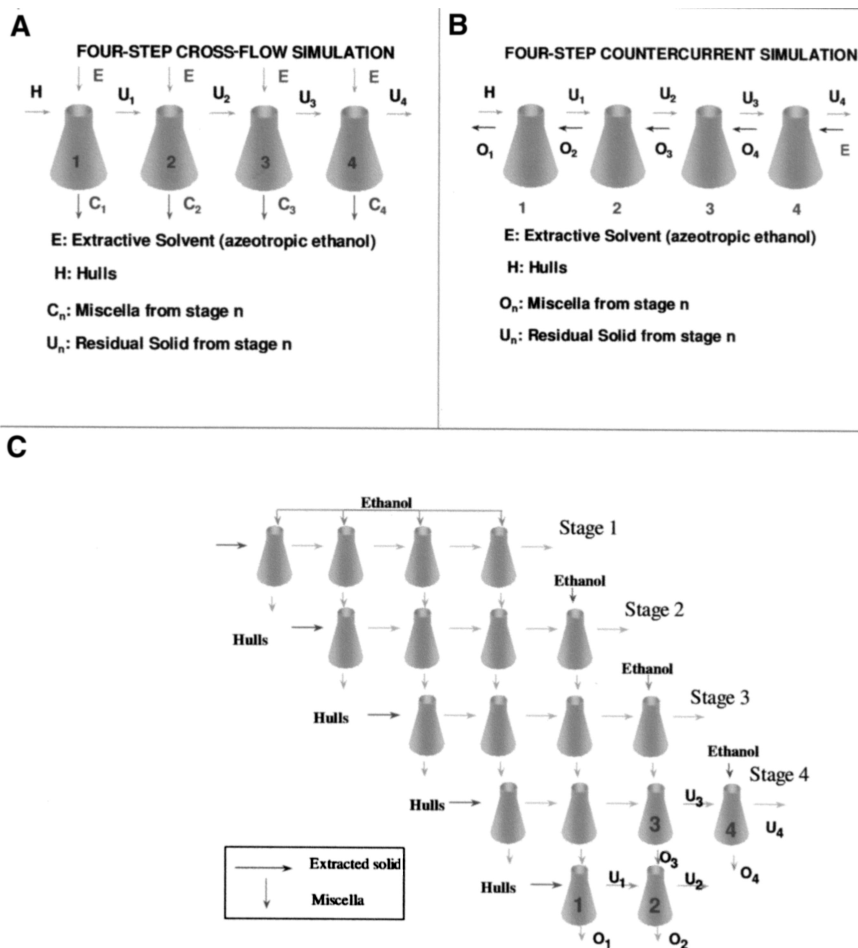

FIG. 1. Cross-flow extraction in four stages (A), simulation of countercurrent extraction in four stages (B), and scheme for simulation of countercurrent extraction of *Gevuina avellana* hulls with 96% ethanol (C).

Cross-flow extraction. Four stages of cross-flow extraction (Fig. 1A), each lasting 30 min, were carried out by using 3 g of defatted hulls, which were contacted with fresh 96% ethanol at a ratio of 20 g of solvent per gram of hulls in capped bottles. These bottles were shaken at 120 rpm in an air-heated New Brunswick G24 rotary incubator at 40°C. Each stage lasted 30 min. Solid–liquid separation was accomplished by filtration as described above, and the separated solids were contacted with 96% ethanol in three additional stages. An aliquot of the miscella separated in each stage (C_i) was analyzed for polyphenolics content and for antioxidant activity as described in the succeeding sections. The remaining miscella was vacuum-evaporated to determine the total extractables by weight.

