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Detection of bound phenolic acids: prevention by ascorbic acid and ethylenediaminetetraacetic acid of degradation of phenolic acids during alkaline hydrolysis

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Abstract

The experimental conditions commonly used to detect bound phenolic acids by alkaline hydrolysis result in loss of several phenolic acids, particularly dihydroxy-derivatives (caffeic acid, dihydrocaffeic acid, homogentisic acid). In this study we show that the addition of ascorbic acid, a strong antioxidant, and ethylenediaminetetraacetic acid, a metal chelator, totally prevent the loss of phenolic acids during alkaline hydrolysis. In these conditions, a complete recovery of caffeic acid following hydrolysis of chlorogenic acid (5′-caffeoylquinic acid, an ester of caffeic acid with quinic acid) was found. This procedure has been successfully applied to quantitatively detect bound phenolic acids in coffee brew and apple.

Keywords: Hydrolysis; Bound phenolic acids; Coffee; Apple; Chlorogenic acid; Caffeic acid

1. Introduction

Dietary plant phenolic compounds received particular attention in the last 10 years due to their powerful antioxidant activity and their putative role in the prevention of several human pathologies, particularly atherosclerosis and cancer.

Among the several thousand phenolic compounds described in plants, phenolic acids derivatives are of particular interest for their abundance and diversity.

The free forms of phenolic compounds are very rarely present in plants. More often, they occur as esters, glycosides and bound complexes. For this reason, several hydrolytic procedures have been described to quantify total phenolics. Most of these procedures are based on alkaline hydrolysis with NaOH (ranging from 2 to 4 N) using incubation times up to 6 h, sometimes under nitrogen (Fenton, Leung, & Clandinin, 1980; Kozlowska, Rotkiewicz, & Zadernowski, 1983; Krygier, Sosulski, & Hogge, 1982; Maillard & Berset, 1995). However, loss of phenolic acids during alkaline hydrolysis have been observed (Krygier et al., 1982; Maillard & Berset, 1995). For a range of hydroxycinnamic acids the loss has been estimated not to exceed 10% of initial values (*o*-coumaric, *p*-coumaric, isoferulic, ferulic acids; 4 N NaOH, 4 h under nitrogen) (Krygier et al., 1982). In the same experimental conditions the loss of caffeic acid and sinapic acid was 67% and 36.5% respectively (Krygier et al., 1982). When exposed to acidic hydrolysis, the losses for hydroxycinnamic acid derivatives has been described to be even more dramatic (Krygier et al., 1982).

This study reports the effect of ascorbate, a strong antioxidant, and ethylenediaminetetraacetic acid (EDTA), a metal chelator, in preventing degradation of phenolic acids during alkaline hydrolysis.

2. Materials and methods

2.1. Materials

Ethylenediaminetetraacetic acid, ascorbic, caffeic, chlorogenic (5′-caffeoylquinic acid), ferulic, dihydroferulic, *p*-coumaric, syringic, vanillic, homovanillic, homogentisic, sinapic and 3-(*p*-hydroxy-phenyl)-propionic acids were from Sigma (St. Louis, MO, USA). *o*-Coumaric and isoferulic acids were from Extrasynthese

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(Genay Cedex, France). Ferulic acid methylester was from Indofine Chemical Company (ICC, Belle Mead, NJ, USA). Dihydrocaffeic acid was from Avocado (Heysham, Lancashire, England). Stock solutions were prepared in MeOH (1 mg/ml), stored at −80 °C and used within 2 weeks. Working standard solutions were prepared daily by dilution in running buffer (1.25% glacial acetic acid, 7% MeOH in water).

