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Determination of free and bound phenolic acids in beer

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Abstract

The aim of this study was the qualitative and quantitative determination of free and bound phenolic compounds, mainly phenolic acids, in beer. In spite of the wide literature describing the content of free phenolic acids in beer, data concerning its content of bound forms are scarce or missing. Moreover, the experimental conditions commonly reported in the literature to detect bound phenolic acids by alkaline hydrolysis result in loss of several phenolic acids, particularly dihydroxy-derivatives. Recently, we described that the addition of ascorbic acid, a strong antioxidant, and ethylenediaminetetraacetic acid, a metal chelator, totally prevents the loss of phenolic acids during alkaline hydrolysis. On this basis, we developed a hydrolytic procedure based on alkaline hydrolysis with 2 N NaOH containing ascorbic acid (1% w/v) and ethylenediaminetetraacetic acid (10 mM). In these conditions, a complete recovery of caffeic acid (98.7±4.3% of expected value) following hydrolysis of chlorogenic acid (5′-caffeoylquinic acid, an ester of caffeic acid with quinic acid) was obtained. In the present study we took advantage of this hydrolytic procedure to quantitatively measure free and total (free plus bound) phenolic acids in beer. After alkaline hydrolysis, which released bound phenolic acids, a remarkable increase in the content of 4-hydroxyphenylacetic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and sinapic acid was observed in beer from three different brands. Our results show that the most of phenolic acids in beer are present as bound forms and only a small portion can be detected as free compounds.

Keywords: Beer; Free phenolic acids; Bound phenolic acids; Alkaline hydrolysis; HPLC–ECD

1. Introduction

Dietary plant phenolic compounds have been described to exert a variety of biological actions, such as free radical scavenging, metal chelation, modulation of enzymatic activity (Editorial, 1994; Koshihara, Neichi, Murota, Lao, Fujimoto, & Tatsumo, 1984; Reddy & Aggarwal, 1994; Sud'ina, Mirzoeva, Pushkareva, Korshunova, Sumbatyan, & Varfolomeev, 1993) and, more recently, to affect signal transduction, activation of transcription factors and gene expression (Bito, Roy, Sen, & Packer, 2000; Natarajan, Singh, Burke, Grunberger, & Aggarwal, 1996; Singh & Aggarwal, 1995; Ursini, Maiorino, Morazzoni, Roveri, & Pifferi, 1994; Yoshioka, Deng, Cavigelli, & Karin, 1995). They received particular attention in the past 10 years due to their putative role in the prevention of several human diseases, particularly atherosclerosis and cancer.

The total polyphenols intake has been reported to be in the order of 1 g/day (Scalbert & Williamson, 2000). For individuals regularly consuming wine, coffee, beer and tea, these beverages will likely be the major sources of phenolic compounds, mainly cinnamates. The free forms of phenolic compounds are very rarely present in plants. More often, they occur as esters, glycosides and bound complexes. Despite extensive literature describing the content of the free forms of phenolic acids of these beverages (Achilli, Cellerino, & Gamache, 1993; Gorinstein, Caspi, Zemser, & Trakhtenberg, 2000; Hayes, Smyth, & Mc Murrough, 1987; Shahidi & Naczk, 1995), our knowledge about their content of bound forms is scarce or missing. One major reason probably consists in the lack of suitable hydrolysing procedures to quantitatively detect bound phenolic acids. Most of the procedures described in the literature to detect bound phenolic acids in food take advantage of alkaline hydrolysis to release bound forms of phenolic acids (Fenton, Leung, & Clandinin, 1980; Kozlowska, Rotkiewicz, & Zadernowski, 1983; Krygier, Sosulski, & Hogge, 1982; Maillard & Berset, 1995).