Simulation of a multistage, continuous countercurrent extraction. A sequence of batch extractions (Figs. 1B and 1C) was carried out, according to the procedure reported by Adu-Peasah *et al.* (4). It allows one to simulate the conditions of any of the four stages in a countercurrent process. Each extraction stage consisted of a mixing stage where 2 g (dry weight basis) of ground defatted hulls and 96% ethanol, at a solvent-to-hull ratio of 20 g/g, were placed in capped bottles, which were shaken in a New Brunswick G24 rotary shaker maintained at 40°C at 120 rpm for 30 min. After this time, the phases were separated by gravity. Aliquots of the miscella from each simulated countercurrent extraction stage (O_i) were taken to determine the polyphenols content, the antioxidant activity, and the total extractables content as described in the following section.

Determination of total extractable phenolics. The amount of total extractable phenolics (TEP) was determined spectrophotometrically according to the Folin–Denis method (5), with chlorogenic acid (Sigma Chemical Co., St. Louis, MO) as standard.

Determination of the α,α*-diphenyl-*β*-picrylhydrazyl (DPPH) radical scavenging activity.* A minor modification of the method described by von Gadow *et al.* (6) was used. This involved mixing 2 mL of a 3.6×10^{-5} M solution of DPPH (Fluka, Buchs, Switzerland) in methanol with 50 µL of a methanol solution of the antioxidant. The decrease in absorbance at 515 nm was recorded in a Hitachi U-2000 spectrophotometer for 16 min. The hydrogen-donating ability of

TABLE 1
Concentrations and Yields After Every Stage During Cross-flow Extraction of *Gevuina avellana* Hulls with 96% Ethanol[a]

	Cross-flow extraction			
Stage	C_1	C_2	C_3	C_4
Extractables concentration (g/L)	5.66 ± 0.03[a]	2.16 ± 0.01[b]	1.14 ± 0.08[c]	1.05 ± 0.06[c]
Accumulated extractables yield (%)	13.32 ± 0.06[a]	18.40 ± 0.028[b]	21.07 ± 0.20[c]	23.48 ± 0.28[c]
Polyphenols (ppm as CA equivalents)	41.04 ± 0.62[a]	27.84 ± 1.34[b]	14.09 ± 0.67[c]	7.61 ± 0.79[d]
Polyphenols yield (% initial matter)·100	9.66 ± 1.5[a]	19.21 ± 4.6[b]	22.53 ± 6.2[b]	24.32 ± 8.1[b]
DPPH[b] radical scavenging activity (IP%)	86.17 ± 3.87[a]	80.82 ± 0.47[b]	56.13 ± 2.65[c]	45.74 ± 3.44[d]

[a]Different roman letter superscripts indicate that the values in the row are significantly different at a 95% confidence level. CA, chlorogenic acid; IP, inhibition percentage.
[b]DPPH (α,α-diphenyl-β-picrylhydrazyl) radical scavenging activity was directly determined in the miscella. Maximum activity $DPPH_{\infty}$ = 92.62 ± 0.26 (for the extract obtained after one extraction stage that lasted 100 h).

the crude extracts was determined as the inhibition percentage (IP) of the DPPH radical and was calculated as:

$$IP = \frac{(\text{Absorbance}_{t=0\text{ min}} - \text{Absorbance}_{t=16\text{ min}})}{\text{Absorbance}_{t=0\text{ min}}} \times 100 \qquad [1]$$

The chemical antioxidants BHA and BHT (Analema, Vigo, Spain) were also tested for antioxidant activity and used as reference standards.

NIR and UV-vis spectra. For recording of NIR spectra, the samples were placed into a standard cup and scanned with Fourier transform-NIR Bomem MB-160 spectrometer. UV-vis spectra were obtained by using a Shimadzu 160A spectrophotometer.

Statistical analysis. Cross-flow extraction was performed in triplicate. Duplication of the simulation of countercurrent extraction was achieved by performing it twice, because of the large set of contacting flasks needed. The values in Tables 1 and 2 show mean ± SD. Statgraphics Software, from Manugistics (Rockville, MD) was used to develop statistical analyses.