All organic solvents (methanol, HPLC grade (MeOH); ethyl acetate) 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 gradient pump, column thermoregulator, autosampling injector (Gilson, Beltline, Middleton, WI, USA) equipped with electrochemical coulometric detector (Coulochem II, ESA, Bedford, MA, USA). A Turbochrom cromatography 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 ID) including a guard column (C_{18}, 5.0 μm particle size, 20×4.0 mm ID; 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 93% A, 7% 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, 93% A, 7% 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. Samples

Coffee brew was prepared using a commercial automatic brewing machine (60 g of roasted and ground coffee from an Italian brand per litre water) and used within 10 min after preparation. Fresh apples (Classic Delicious) were obtained from local market.

2.4. Hydrolytic procedure and extraction

2.4.1. Hydrolytic procedure

The hydrolytic procedure was performed essentially according to Fenton et al. (1980), except that incubation was performed for 30 min at 30 °C instead of 6 h at RT. Briefly, phenolic compounds (0.5 mg in 0.5 ml) were subjected separately to alkaline hydrolysis with 4.5 ml 2 N NaOH at 30 °C for 30 min, unless otherwise specified. Isoferulic acid (0.2 mg) was added as internal standard before hydrolysis. Blanks were run in the same conditions in 5 ml water. The validation of the above reported hydrolytic treatment was tested using ferulic acid methylester as substrate for hydrolysis. After 30 min incubation at 30 °C, the substrate ferulic acid methylester was completely disappeared, while 96.9±3.7% of expected ferulic acid liberated upon hydrolysis was found. Similar results were obtained for longer incubation times (up to 4 h). On the basis of these results, an incubation time of 30 min at 30 °C was chosen for our experiments. To study the effect of ascorbic acid and EDTA on phenolic acids degradation, in another set of experiments hydrolysis was perfomed in the same experimental conditions above reported, but in the presence of 1% ascorbic acid and 10 mM EDTA.

2.4.2. Extraction

Extraction of phenolic acids was carried out essentially as previously described (Woodring, Edwards, & Chisholm, 1990). At the end of the incubation, 0.5 ml solution were acidified 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 MeOH and vortexed for 5 min, then diluted with running buffer (1.25% glacial acetic acid, 7% MeOH 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 (mean 96.5±5.8).

2.5. Treatment of coffee brew samples

2.5.1. Non-hydrolysed coffee brew samples

Coffee brew (0.5 ml) was added to 0.1 mg 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 (Section 2.4). The final residue was dissolved in 5 ml MeOH, vortexed for 5 min, then diluted 1:100 with running buffer prior to HPLC-ECD analysis. Recovery experiments were performed by adding known amounts of pure chlorogenic acid and caffeic acid, as representative coffee phenolic acids, to coffee brew. Recovery was found to be 97.2±3.1% and 98.5±4.4% of expected value for chlorogenic acid and caffeic acid respectively.

2.5.2. Hydrolysed coffee brew samples

Coffee brew (0.5 ml) was subjected to alkaline hydrolysis with 4.5 ml 2 N NaOH containing 10 mM EDTA,

1% ascorbic acid at 30 °C for 30 min, in the presence of 0.1 mg isoferulic acid as internal standard. At the end of incubation, samples were treated as reported earlier (Section 2.4). The final residue was dissolved in 0.5 ml MeOH, vortexed for 5 min, then diluted 1:100 or 1:1500 as specified with running buffer prior to HPLC-ECD analysis. In these conditions, alkaline hydrolysis of known amount of pure chlorogenic acid resulted in complete recovery of liberated caffeic acid (97.0±2.8% of expected value). Moreover, addition of known amount of pure chlorogenic acid to coffee brew resulted in total recovery of liberated caffeic acid upon hydrolysis (95.8±3.5% of expected value).

2.6. Treatment of apple samples

Apples were peeled and sliced with plastic knife and homogenized in potter with PTFE pestle with ice-cold Na-acetate buffer pH 5.0, 5 mM containing 1% ascorbic acid and 10 mM EDTA for 90 s. In a parallel experiment, ascorbic acid and EDTA were omitted. 0.5 ml aliquots were added with isoferulic acid as internal standard and treated for alkaline hydrolysis in the presence of ascorbic acid and EDTA as reported above for coffee brew samples (Section 2.5).