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However, the experimental conditions commonly used result in loss of several phenolic compounds, particularly dihydroxy-derivatives of phenolic acids (Krygier et al., 1982; Maillard & Berset, 1995). For caffeic acid and sinapic acid, the loss has been estimated to be 67 and 36% of initial values, respectively (Krygier et al., 1982). When exposed to acidic hydrolysis, the loss of phenolic acids derivatives has been described to be even more dramatic (Krygier et al., 1982). Recently, we described that the addition of ascorbic acid, a powerful antioxidant, and ethylenediaminetetraacetic acid (EDTA), a metal chelator, completely prevents the degradation of phenolic acids during alkaline hydrolysis (Nardini, Cirillo, Natella, Mencarelli, Comisso, & Scaccini, 2002a). On this basis, a procedure has been developed to quantitatively detect total phenolic acids in food. This procedure has been successfully applied to measure total phenolic acids in coffee brew (Nardini, Cirillo, Natella, & Scaccini, 2002b) and apple (Nardini et al., 2002a). In the present study we took advantage of this procedure to qualitatively and quantitatively measure the content of free (nonhydrolysed) and total (free plus bound, hydrolysed) phenolic acids in beer.

2. Materials and methods

2.1. Materials

Ascorbic acid, EDTA, caffeic acid, chlorogenic acid (5′-caffeoylquinic acid), gallic acid, protocatechuic acid, vanillic acid, ferulic acid, *p*-coumaric acid, syringic acid and sinapic acid were from Sigma (St. Louis, MO, USA). *o*-Coumaric acid, 4-hydroxyphenylacetic acid and isoferulic acid were from Extrasynthese (Genay Cedex, France). 4-Hydroxybenzoic acid was from Fluka (Buchs, Switzerland). Stock solutions were prepared in methanol (1 mg/ml), stored at −80 °C and used within 2 weeks. Working standard solutions were prepared daily by dilution in sample buffer (1.25% glacial acetic acid, 7% methanol in water).

All organic solvents were obtained from Carlo Erba (Milano, Italy). For HPLC analysis, ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA) was used.

2.2. HPLC instrumentation

The HPLC consists of a Perkin-Elmer Series 4 Liquid Chromatograph (Perkin-Elmer Norwalk, CT, USA) with a gradient pump, column thermoregulator and autosampling injector (Gilson, Beltline, Middleton, WI, USA) equipped with an electrochemical coulometric detector (Coulochem II, ESA, Bedford, MA, USA). Turbochrom chromatography work station software was used for data processing. Operating conditions were as follows: column temperature, 30 °C; flow rate: 1 ml/min; injection volume, 50 μl; electrochemical detection at +600 mV, sensitivity range 200 nA, filter 2 s.

Chromatographic separations were performed on a Supelcosil LC-18 C_{18} column (5.0 μm particle size, 250×4.6 mm i.d.) including a guard column (C_{18}, 5.0 μm particle size, 20×4.0 mm i.d.; both Supelco, Bellefonte, PA, USA). For gradient elution mobile phase A and B were employed. Solution A contained 1.25% glacial acetic acid in water; solution B was absolute methanol. The following gradient was used: 0–25 min, from 98% A, 2% B to 76% A, 24% B, linear gradient; 26–45 min, 76% A, 24% B; 46–53 min, from 76% A, 24% B to 55% A, 45% B, linear gradient; 54–55 min, 55% A, 45% B; 56–86 min, 98% A, 2% B. Prior to HPLC analysis, all samples were filtered using Millex-HV filters (Millipore, Bedford, MA, USA) with 0.45-μm pore size.

2.3. Hydrolytic procedure and extraction

The hydrolytic procedure was performed essentially as previously described (Nardini et al., 2002a, 2002b). Briefly, phenolic compounds (0.5 mg in 0.5 ml) were subjected separately to alkaline hydrolysis with 4.5 ml 2 N NaOH containing ascorbic acid (1% w/v) and EDTA (10 mM) at 30 °C for 30 min. Isoferulic acid (0.2 mg) was added as internal standard before hydrolysis. Blanks were run in the same conditions, replacing NaOH solution with water. As previously reported, this hydrolytic procedure allows the total recovery of ferulic acid and caffeic acid liberated upon hydrolysis from ferulic acid methylester and chlorogenic acid, respectively (Nardini et al., 2002a). In a parallel set of experiments, hydrolysis was performed in the same experimental conditions above reported but in the absence of ascorbic acid and EDTA.