General linear models were established at a 95% confidence level by a Student *t*-test to determine the significance of the effects of operational variables (temperature, extraction time) on extraction yield. A one-way ANOVA was used to determine the significance of the effect of temperature on the antioxidant activity, analyzing the antioxidant activity values for each concentration separately.

A standard test for the means, considering the sample size and the SD, was done to compare values (extractables yield, polyphenolics extraction yield, DPPH inhibition, etc.) at a 95% significance level.

RESULTS AND DISCUSSION

Effect of temperature on the extraction yield. The extraction kinetics were evaluated at each temperature to determine the evolution of polyphenol concentrations at contact times compatible with an industrial process. The effect of temperature on the extraction yield was found to be more important than that of extraction time. Yield of extractables doubled when going from 25 to 45°C (Fig. 2). After 1 h, the yield increased almost linearly with extraction time for all temperatures, with the slope determined to be 0.22 and 0.38 g polyphenols/(100 g·h). On average, a 30% increase in the extraction yield was obtained when going from 1 to 7 h of extraction, although this increase was 70% for the assay at 40°C. Extraction yields were comparable with those obtained by Moure *et al*. (2).

Influence of extract concentration on antioxidant activity. Because the prooxidant action of antioxidants has been reported frequently, experiments were done to determine

TABLE 2
Concentrations and Yield Data After Every Stage During Simulation of Countercurrent Extraction of *Gevuina avellana* Hulls with 96% Ethanol

	Simulation of countercurrent extraction[a]			
Stage	O_1	O_2	O_3	O_4
Extractables concentration (g/L)	8.93 ± 0.33[a]	6.51 ± 0.24[b]	No data[b]	4.47 ± 0.13[c]
Extractables yield per stage (% initial matter)	21.02 ± 0.78[a]	15.32 ± 0.56[b]	No data[b]	10.53 ± 0.32[c]
Polyphenols (ppm as CA equivalents)	156.96 ± 15.12[a]	65.84 ± 4.78[b]	23.55 ± 3.56[c]	5.69 ± 1.24[d]
Polyphenols yield (% initial matter)·100	36 ± 3.5[a]	15 ± 1.1[b]	5.55 ± 0.84[c]	1.34 ± 0.29[d]
DPPH[c] radical scavenging activity (IP%)	94.44 ± 4.87[a]	91.29 ± 1.63[a]	56.75 ± 4.38[b]	36.36 ± 2.64[c]

[a]Different roman letter superscripts indicate that the values in the row are significantly different at a 95% confidence level.
[b]Not available, because this extract is needed for use with another contacting stage (see Fig. 2C).
[c]DPPH (α,α-diphenyl-β-picrylhydrazyl) radical scavenging activity was directly determined in the miscella. For other abbreviations see Table 1.

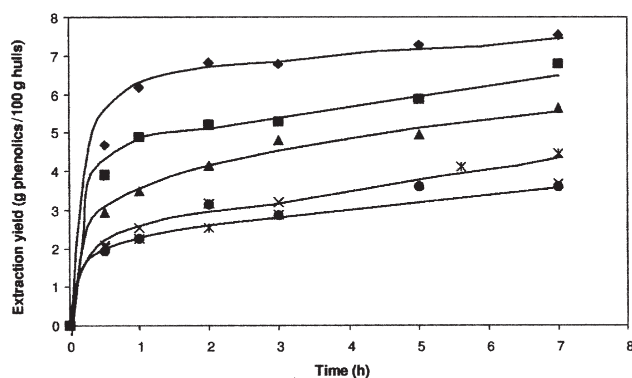

FIG. 2. Kinetics of the ethanol extraction of total phenolic compounds, calculated as grams of chlorogenic acid equivalents/100 g hulls. (●) 25°C, (×) 35°C, (▲) 40°C, (■) 45°C, (◆) 50°C.

whether the extract concentration influences antioxidant activity or if a prooxidant effect occurs (2,7). Strong antioxidants can undergo autoxidation, thereby generating reactive substances and acting as prooxidants. The polarity of the system (7–9), the presence of metals (7,8,10), and the use of either too low (8,11) or too high a concentration of antioxidants (12) can influence this behavior. The graph of DPPH radical-scavenging activity of the extracts, as a function of both the extraction time and temperature (Figs. 3A–3F) shows that temperature did not greatly affect the antioxidant activity in the ranges studied.