2.7. Quantitation and statistical analysis

For calibration curve, 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.

All data presented are mean ± standard deviation of at least three independent experiments ($n \geqslant 3$). Statistical analysis was performed using a one-factor analysis of variance (ANOVA) and Scheffe's method for multiple comparison. Probability of $P < 0.05$ was considered statistically significant.

3. Results and discussion

Caffeic acid derivatives (chlorogenic acids, a family of esters formed between certain cinnamic acids and quinic acid, and caftaric acid) are found at relatively high concentrations as normal metabolites in many plant-derived foods. For this reason, in the first phase of the study we used caffeic acid and chlorogenic acid (5′-caffeoylquinic acid, the only one chlorogenic acid isomer available commercially) as model compounds.

Fig. 1 shows the time course of caffeic acid degradation during alkaline hydrolysis. A fast and dramatic degradation of caffeic acid occurred in these conditions, with only 60.9±2.3% and 22.9±5.5% of initial caffeic acid recovered after 15 and 30 min incubation, respectively. Similar results were obtained when hydrolysis was driven under nitrogen flow (60.7±3.9% and 26.1±2.1% at 15 and 30 min incubation respectively). In these experimental conditions, hydrolysis of chlorogenic acid, which is complete after 30 min incubation at 30 °C, gave only 22.0±11.5% of expected caffeic acid.

Caffeic acid has been already reported to undergo spontaneous oxidation, particularly at alkaline pH (Cilliers & Singleton, 1989, 1991). Ascorbic acid has been described to prevent caffeic acid oxidation at pH 7.0 (Cilliers & Singleton, 1990) and to regenerate caffeic acid from phenoxyl radical (Laranjinha, 2001; Laranjinha & Cadenas, 1999), while cupric ions have been reported to catalyse the oxidation of caffeic acid at neutral pH (Nardini et al., 1995). Therefore we investigated the effects of ascorbic acid and EDTA on caffeic acid stability during alkaline hydrolysis. As shown in Table 1, the presence of EDTA (10 mM) resulted in higher caffeic acid recovery (55.4±6.0% of initial value) in respect to standard alkaline hydrolysis (19.7±3.4). However, EDTA failed to completely prevent the loss of caffeic acid, even when present at high concentration (100 mM). The presence of ascorbic acid (1%) either alone or together with EDTA completely prevented caffeic acid degradation. Moreover, we couldn't find any significant loss of caffeic acid up to 2 h incubation in the presence of 1% ascorbic acid and 10 mM EDTA. On the basis of the results obtained, 10 mM EDTA and 1% ascorbic acid were added during alkaline hydrolysis thereafter.

Table 2 shows the effect of EDTA-ascorbate on the recovery of several common phenolic acids subjected to alkaline hydrolysis. Among these, dihydrocaffeic acid (3-(3,4-dihydroxy-phenyl)-propionic acid) and homogentisic acid (2,5-dihydroxy-phenyl-acetic acid), in addition to caffeic acid (3,4-dihydroxy-cinnamic acid), almost completely disappeared during alkaline hydrolysis. The loss of these compounds was totally prevented by the addition of EDTA-ascorbate. Dihydroferulic acid (3-(4-hydroxy-3-methoxy-phenyl)-propionic acid) and sinapic acid (3,5-dimethoxy-4-hydroxy)-cinnamic acid) showed a somewhat uncomplete recovery after alkaline hydrolysis ($P = 0.0608$ and $P = 0.0276$ respectively), which was totally restored in the presence of EDTA-ascorbate. In these conditions (10 mM EDTA, 1% ascorbic acid), alkaline hydrolysis of chlorogenic acid resulted in complete recovery of liberated caffeic acid (97.0±2.8% of expected value against 22.0±11.5% in the absence of EDTA-ascorbate).