Extraction of phenolic compounds was carried out essentially as previously described (Nardini et al., 2002a, 2002b). At the end of incubation, 0.5 ml solution were brought to pH 3.0 using 4 N HCl and added with 300 mg NaCl. Samples were extracted three times with ethyl acetate (×4 vols) by vortexing for 5 min. After each extraction, samples were centrifuged (3000×*g*, 10 min) and supernatants collected. The organic phase was dried under nitrogen flow. The residue was dissolved in a final volume of 0.5 ml methanol and vortexed for 5 min, then diluted with sample buffer (1.25% glacial acetic acid, 7% methanol in water) prior to HPLC–ECD analysis. The recovery of single phenolic acids using the above reported extraction procedure was found to range from 92.1 to 107.0% of expected value (Nardini et al., 2002a).

2.4. Samples preparation and treatment

Beers from three different commercial brands were used in this study. Beer samples were degassed by sonication

and treated for free (nonhydrolysed samples) and total (hydrolysed samples) phenolic compounds determination as follows.

Nonhydrolysed beer samples. Beer (0.5 ml) was added with 1 μg isoferulic acid as internal standard and acidified with 1 N HCl to pH 3.0. After addition of 300 mg NaCl, samples were extracted three times as reported earlier. The final residue was dissolved in a final volume of 0.1 ml methanol and vortexed for 5 min; then 0.4 ml of sample buffer was added, followed by 5 min vortexing. Samples were centrifuged at 17 500×*g* for 5 min and filtered prior to HPLC–ECD analysis. After appropriate dilution, 50 μl sample were analysed. Recovery experiments were performed by adding known amounts (2.5–50 μg) of pure phenolic compounds (protocatechuic, chlorogenic, vanillic, caffeic, siringic, *p*-coumaric, ferulic, sinapic, *o*-coumaric, 4-hydroxyphenylacetic acids, as representative of beer phenolics) to beer sample.

Hydrolysed beer samples. Beer (0.5 ml) was subjected to alkaline hydrolysis with 4.5 ml 2 N NaOH containing 10 mM EDTA and 1% ascorbic acid at 30 °C for 30 min, in the presence of 1 μg isoferulic acid as internal standard. At the end of incubation, 0.5 ml sample were acidified to pH 3.0 with 4 N HCl, added with 300 mg NaCl and treated for extraction as reported above. The final residue was dissolved in 0.1 ml methanol, vortexed for 5 min, and 0.4 ml of sample buffer were added, followed by 5 min vortexing. Samples were centrifuged at 17 500×*g* for 5 min and filtered prior to HPLC–ECD analysis. Fifty microlitres sample were analysed as such or after appropriate dilution. Recovery experiments were performed by adding known amounts (2.5–50 μg) of pure compounds (protocatechuic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, *o*-coumaric, 4-hydroxyphenylacetic acids, as representative of beer phenolics) to beer sample. Moreover, to validate the hydrolytic procedure, known amounts of pure chlorogenic acid were added to beer sample and caffeic acid liberated upon hydrolysis was measured.

2.5. Quantitation and statistical analysis

For calibration curves, appropriate volumes of the stock solutions were diluted with running buffer. Three replicates of standards at four concentration levels (20, 100, 200 and 500 ng/ml) were analysed. Calibration curve was determined on each day of analysis. For quantitative determination, peak areas in the sample chromatograms were correlated with the concentrations according to the calibration curve.

Data presented are mean ± standard deviation. Statistical analysis was performed using a one-factor analysis of variance (ANOVA, Scheffe's method) for multiple comparison. Probability of $P < 0.05$ was considered statistically significant.

3. Results and discussion

Table 1 shows the recovery of several common phenolic compounds subjected to alkaline hydrolysis. Most of these compounds have been selected on the basis of their previously reported presence in beer (Achilli et al., 1993; Gorinstein et al., 2000; Hayes et al., 1987; Shahidi & Naczk, 1995). In the experimental conditions commonly used in the literature, without addition of antioxidants (Fenton et al., 1980; Kozlowska et al., 1983; Krygier et al., 1982; Maillard & Berset, 1995), caffeic acid, protocatechuic acid dramatically decreased, while gallic acid totally disappeared. The presence of ascorbic acid and EDTA during alkaline hydrolysis completely prevented the loss of gallic acid, protocathecuic acid and caffeic acid, which is probably due to spontaneous oxidation (Cilliers & Singleton, 1989–1991) and allows a quantitative recovery of all the phenolic compounds tested.