No significant effect of extraction time on IP after 1-h extraction, obtained by the general linear model at $P < 0.05$, was observed, which suggests that the active compounds are extracted during short extraction times. The increase of IP values with the extraction time at 40°C for 0.5 g/L (Fig. 3D) was measured as positive effect, but P was only 0.072.

ANOVA analysis showed that only the experiment at 25°C was significantly different from those at other temperatures ($P = 0.0279$ in the Fisher test) at 10 g/L, although this analysis did not pass the Cochran test: The variance at 25°C was significantly higher ($P = 0.00296$) than the variance of the values from the other temperatures. For the experiments with 2.5 g/L, temperature had no significant effect ($P = 0.5679$ for

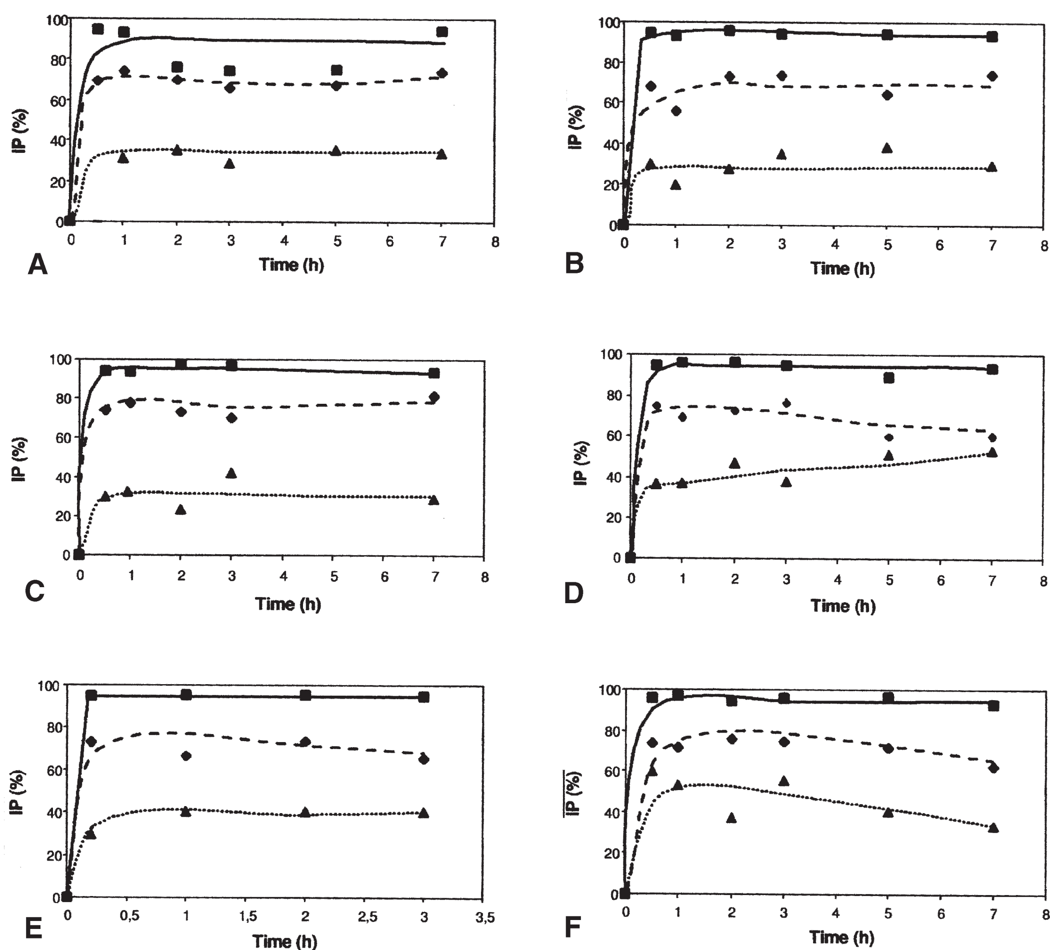

FIG. 3. Effect of temperature and extraction time on the DPPH radical scavenging activity of ethanol extracts of *Gevuina avellana* hulls (▲) 0.25 g/L, (◆) 2.5 g/L, (■) 10 g/L. (A) 25°C, (B) 30°C, (C) 35°C, (D) 40°C, (E) 45°C, and (F) 50°C; IP, inhibition percentage (see Materials and Methods section for experimental details). DPPH, α,α-diphenyl-β-picrylhydrazyl radical.

the Fisher test) on IP. For the experiments at 0.5 g/L there was a significant effect of temperature ($P = 0.001$ in the Fisher test) on antioxidant activity. The highest IP values were obtained for 40 and 50°C, but a decrease in activity with concentration was observed at 50°C, while an increase was observed at 40°C. Therefore, 40°C was chosen for further experiments.

The concentration of an antioxidant significantly influences its behavior, and when the antioxidant activity is measured as the DPPH radical scavenging capacity, saturation of the radical can occur at high concentrations (2). Significant differences among the three extract concentrations were observed (Fig. 3). The recommended extract shows a DPPH inhibition of over 80% with the shorter extraction time. The concentration of 10 g/L was so high that DPPH was saturated, thereby giving IP over 95%. From these results, extraction times longer than 1 h are not recommended.

IP values obtained from *G. avellana* hull extracts were similar to those of BHA at concentrations higher than 1 g/L. At lower concentrations (0.5 g/L) BHA had higher IP, about 50 vs. 40% for hull extracts.