The content of bound phenolic acids of coffee brew and apple samples has been measured in the presence of EDTA-ascorbate during the hydrolytic treatment. Coffee and apple were chosen on the basis of their high

content of bound phenolic acids, particularly chlorogenic acids (Clifford, 1985; Clifford & Wight, 1976; Macheix, Fleuriet, & Billot, 1990). Fig. 2 shows typical chromatograms obtained from non-hydrolysed (Fig. 2B) and hydrolysed (Fig. 2C, D) coffee brew samples. Single phenolic acids were identified by coelution with standard compounds. Table 3 summarises the results obtained. Chlorogenic acid (5′-caffeoylquinic acid) was present in non-hydrolysed coffee brew samples (478.9±23.2 μg/ml), while free caffeic, *p*-coumaric and ferulic acids were undetectable. The hydrolytic procedure liberated ferulic acid, *p*-coumaric acid and relevant amounts of caffeic acid. Caffeic acid liberated upon hydrolysis in the presence of EDTA-ascorbate amounted to 830.0±69.9 μg/ml. This amount was higher than the amount expected from hydrolysis of chlorogenic acid (5′-caffeoylquinic acid) on the basis of 1 to 1 stoichiometry (molecular weights: 354.3 and 180.2 for chlorogenic and caffeic acid respectively). This result is explained by the fact that coffee contains also dicaffeoylquinic derivatives and different isomer of caffeoylquinic acids besides 5-caffeoylquinic acid, the one detected in our experiments (Hughes & Thorpe, 1987).

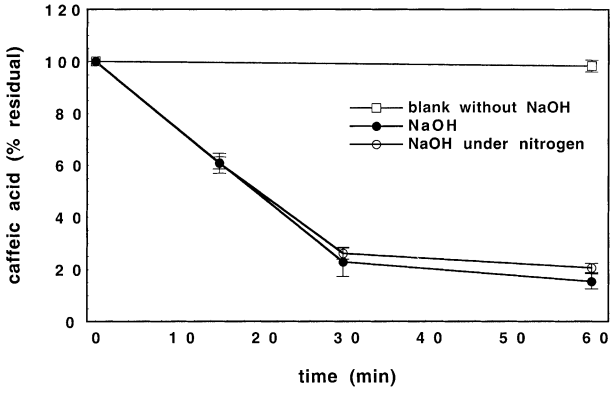

Fig. 1. Recovery of caffeic acid during alkaline hydrolysis. Caffeic acid (0.5 mg/ 0.5 ml) was subjected to alkaline hydrolysis with 4.5 ml 2N NaOH at 30 °C, with and without nitrogen flow. Blanks were run without NaOH. At the indicated times, aliquots were withdrawn, extracted and analyzed by HPLC-ECD as reported in Section 2. Values are means ± SD of three independent experiments and are expressed as % residual in respect to initial values.

Table 1
The effect of ascorbic acid and EDTA on caffeic acid recovery during alkaline hydrolysis[a]

Treatment	Caffeic acid (% residual)
Blank without NaOH	100
NaOH	19.7±3.4
NaOH + EDTA (10 mM)	55.4±6.0
NaOH + EDTA (100 mM)	54.4±6.0
NaOH + ascorbic acid (1%)	104.1±5.8
NaOH + EDTA (10mM) + ascorbic acid (1%)	106.6±4.8

[a] Values are means ± S.D. of three independent experiments.

