

Oxidation of phenolic compounds from *Aloe barbadensis* by peroxidase activity: Possible involvement in defence reactions

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Abstract – Sephadex LH-20 chromatography and reverse phase-high performance liquid chromatography (RP-HPLC) have been combined to analyse different phenolics in *Aloe barbadensis* Mill. Among them, a new chromone peak was found. Whole phenolics, and anthrone and chromone fractions were assayed as substrates of endogenous peroxidases (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) and polyphenol oxidases (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) by following the disappearance of specific RP-HPLC peaks after appropriate incubations in the presence and in absence of H₂O₂. Chromones, but not anthrones, were found to be good substrates of peroxidases. It is postulated that peroxidase oxidation of chromones may have a protective and sealing effect against infection after wounding. Polyphenol oxidases may have a secondary, if any, effect. © 2001 Éditions scientifiques et médicales Elsevier SAS

Aloe barbadensis / defence reaction / peroxidase / phenolic compounds / polyphenol oxidase / reactive oxygen species

RP-HPLC, reverse phase-high performance liquid chromatography / TLC, thin layer chromatography / R_t, retention time

1. INTRODUCTION

Although medicinal properties and active components of Aloe extracts are extensively studied, little is known about the function of the secondary metabolism in this plant.

The main secondary metabolites described in *Aloe* species are phenolics of the anthrone, chromone and phenyl pyrone type (figure 1). Among them, only anthrones and chromones have been identified in *Aloe barbadensis*, also called *Aloe vera* [15, 17], and their biological activities are being investigated. Some of the biological activities attributed to phenolic compounds of Aloe may be related with the defence mechanisms of the plant. For example, barbaloin content and distribution have been recently proposed as a part of a defence mechanism against herbivores [7, 8].

Plants have developed a broad range of strategies to protect themselves against biotic and abiotic stress [21]. The first step of defence is based on the activa-

tion of pre-existing components, such as the liberation of toxic compound like phenolics, or the rapid generation of reactive oxygen species and subsequent oxidative reactions. Peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) and polyphenol oxidase (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) may play a key role in this oxidative reactions to yield radical, oxidative-coupled oligomers or tannin and melanin-type compounds [12].

Peroxidase activity has been described in *Aloe* spp. [4], but no relation with endogenous phenolic compounds has been established. In this work, the oxidation of these phenolics by peroxidase and polyphenol oxidase activities has been studied and the possible involvement of these reactions in the defence mechanism of Aloe plants is discussed.

2. RESULTS

2.1. Analysis by RP-HPLC of *A. barbadensis* phenolic compounds

We have developed a RP-HPLC method using a gradient of acetic acid in water and acetonitrile. The

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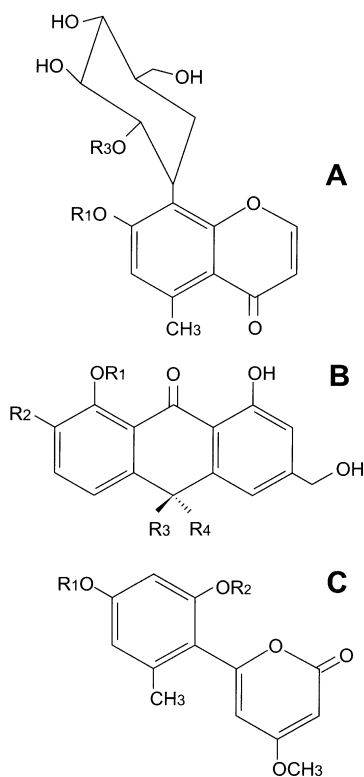


Figure 1. Chromones (A), anthrones (B) and phenyl pyrones (C) in *Aloe* spp.

optimised method allowed to distinguish eight peaks (figure 2A). A commercial standard allowed the identification of peak 7 ($R_t = 25.99$ min) as the anthrone β -barbaloin. Incubation of commercial β -barbaloin, at 30 °C in sodium acetate buffer (pH 5.0), gave (following a second order kinetics) a new peak detected by RP-HPLC with less retention time ($R_t = 23.64$ min), peak 4 in figure 2A, other than that of β -barbaloin and with the same UV-VIS spectrum (data not shown). The co-elution of both peaks in a Sephadex LH-20 chromatography with methanol as mobile phase, the simultaneous migration in a TLC analysis and the comparative analysis with other reported chromatograms using similar HPLC system [15] allowed the identification of peak 4 (figure 2A) as α -barbaloin.