Cross-flow extraction. Concentration and yield data after each cross-flow extraction stage (C_i) showed that both the total extractables and the polyphenolic compounds in the extract decreased progressively with each successive stage. The total extractables content decreased faster than did the polyphenolics between the first and second stages, whereas the opposite occurred in the stages. Polyphenolics were preferentially extracted in the earlier stages. Antioxidant activity was greater during the first two stages, but despite the reduced activity in the later stages, where concentration of polyphenolics was low, compounds with a high antioxidant power were still present.

The high variation in extractables content from the first to the second stage was probably due to the increased liquid/solid ratio, because the solids entering the second stage were already soaked with miscella and the resulting "free" solvent/solid ratio is higher than in the first stage. DPPH inhibition in the first stage was 86.2%, which was slightly lower than the 92.6% of $DPPH_\infty$ (Table 1), which took 100 h of extraction time. A longer extraction time is not expected to render a significant increase in the antioxidant activity of the extract.

Simulation of countercurrent extraction. The flow diagram of the simulated countercurrent contact in four stages and the nomenclatures for the stages and streams are shown in Figure 1B. The extractables concentration, polyphenol concentration, and yield are summarized in Table 2. Complete data from the third stage are not available, because this sample was not discarded but added to flask 2 (Fig. 1B). Small aliquots were taken to analyze the concentration of polyphenols and IP.

The extraction yield of total soluble material diminished with successive stages, but the decrease was faster for the polyphenolics (Table 2). The first stage of countercurrent extraction (O_1) was more selective with regard to polyphenolic compounds extraction, and the differences among stages were more noticeable than with cross-flow extraction. The O_1 stage had the highest antioxidant and polyphenolics contents. Stage 2 also had a high DPPH inhibition activity even with only half the polyphenolics content. This result suggests that a 3-stage countercurrent extraction can produce an extract that is better than that obtained by a one-stage batch extraction. The last stage (O_4) of countercurrent extraction (in which fresh solvent was added to previously extracted hulls) gave a significant antioxidant activity despite the marked decrease in polyphenolics.