On the basis of these results, the content of free (nonhydrolysed beer) and total (free plus bound, hydrolysed beer) phenolic acids was measured in three different brands of beer. The alkaline hydrolysis procedure in the presence of EDTA-ascorbate has been used to detect total phenolic acids. Fig. 1A shows the chromatographic profile obtained in our experimental conditions of a standard mixture of phenolic compounds. Typical chromatograms obtained from nonhydrolysed and hydrolysed beer samples are shown in Fig. 1B and C, respectively. Single phenolic compounds were identified

Table 1
Stability of phenolic compounds during alkaline hydrolytic treatment[a]

	NaOH (% residual)	NaOH + EDTA + ascorbic acid (% residual)
Gallic acid	0*	111.7±1.1
Protocatechuic acid	53.2±4.0*	97.6±4.8
p-Hydroxy-benzoic acid	108.9±11.6	103.8±7.6
4-Hydroxyphenylacetic acid	89.8±10.4	106.6±8.2
Vanillic acid[b]	104.2±2.0	101.0±1.2
Caffeic acid[b]	19.7±3.4*	106.6±4.8
Syringic acid[b]	101.7±3.6	101.6±3.7
p-Coumaric acid[b]	101.1±1.8	103.9±9.8
Ferulic acid[b]	106.5±7.0	103.2±1.0
Sinapic acid[b]	92.1±3.6*	110.7±12.2
Isoferulic acid[b]	94.6±6.2	97.5±0.9
o-Coumaric acid[b]	100.1±11.6	97.3±5.0

[a] Phenolic compounds were subjected to hydrolytic treatment as reported in Method section. Values are means ± S.D. ($n = 3$).

[b] Nardini et al. (2002a).

* Values within a row are significantly different ($P \leq 0.05$) from blanks without NaOH (100%).