The UV-VIS spectra and the relative retention times, as compared with the results obtained by Okamura et al. [15], allowed us to assign tentatively peak 1 ($R_t = 4.81$ min) as aloesin and peak 8 ($R_t = 37.83$ min) as aloeresin E.

In our first optimisation assays, we found, as Okamura et al. [15], only one peak with a retention time

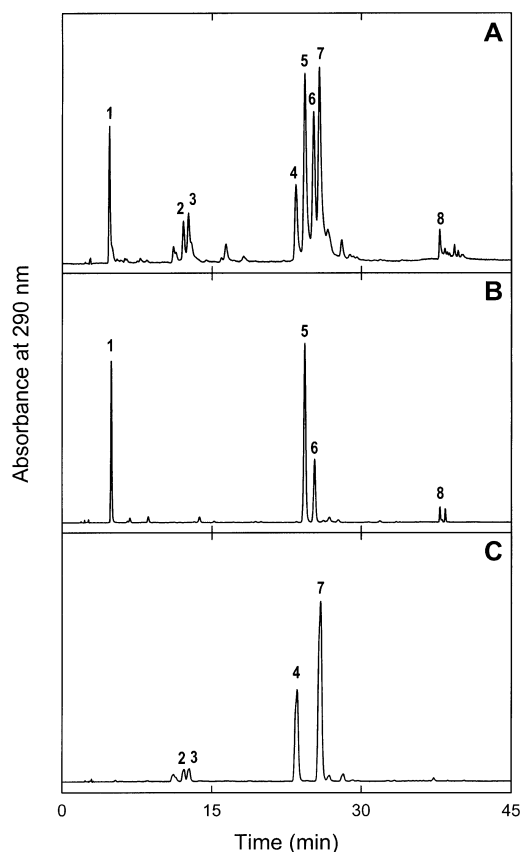


Figure 2. RP-HPLC profiles of *Aloe barbadensis* phenolic compounds. Total phenolics (A), first fraction eluted after Sephadex LH-20 chromatography (B) and last fraction eluted after Sephadex LH-20 chromatography (C).

between α - and β -barbaloin, that these authors had identified as isoaloeresin D. Nevertheless, a better adjustment of the gradient and the use of acetic acid in water (and not water alone) showed the initial co-elution of another peak with α -barbaloin. Our optimised gradient, as it can be seen in figure 2A, resolved two peaks (5 and 6) with retention times between α - and β -barbaloin ($R_t = 24.48$ and 25.40 min, respectively), peak 6 being tentatively isoaloeresin D.

We cannot give any additional data at the moment about peaks 2 and 3 ($R_t = 11.22$ and 12.70 min, respectively).

2.2. Partial purification of *A. barbadensis* phenolic compounds

Through Sephadex LH-20 low pressure chromatography, using methanol as mobile phase, the total *A. barbadensis* phenolics could be separated into two

main fractions. RP-HPLC analysis showed that the first fraction eluted from the column mainly contained the peaks 1, 5, 6 and 8 (figure 2B). Analysis of UV-VIS spectra, with absorption maxima at 205–207, 210–217, 225–228, 242–244, 252 and 283–300 nm, indicated the chromone nature of these components [22].

On the other hand, RP-HPLC analysis of the second eluted fraction from Sephadex LH-20 column, showed that it mainly contained peaks 4 and 7, and low amounts of peaks 2 and 3 (figure 2C). The UV-VIS spectra, with absorption maxima at 209, 260, 268, 296 and 357 nm, indicated the anthrone nature of the main components [22].

Thus, Sephadex LH-20 chromatography allowed to separate the *A. barbadensis* phenolic compounds between a chromone and an anthrone fraction.

2.3. Oxidation of total phenolics from *A. barbadensis*

Once the phenolic compounds were characterised by an appropriate RP-HPLC method, we studied their possible involvement in endogenous enzymatic oxidative reactions.