In the absence of EDTA-ascorbate during hydrolytic treatment, a value of 433±22.2 μg/ml of liberated caffeic acid was found, a 52% value in respect to the amount detected in the presence of EDTA-ascorbate. Chlorogenic acid (5′-caffeoylquinic acid) was also detected in apple at high level (175.7±9.2 μg/g fresh weight), while free caffeic acid and ferulic acid were 1.5±0.2 and 0.47±0.1 μg/g respectively. The hydrolytic procedure liberated considerable amounts of caffeic acid (84.6±6.8 μg/g) and *p*-coumaric acid (10.3±0.9 μg/g). Interestingly, chlorogenic acid content was found to be about 9 fold lower when homogenization was performed in the absence of ascorbic acid and EDTA (20.2±2.9 μg/g compared to 175.7±9.2 μg/g obtained in the presence of ascorbic acid and EDTA). Due to the quite low pH of homogenization buffer (5.0), the loss of chlorogenic acid during homogenization in the absence of ascorbic acid and EDTA is unlikely to be due to spontaneous autoxidation. More likely, the loss is due to enzymatic oxidation, occurring in fresh fruits by the action of polyphenol oxidase (Coseteng & Lee, 1987; Matheis, 1987). The ability of ascorbic acid to reduce back the phenoxyl radical intermediate produced during oxidation of phenolic compounds is probably responsible for the observed effect. Such a mechanism has been already demonstrated for the phenoxyl radical intermediate derived from caffeic acid oxidation, which is reduced back to caffeic acid by ascorbate (Laranjinha, 2001; Laranjinha & Cadenas, 1999).

The results presented in this study show that the addition of EDTA-ascorbate during alkaline hydrolysis completely prevents the degradation of those phenolic acids particularly susceptible to oxidative degradation,

Table 2
The effect of ascorbic acid and EDTA phenolic acids recovery during alkaline hydrolysis[a]

	NaOH (% residual)	NaOH + EDTA + ascorbic acid (% residual)
Caffeic acid	19.7±3.4a	106.6±4.8b
m-Coumaric acid	96.7±5.2a	98.1±9.6a
o-Coumaric acid	100.1±11.6a	97.3±5.0a
p-Coumaric acid	101.1±1.8a	103.9±9.8a
Dihydrocaffeic acid	0a	96.0±5.1b
Dihydroferulic acid	82.8±5.5a	94.3±10.2a
Ferulic acid	106.5±7.0a	103.2±1.0a
Homogentisic acid	4.4±0.3a	106.2±11.4b
Homovanillic acid	95.4±5.5a	105.9±12.9a
3-(*p*-Hydroxy-phenyl)-propionic acid	95.0±7.7a	101.5±12.9a
Isoferulic acid	94.6±6.2a	97.5±0.9a
Sinapic acid	92.1±3.6a	110.7±12.2b
Syringic acid	101.7±3.6a	101.6±3.7a
Vanillic acid	104.2±2.0a	101.0±1.2a

[a] Values (means ± S.D., $n=3$) within a row with different letters are significantly different ($P \leqslant 0.05$) from blanks without NaOH (100%).