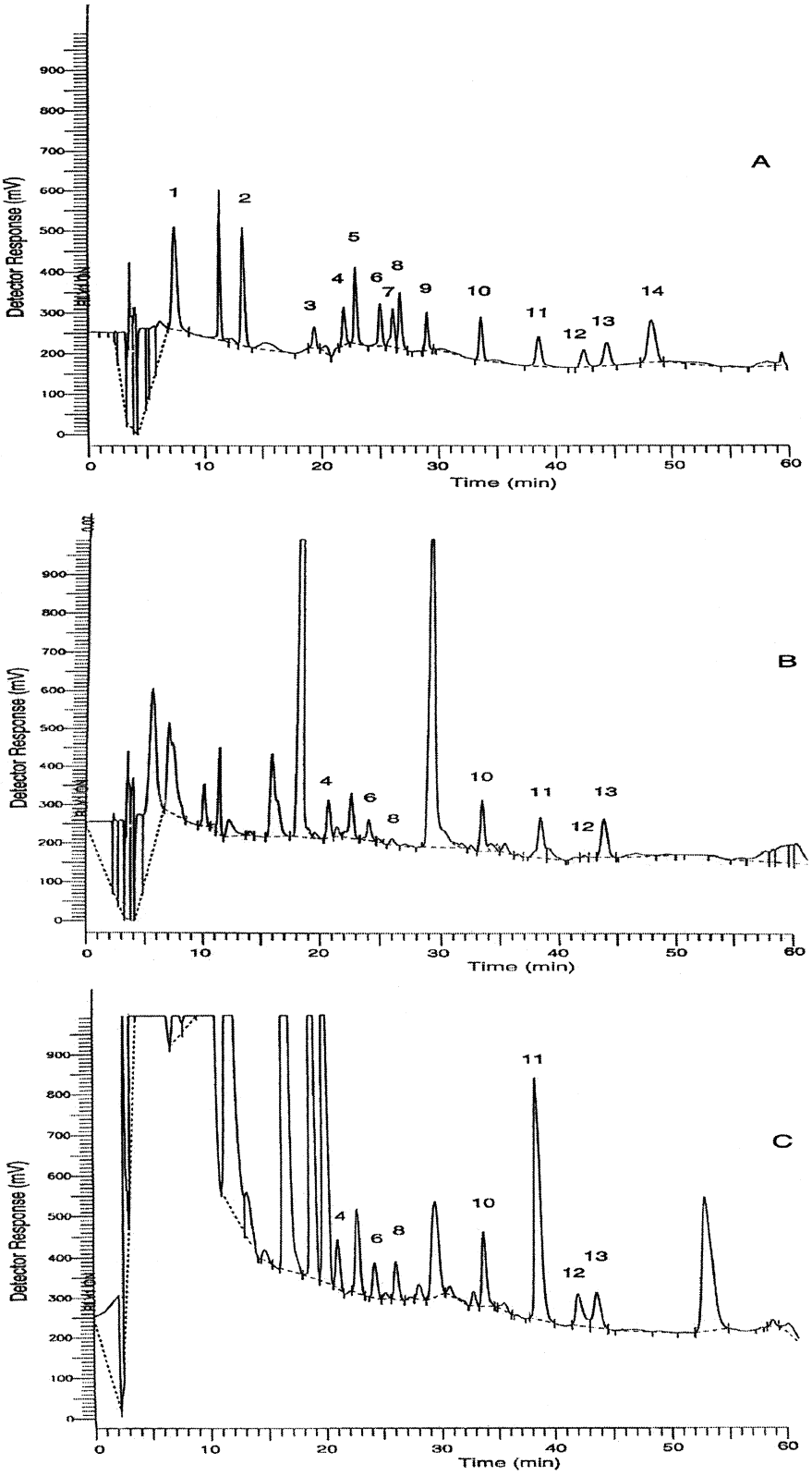

Fig. 1. High-performance liquid chromatograms of (A) standard mixture of phenolic compounds; (B) diluted nonhydrolysed beer sample; (C) hydrolysed beer sample. Chromatographic separations are obtained on a Supelcosil LC-18 C_{18} column (5.0 μm particle size, 250×4.6 mm i.d.) using a binary gradient (mobile phase A, 1.25% glacial acetic acid in water; mobile phase B, absolute methanol). The operating conditions are: flow rate, 1 ml/min; column temperature, 30 °C; detector potential, +600 mV; sensitivity range, 200 nA. Peaks identification: 1, gallic acid; 2, protocatechuic acid; 3, 4-hydroxybenzoic acid; 4, 4-hydroxyphenylacetic acid; 5, catechin; 6, vanillic acid; 7, chlorogenic acid; 8, caffeic acid; 9, syringic acid; 10, *p*-coumaric acid; 11, ferulic acid; 12, sinapic acid; 13, isoferulic acid; 14, *o*-coumaric acid.

by retention time and coelution with standard compounds. The amount of vanillic, caffeic, ferulic and sinapic acid (peaks 6, 8, 11 and 12, respectively) was dramatically increased in hydrolysed samples in respect to nonhydrolysed samples, taking into account that the amount of the injected sample corresponded to 0.005 ml of original beer for both samples. A moderate increase of 4-hydroxyphenylacetic acid and *p*-coumaric acid after hydrolysis was also observed (peaks 4 and 10, respectively). Table 2 summarizes the results obtained from three different brands of beer. The content of free phenolic compounds measured in our nonhydrolysed beer is in agreement with data available in the literature (Achilli et al., 1993; Gorinstein et al., 2000; Hayes et al., 1987; Shahidi & Naczk, 1995). Chlorogenic acid and syringic acid are present in traces in Italian beer and at low levels in Austrian and German beers. The content of caffeic acid is quite similar in all the beers tested, while the content of the remaining phenolic compounds is somewhat different in the three beers tested. Upon alkaline hydrolysis, significant amount of phenolic acids were released in all the beers tested. The content of 4-hydroxyphenylacetic acid and *p*-coumaric acid increased moderately (the increase is $\leqslant 50\%$ of the value measured in nonhydrolysed samples) after hydrolysis in all beers, except German beer, which showed 469.1% increase in *p*-coumaric content after hydrolysis. Considerable increases ($\geqslant 100\%$ of the value measured in nonhydrolysed samples) were observed for vanillic acid, caffeic acid, syringic acid, ferulic acid and sinapic acid. Again, caffeic acid content measured after hydrolysis is quite similar in all beers tested.