To do this, we analysed the RP-HPLC phenolic patterns after 1 h incubation of four reaction media with total phenolic compounds, the presence and the absence of peroxidase and polyphenol oxidase activities and with or without H_2O_2 (see Methods). The presence of peroxidase and polyphenol oxidase activities with H_2O_2 (peroxidase + polyphenol oxidase reaction medium) produced the higher changes in the chromatographic profile (figure 3B), compared with control medium in absence of peroxidase, polyphenol oxidase and H_2O_2 (figure 3A). In these conditions, peaks 2, 3, 5 and 6 strongly decreased, peak 1 moderately decreased, while peaks 4 and 7 were slightly affected. At the same time, compounds, probably with a polymeric nature, were formed and eluted as a hump at the end of the gradient (figure 3B).

The reaction medium in the presence of peroxidase and polyphenol oxidase activities and the absence of H_2O_2 (polyphenol oxidase reaction medium) did not show qualitative changes in the chromatographic profile, but showed significant quantitative changes. Appropriate controls of chemical oxidation by H_2O_2 did not show significant changes.

By integration of the peaks, and its relative quantification taking the area of each peak in the control medium with phenolics alone as reference, we analysed in detail reactions in each medium. As table 1 shows in the polyphenol oxidase reaction medium (1T, without H_2O_2), there were moderate decreases of all

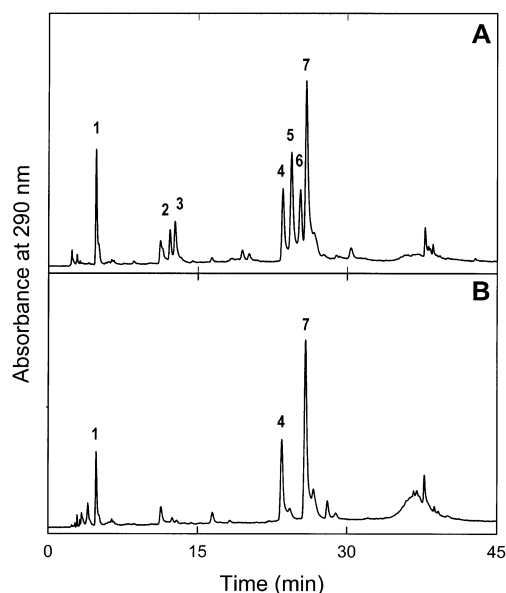


Figure 3. RP-HPLC profile of reaction media with total phenolics from *A. barbadensis*. Control with total phenolics in absence of H_2O_2 , peroxidase and polyphenol oxidase activities (A) and reaction medium in presence of 0.5 mM H_2O_2 and peroxidase (1 nkat) and polyphenol oxidase (2 nkat) activities (B).

peaks (between 23 and 50 %). However, in the presence of H_2O_2 (peroxidase + polyphenol oxidase reaction medium) peaks 2, 3, 5 and 6 had a drastic decrease (82, 93, 95 and 100 %, respectively), peaks 1 and 7 a moderate decrease (52 and 28 %, respectively) and peak 4 (17 %) a slight decrease.

Similar results were obtained using commercial horseradish peroxidase (type X) in presence of H_2O_2 (data not shown).

2.4. Oxidation of partially purified *A. barbadensis* phenolics

We carried out similar reaction incubations with the purified phenolic fractions obtained with Sephadex LH-20 (chromone and anthrone fractions).

As table 1 shows, polyphenol oxidase reaction medium with chromones produced a small decrease of peaks 1 and 5 (12 and 20 %, respectively), while in the presence of H_2O_2 (peroxidase + polyphenol oxidase reaction medium with chromones) peaks 5 and 6 completely disappeared and peak 1 decreased some (29 %). At the same time, polymeric compounds in the final part of the HPLC gradient appeared, as illustrated in figure 4.

With respect to anthrone reaction media, a moderate decrease of peak 2 and a small decrease of peak 3 (31

Table I. Integration results of relevant peaks in the different reaction media assayed containing peroxidase (1 nkat) and polyphenol oxidase (3.616 nkat) activities. Total phenolics in absence of H_2O_2 (medium 1T), total phenolics in presence of 0.5 mM H_2O_2 (medium 2T), chromones in absence of H_2O_2 (medium 1C), chromones in presence of 0.5 mM H_2O_2 (medium 2C), anthrones in absence of H_2O_2 (medium 1A) and anthrones in presence of H_2O_2 (medium 2A). The results are expressed as % of decrease in peak area, taking 100 % as the area of each peak in the respective control medium (with total phenolics, chromones or anthrones) in absence of H_2O_2 and absence of peroxidase and polyphenol oxidase activities. Controls with H_2O_2 and heat-denatured enzyme preparations did not show significant area decreases.