Cross-flow vs. countercurrent extraction. Figure 4 shows the specific activity of the extracts for both cross-flow and countercurrent extraction with respect to extractable matter (A) and polyphenolics (B). In cross-flow extraction the specific activity increased as the extraction progressed, whereas this increase was not observed with countercurrent simulation. The high specific antioxidant activity of the miscella from the third and fourth stages may be explained by the presence of higher M.W. compounds that have a lower extractability but are more active. Regarding this observation, the higher antioxidant activity of polymeric phenols compared to monomeric ones has been reported for condensed tannins (13), flavanols (14), procyanidins (15), and dimers of ferulic acid (16).

During simulated countercurrent extraction, the differences in the specific activity were less than those observed during cross-flow extraction (Fig. 4). Countercurrent extraction appears to achieve a selective extraction. It is conceivable that some amount of other compounds, possibly sugars,

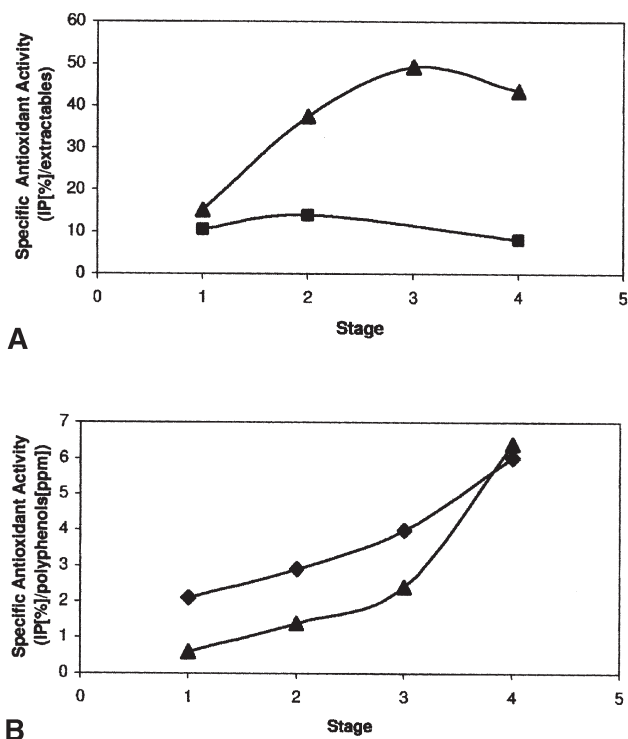

FIG. 4. Specific antioxidant activity with respect to the total extractable matter (A) and total polyphenols (B) of the extracts from the four simulated stages of countercurrent extraction (▲) and from cross-flow extraction (◆). For abbreviation see Figure 3.

were extracted during the first stages, thus giving a lower specific activity. A different profile of compounds is evident in the countercurrent process compared with the cross-flow extraction or batch process.

Different UV-vis spectra were produced by the extracts from cross-flow and countercurrent extraction (Figs. 5A,B). Absorbances were higher for the extracts from the first stages (C_1, C_2, O_1, and O_2). During cross-flow extraction, absorbance progressively declined with each stage, whereas in the simulated countercurrent extraction, the first two stages showed similar absorption maxima. A notable difference was observed between the two graphs, where O_2 showed a different profile and a higher UV absorbance than did C_2. This finding is in accordance with the high DPPH inhibition and polyphenolics content of O_2 (Table 2) as compared with those of C_2 (Table 1). On the other hand, compounds having absorption maxima at 380 nm and higher appeared in the first stage of cross-flow extraction and in the first three stages of countercurrent extraction. The first extract showed peaks between 420 and 480 nm, which could possibly be carotenoids. In cross-flow extraction this peak was considerably lower than in countercurrent extraction and almost disappeared in the second stage. Further characterization is needed, because these compounds could contribute to the antioxidant activity. The shape and the absorbance of the extracts from the fourth stage of both contacting schemes were very similar, and the antioxidant activities were also very close. The apparent similarity may have resulted from a common feature in both contacting schemes, where a solid, which was previously extracted three times, is contacted with fresh solvent.