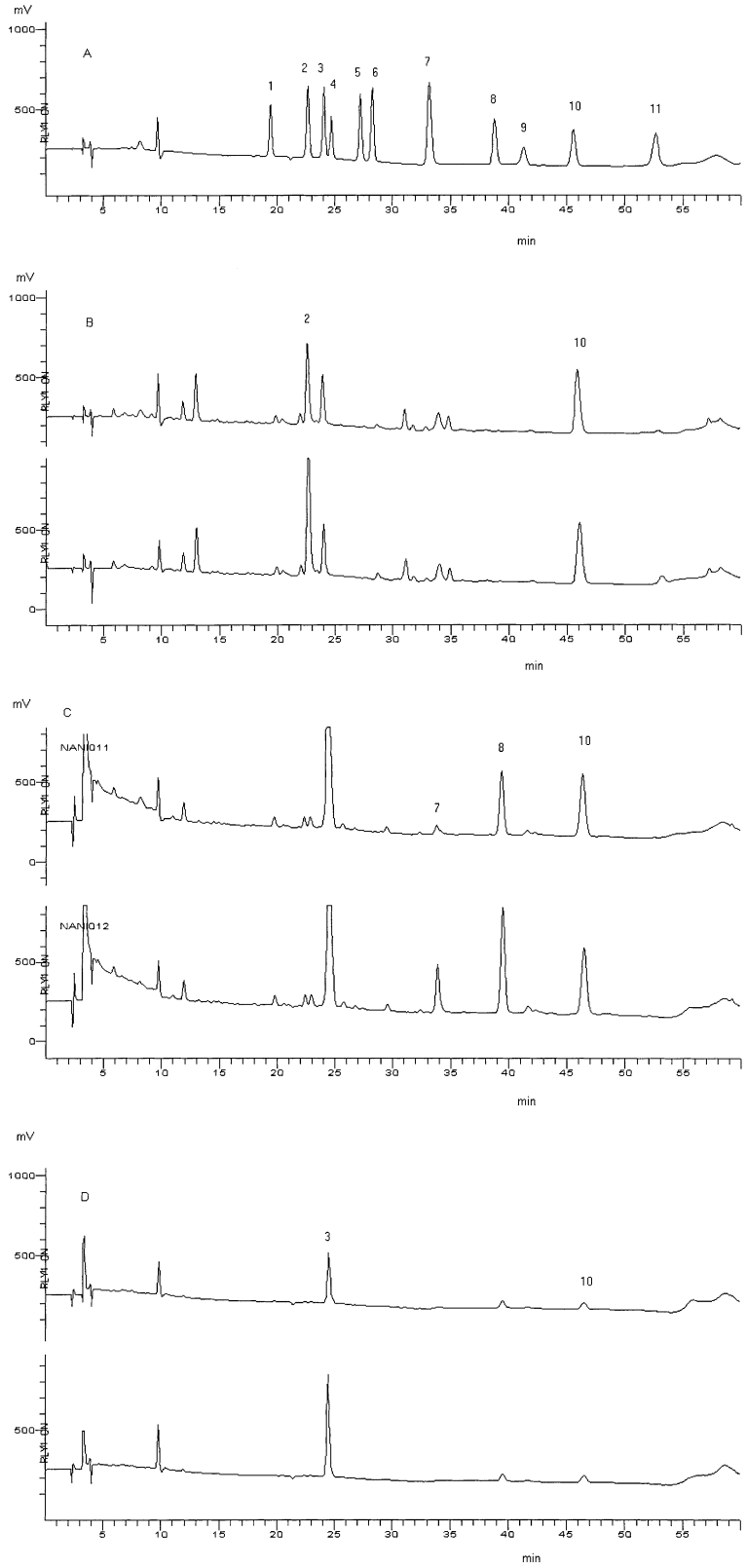

Fig. 2. High-performance liquid chromatograms of (A) standard mixture of phenolic acids; (B) upper: diluted non-hydrolysed coffee brew sample; lower: same sample + standard chlorogenic acid; (C) upper: diluted hydrolysed coffee brew sample; lower: same sample + standard ferulic and p-coumaric acids; (D) upper: hydrolysed coffee brew sample in (C) was further diluted; lower: same sample + standard caffeic acid. The operating conditions are reported in Section 2.2. Peaks identification: 1, dihydrocaffeic acid; 2, chlorogenic acid; 3, caffeic acid; 4, homovanillic acid; 5, syringic acid; 6, 3-hydroxy-phenyl-propionic acid; 7, *p*-coumaric acid; 8, ferulic acid; 9, *m*-coumaric acid; 10, isoferulic acid; 11, *o*-coumaric acid.

Table 3
Phenolic acids content of coffee brew and apple before and after hydrolysis[a]

	Chlorogenic acid	Caffeic acid	*p*-Coumaric	Ferulic acid
Non hydrolysed coffee (μg/ml)	478.9±23.2	n.d.	n.d.	n.d.
Hydrolysed coffee (μg/ml)	n.d.	830.0±69.9	14.0±1.1	142.8±12.3
Non hydrolysed apple (μg/g)[b]	175.7±9.2	1.5±0.2	n.d.	0.47±0.1
Hydrolysed apple (μg/g)[b]	n.d.	84.6±6.8	10.3±0.9	3.0±0.2

[a] Values are means ± S.D. of four independent experiments.
[b] Values are expressed as μg/g fresh weight.

such as *o*-dihydroxyphenols (Sosulski, 1979), allowing quantitative estimation of these compounds.

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