Peak	R_t (min)	Compound	Area decrease (%)					
			Total phenolics		Chromones		Anthrones	
			1T	2T	1C	2C	1A	2A
1	4.81	Aloesin	25	52	12	29	–	–
2	12.22	?	40	82	–	–	31	100
3	12.70	?	23	93	–	–	18	100
4	23.64	α -Barbaloin	28	17	–	–	0	0
5	24.48	Chromone x?	49	95	20	100	–	–
6	25.40	Isoaloesin D	50	100	0	100	–	–
7	25.99	β -Barbaloin	25	28	–	–	0	0

and 18 %, respectively) took place in the polyphenol oxidase reaction medium, while peaks 4 and 7, were not affected. In the presence of H_2O_2 (peroxidase + polyphenol oxidase reaction medium), peaks 2 and 3 completely disappeared while again, peaks 4 and 7 were not affected.

Similar results were obtained using commercial horseradish peroxidase (type X) (data not shown).

3. DISCUSSION

The optimisation of a RP-HPLC method allowed us to find a new peak in the profile of *A. barbadensis* phenolic compounds. Although still requiring further characterisation, UV-VIS data indicated the chromone nature of this peak.

As table I summarises, the seven Aloe phenolics investigated are differently affected by peroxidase and polyphenol oxidase reactions. The reactions in the medium without H_2O_2 , imputable at first to polyphenol oxidase activity, had only moderate effects in all peaks, especially peaks 2, 5 and 6, with an area decrease between 40 and 50 %. Whereas incubation in the presence of H_2O_2 , by reactions imputable to both peroxidase and polyphenol oxidase activities, strongly decreased peaks 2, 3, 5 and 6, and moderately peak 1. The separation of phenolics in two fractions (chromones and anthrones) allowed us a more precise analysis.

The incubation with chromones, in the absence of H_2O_2 suggested that only (and weakly) peaks 1 and 5 were substrates of polyphenol oxidase. In the presence of H_2O_2 , peaks 5 and 6 (totally) and peak 1 (some 29 %) were transformed during the 1-h incubation time. On the other hand, among anthrones, only the unknown peaks 2 and 3 were transformed (weakly in the absence of H_2O_2 and totally in its presence), while peaks 4 and 7 were not affected. These results suggest

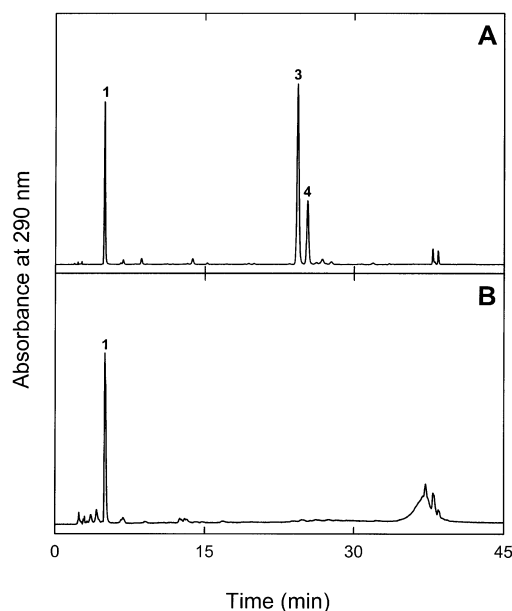


Figure 4. RP-HPLC profile of reaction media with chromone from *A. barbadensis* fraction obtained from Sephadex LH-20 chromatography. Control with chromones in absence of H_2O_2 , peroxidase and polyphenol oxidase activities (A) and reaction medium with chromones in presence 0.5 mM of H_2O_2 , peroxidase (1 nkat) and polyphenol oxidase (2 nkat) activities (B).

that chromone compounds, like peaks 5 and 6 (one of these could be isoaloesin D), are very good substrates of endogenous peroxidase (probably by the presence of a *p*-coumaryl group in the molecule) but not of endogenous polyphenol oxidase. Furthermore, *p*-coumaric acid inhibits polyphenol oxidase activity [16]. The same is true for the unknown peaks 2 and 3, but more information is necessary about the nature of these compounds. The anthrone compounds α - and β -barbaloin were not substrates of peroxidase or polyphenol oxidase. These results were confirmed with the incubation of commercial barbaloin with peroxidase and polyphenol oxidase from Aloe in the absence and in the presence of H₂O₂ and also with commercial horseradish peroxidase type X (data not shown).