NIR spectra (Figs. 6A,B) support the observation that the extracts from countercurrent and cross-flow are different in their chemical content. The countercurrent spectra are different between 6460 and 7230 cm^{-1}, and the spectra of the extracts from cross-flow extraction are different in the range between 6000 and 7230 cm^{-1}.

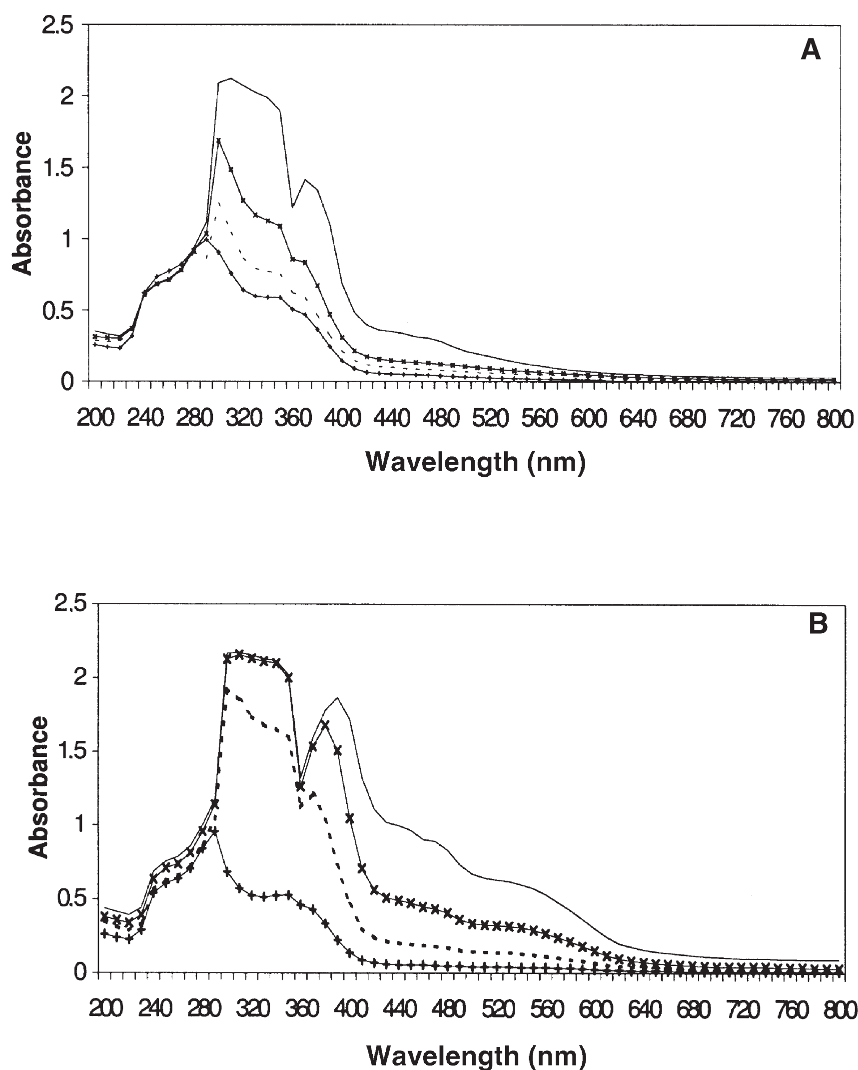

FIG. 5. UV-vis spectra of the extracts after cross-flow extraction (A) and simulation of countercurrent extraction (B). (A) C_1 (—), C_2 (X), C_3 (---), C_4 (+); (B) O_1 (—), O_2 (X), O_3 (---), O_4 (+).