Method performance was validated by recovery experiments, adding known amounts of pure phenolic acids to beer samples. Recovery was found to be in the range of 95.4–104.0% and 94.0–102.9% in nonhydrolysed beer samples and hydrolysed beer samples respectively. Moreover the addition of known amount of chlorogenic acid to beer resulted in total recovery of caffeic acid released upon hydrolysis (98.7±4.3% of the expected value).

In conclusion, our results demonstrated that the most of phenolic acids in beer are present in bound forms.

Conjugated forms of phenolic compounds, although being widely present and consumed in a Western diet, have been supposed not to be bioavailable in humans.

Recently, esterases with the ability to hydrolyse hydroxycinnamate esters at appreciable rates have been described in humans and rats (Andreason, Kroon, Williamson, & Garcia-Conesa, 2001a). The cynnamoyl esterase activity is distributed all along the small and large intestine and is present both in the mucosa cells and in the lumen. Further, bacteria in the gastrointestinal tract of mammals are also capable of releasing free phenolic acids from bound complexes into the gastrointestinal tract (Andreason, Kroon, Williamson, &

Table 2
Phenolic compounds content (mg/l) of beer before and after hydrolysis[a]

	Italian beer			Austrian beer			German beer		
	No hydrolysis	Plus hydrolysis	Increase%[b]	No hydrolysis	Plus hydrolysis	Increase%[b]	No hydrolysis	Plus hydrolysis	Increase%[b]
Protocatheceuic acid	tr[c]	tr[c]	–	tr[c]	tr[c]	–	tr[c]	tr[c]	–
4-OH-phenylacetic acid	0.61±0.04	0.81±0.10[d]	33.5	0.60±0.02	0.72±0.04[d]	19.8	0.27±0.02	0.40±0.03[d]	45.2
Chlorogenic acid	tr[c]	–	–	0.21±0.03	–	–	0.26±0.02	–	–
Vanillic acid	0.59±0.07	1.17±0.17[d]	98.9	0.85±0.01	2.10±0.13[d]	146.5	0.80±0.02	1.17±0.09[d]	47.1
Caffeic acid	0.20±0.03	1.16±0.10[d]	470.2	0.20±0.02	1.21±0.11[d]	508.7	0.15±0.01	1.09±0.05[d]	644.0
Syringic acid	tr[c]	tr[c]		0.23±0.01	0.46±0.01[d]	102.3	0.17±0.02	0.43±0.02[d]	155.8
p-coumaric acid	0.72±0.03	1.09±0.13[d]	51.1	0.76±0.04	0.91±0.04[d]	18.7	0.34±0.02	1.96±0.08[d]	469.1
Ferulic acid	2.31±0.03	13.88±0.87[d]	499.7	1.89±0.13	12.64±0.42[d]	567.7	1.36±0.07	16.75±0.30[d]	1131.6
Sinapic acid	0.20±0.02	3.00±0.34[d]	1381.7	0.84±0.06	6.16±0.10[d]	633.2	0.54±0.03	5.05±0.06[d]	826.5

[a] Phenolic acids content was measured in nonhydrolyzed samples and in samples submitted to alkaline hydrolysis as reported in Section 2. Values are means±S.D. ($n = 3$).
[b] Increase is expressed as% of the value measured in nonhydrolysed samples.
[c] tr, traces (<0.1 mg/l).
[d] Values are significantly different from those measured in nonhydrolysed samples ($P < 0.05$ by ANOVA).

Garcia-Conesa, 2001b; Buchanan, Wallace, & Fry, 1996; Couteau, McCartney, Gibson, Williamson, & Faulds, 2001; Kroon Faulds, Ryden, Robertson, & Williamson, 1997; Plumb, Garcia-Conesa, Kroon, Rhodes, Saxon, & Williamson, 1999; Wende, Buchanan, & Fry, 1997). Moreover, recent absorption studies suggest that bound forms of phenolic acids, are still bioavailable to humans and rats (Andreason et al., 2001b; Azuma, Ippoushi, Nakayama, Ito, Higashio, & Terao, 2000; Bourne & Rice-Evans, 1998; Camarasa, Escubedo, & Adzet, 1988; Nardini et al., 2002b). On these bases, to properly evaluate absorption processes in vivo, the qualitative and quantitative determination of total (free plus bound) phenolic acids in food should be recommended.

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