Although these reactions are still being studied with purified phenolics and enzymes, it seems that polyphenol oxidase plays a minor role in the oxidative reactions of Aloe phenolics. The reactions observed in the absence of exogenous H₂O₂ with total phenolics could be explained by considering that some peroxidases are capable of producing H₂O₂, especially the basic peroxidases with a high isoelectric point [19]. One strongly basic peroxidase has been previously reported in *A. barbadensis* parenchyma [4] and at least five peroxidase isoenzymes (including one strongly basic isoenzyme) have been found in the *A. barbadensis* cortex (data not shown). Additionally, minor reactions due to polyphenol oxidase activity can contribute, via O₂⁻ production, to yield H₂O₂ ready to use by peroxidase [9, 18].

Phenolics, such as aloesin and α - and β -barbaloins, which are not good substrates when assayed in their respective fractions (chromones and anthrones in *table I*) were however transformed when assayed with total phenolic extract. These apparent oxidations could be explained taking into account that peroxidase-catalysed reactions take place in one-electron steps and yields unstable mono-radical species and then quinones. These radicals and quinones, depending on their individual redox potential, may oxidise other phenolics with a lower redox potential by means of an oxidation coupled reaction [3, 12, 14]. Thus, the reaction observed with aloesin and barbaloins may be the result of a coupled oxidation by radicals or quinones from chromones, like peaks 5 and 6, that have previously been oxidised by peroxidase.

These oxidative reactions observed *in vitro* with endogenous phenolics and enzymes from *A. barbadensis*, can serve as a reference model to investigate the defence mechanism of this plant against injuries. Thus,

the liberation of phenolics produced after injury may serve as a primary response against herbivores or microorganisms due to their toxicity *per se*. Thus, barbaloin has been described as a potential antibacterial and antiviral compound [2, 10]. Also, it has been proposed, attending to their characteristic distribution in the plant, as a part of the peripheral defence strategy against herbivores [7, 8].

The defence mechanism must further involve other phenolics and the oxidative reactions that take place after injury. After a number of stress stimuli, the plant produces the so-called oxidative burst, which constitutes in the production of reactive oxygen intermediates (ROIs), primarily superoxide (O₂⁻) and H₂O₂ [6]. The origin of this H₂O₂ is still under investigation, but some potential mechanisms have been proposed, such as the NADPH-dependent oxidase, polyamine oxidases and their own peroxidase [6]. Evidences for the production of H₂O₂ in *A. barbadensis* have been found, since histochemical staining of peroxidase activity with 4-methoxy- α -naphthol or 3,3',5,5'-tetramethylbenzidine have been detected in the absence of exogenous H₂O₂ in sections of leaves (data not shown), as described with strawberry sections [13].

The *in vivo* utilisation of this H₂O₂ by peroxidase to produce the oxidative reactions observed *in vitro* with the endogenous phenolics is rather plausible. Thus, the response to injury may consist first, in the production of H₂O₂ and other reactive oxygen species that *per se* are toxic, second, in the oxidation of phenolics, mainly certain chromones, to produce more toxic species such as radicals and quinones, and last, in the formation of polymers, tannin or melanin-type, which contribute through their toxic properties and by sealing the affected area.

4. METHODS

4.1. Plant material and chemicals

A. barbadensis Miller was purchased in a local garden-centre. All reagents were of analytical grade and were purchased from Sigma-Aldrich Chemical. Chromatography products were obtained from Amersham Pharmacia Biotech.