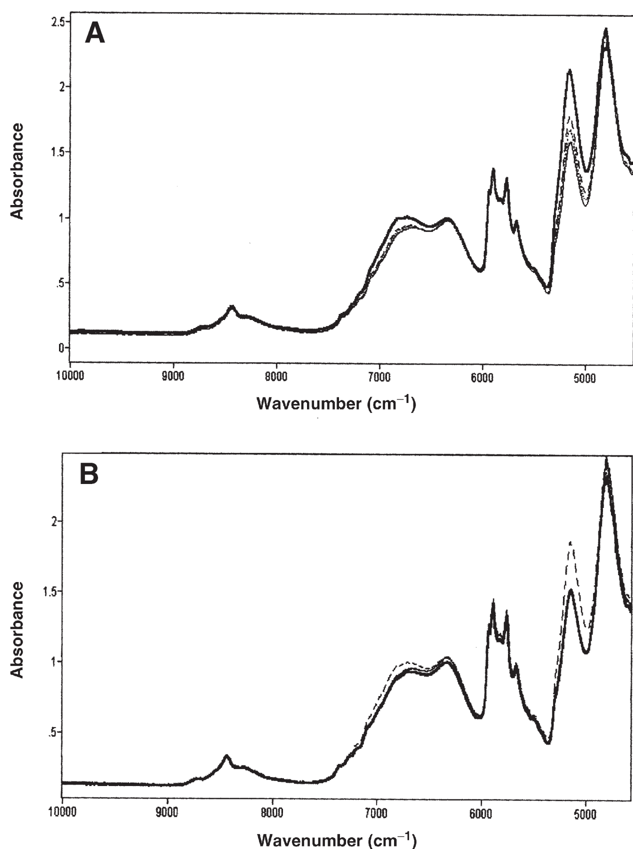

FIG. 6. NIR spectra of the extracts after cross-flow (A) and simulation of countercurrent extraction (B) experiments. —, Stage 1; - - -, stage 2; ......, stage 3; – – –, stage 4.

In countercurrent extraction, the absorbance in the 6460–7000 cm^{-1} decreased from stage O_1 to stage O_4, as would be expected, because this range coincided with the decrease in total polyphenolics content and extractables content. This decrease was reproduced in the 5160 cm^{-1} peak. In contrast, the absorbance of the extract from stage 1 was lower than that from further stages. From stages C_2 to C_4 the absorbance decreased as in countercurrent extraction. This suggests that hydroxyl content in the extract from countercurrent increased from stage 1 to 2, and then decreased until the end.

Although each of the countercurrent contact stages lasted the same time as that of the corresponding stage in cross-flow extraction, the countercurrent stage was the result of some prior contact stages, not with fresh ethanol but an extract from another stage. As a hypothesis, the differences in the polyphenolics profile obtained with different contact times in batch experiments are in this case attenuated because of prior contacting stages, and each stage shows a more regular polyphenolics profile. These differences were parallel to those observed when calculating the specific antioxidant activity during cross-flow and countercurrent extraction (Fig. 6). They can be explained as the existence of a selective extraction with time: In the first stage of a batch process, less hydroxylated polyphenols and possibly other nonpolyphenol compounds were extracted, e.g., carotenoids.

Our findings demonstrated that *G. avellana* hulls are a good source of antioxidants, a great part of which are polyphenolic in nature. The antioxidant power of the extracts was comparable to that of synthetic BHA, as indicated by the DPPH inhibition method, when used at 1 g/L concentration. The temperature optimum for extraction was 40°C. The four-stage simulated countercurrent extraction process showed the highest performance with regard to antioxidant activity. The process can produce an extract with antioxidant activity greater than that of cross-flow extraction, even with only three stages, which means a considerable savings in solvent quantities and cost.

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