4.2. Peroxidase and polyphenol oxidase extraction

Enzymatic activities were extracted as previously described [4] with minor modifications. The cortex tissue of leaves (200 g) were homogenised in an

Omni-mixer (Sorvall, USA) with 100 mL 100 % acetone at -20°C and filtered under vacuum. The protein precipitate was washed with acetone at -20°C and dried. The powder was resuspended twice with 500 mL 50 mM Tris-HCl (pH 7.5), 1 M KCl and 0.1 M CaCl_2 , by gently stirring overnight and, then, it was centrifuged at $20\,000 \times g$ for 20 min. Ammonium sulphate was added to the supernatant up to a 1.7-M concentration. After gently stirring during 2 h, the preparation was centrifuged at $1\,000 \times g$ for 20 min. Wet Phenyl sepharose (100 mL), previously equilibrated with 1.7 M ammonium sulphate in 50 mM Tris-HCl (pH 7.5), was added to the supernatant with occasional stirring with a glass rod. After approximately 1 h, virtually all the peroxidase activity had disappeared from the supernatant portion of the mixture. The precipitate was collected by centrifugation at $300 \times g$ for 20 min and proteins were recovered by washing exhaustively with 50 mM Tris-HCl (pH 7.5) and were dialysed overnight against 50 mM Tris-HCl (pH 7.5) at 5°C . The resulting extract contained around 3.062 nkat peroxidase activity and 6.068 nkat polyphenol oxidase activity per mg of protein and was used as the source of endogenous enzyme activities.

4.3. Enzyme assays and protein determination

Peroxidase activity (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) was determined using 4-methoxy- α -naphthol as substrate, and the enzyme amount (expressed in nkat) was calculated using an $\epsilon_{593} = 21\,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [5]. Polyphenol oxidase activity (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) was determined using catechin as substrate, and the enzyme amount (expressed in nkat) was calculated using an $\epsilon_{390} = 4\,680 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [11]. Protein concentration was measured by the method of Bradford [1] with a Protein Assay Kit (Bio-Rad) using bovine serum albumin as standard.

4.4. Preparation of standards and total phenolics

Phenolic compounds were extracted from Aloe cortex with methanol (1/2, w/v), and the resulting suspensions were centrifuged at $14\,000 \times g$ for 5 min. The supernatants were subjected to HPLC analysis as sample solutions. Standard phenolic compounds were dissolved in methanol at 50 mM. Both the sample and the standard solutions were filtered through a 0.45- μm membrane filter before injection. Concentration of phenolics was estimated by direct spectrophotometric determination at 290 nm using a β -barbaloin calibration curve.

4.5. Partial purification of phenolic compounds by low pressure chromatography on Sephadex LH-20

The phenolic compounds were purified by low pressure chromatography on a Sephadex LH-20 column ($45.0 \times 1.5 \text{ cm I.D.}$) using methanol as a mobile phase, at a flow rate of $0.8 \text{ mL}\cdot\text{min}^{-1}$, at room temperature.

4.6. Thin layer chromatography analysis of phenolic compounds

Thin layer chromatography was performed on aluminium sheets pre-coated with a 0.25-mm layer of silica gel 60 F₂₅₄ (Merck, Spain). The solvent was ethyl acetate/methanol/water (100/13.5/10, v/v/v) [20].

4.7. Oxidation of phenolic compounds

The reactions were carried out at 30°C during 1 h in 1 mL assay volume containing 50 mM sodium acetate (pH 5.0), 0.5 mM phenolics (total phenolics, chromones or anthrones), in presence or absence of peroxidase (1 nkat) and polyphenol oxidase (2 nkat) and 0.5 mM H_2O_2 . The peak area changed linearly with time, at least, up to 1 h (data not shown) which was selected as incubation time to combine accuracy and rapidity with minor side reactions.

4.8. HPLC analysis of phenolic compounds

HPLC analysis was carried out in a Beckman system (San Ramon, CA, USA) comprising a Programmable Solvent Module 126 pump, Scanning Detector Module 167 and manual injector. The data were processed with the GOLD system. Reverse-phase HPLC (RP-HPLC) of phenolic compounds was carried out at 45°C on a Beckman ultrasphere 5 μm ODS column ($25 \text{ cm} \times 4.6 \text{ mm I.D.}$) using a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. Solvent A was 2.5 % acetic acid in water and solvent B was acetonitrile. A linear gradient from 12 to 26 % in 30 min, from 26 to 70 % in 15 min, followed by washing and reconditioning of the column was used.

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