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DISTRIBUTION OF PHENOLIC COMPOUNDS IN THE SEAGRASS *POSIDONIA OCEANICA*

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Key Word Index—*Posidonia oceanica*; Posidoniaceae; marine phanerogam; Mediterranean sea; phenyl propenoids; phenolic compounds.

Abstract—The identification and quantification of phenolic compounds was carried out in various *Posidonia oceanica* foliar tissues using high performance liquid chromatography (HPLC). Samples were taken during the spring and correspond to sites presenting different environmental conditions: over-grazing by herbivores (*Sarpa salpa, Paracentrotus lividus*), presence of anthropogenic waste (chemical and organic), interspecific competition with the alga *Caulerpa taxifolia* and intra-specific competition (dense meadows). A total of 23 compounds were identified and quantitatively analysed; of these, acetosyringone and ferulic acid exhibit the highest concentrations. The level of the compounds found varies with (i) the tissue examined with the highest concentrations being observed in young growing leaves (intermediate leaf blades) and (ii) the environmental conditions (abiotic factors and "stress" conditions). © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The presence of "tannin cells", which are specialized in the production of phenolic compounds, has been noted in a number of marine phanerogams [1]. These phenolic compounds seem to play an important role in the protection of these plants against competitors, predators and pathogens [2], as observed for terrestrial plants [3–6]. *Posidonia oceanica* (L.) Delile is a marine phanerogam endemic to the Mediterranean sea where it forms vast meadows from the water surface to depths up to 40 m. In this phanerogam, the presence of cells rich in phenolic compounds has already been demonstrated [7]. The number of these cells seems to increase with increasing proximity to waste outlets [8] as well as with the existence of high levels of interspecific competition (e.g. *Caulerpa taxifolia* (Vahl) C. Agardh [9]), suggesting an increase with stress.

Here we test if the level of phenolic compounds present in *Posidonia oceanica* foliar tissues could be used as an indicator of the level of stress. To this end, the quantification and identification of phenolic compounds were performed in various foliar tissues originating from different sites presenting different environmental conditions: over-grazing by herbivores (*Sarpa salpa* Linnaeus, 1758, *Paracentrotus lividus* Lamarck, 1816), presence of anthropogenic waste (chemical and organic), interspecific competition with the algae *Caulerpa taxifolia* and intra-specific competition (dense meadows).

RESULTS AND DISCUSSION

All 23 reference compounds were detected in the phanerogam samples (Fig. 1). Of these, acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) are the main compounds. Other compounds exhibit much lower average concentrations: syringaldehyde (3,5-dimethoxy-4-hydroxy benzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), acetovanillone (4-hydroxy-3-methoxyacetophenone), 4-hydroxybenzoic acid, *p*-anisic acid (4-methoxybenzoic acid), protocatechualdehyde (3,4-dihydroxybenzaldehyde) and protocatechuic acid (3,4-dihydroxybenzoic acid). The remaining compounds represent less than 2% of the total phenolic compounds levels, including caffeic acid (3,4-dihydroxycinnamic acid) and gallic acid (3,4,5-trihydroxybenzoic acid) which are present merely as trace compounds (< 0.1% of the total abundance).

Of the 27 compounds previously identified in *Posidonia oceanica* [7–14], 21 were detected in the present study in the five studied sites, and two compounds

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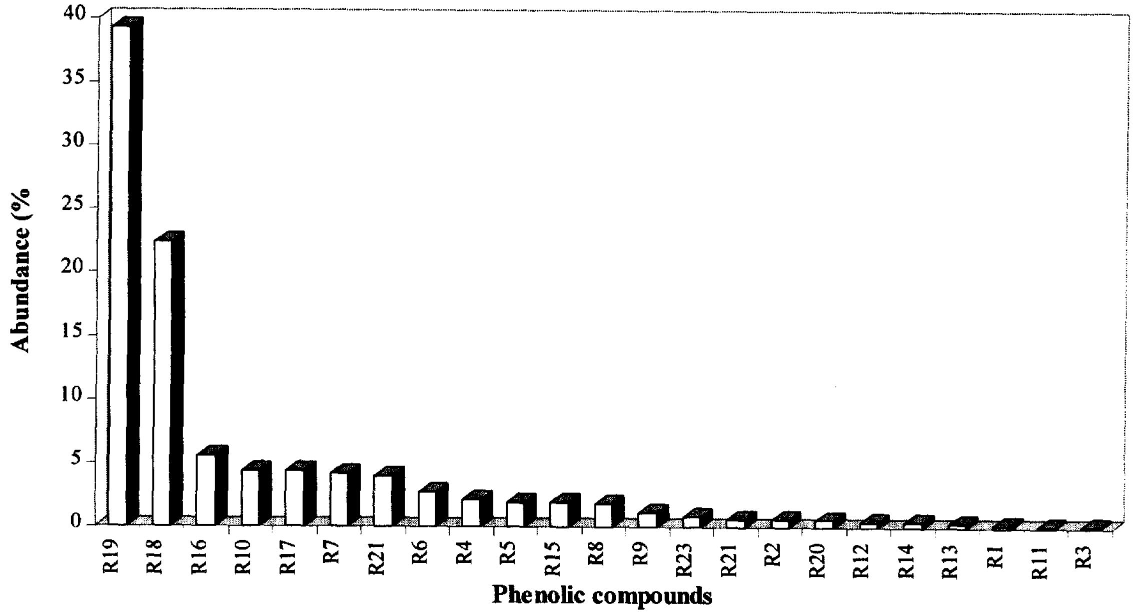
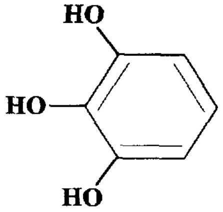
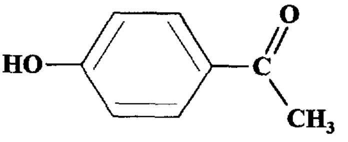


Fig. 1. Mean abundances of the different phenolic compounds identified using HPLC.



1,2,3-trihydroxybenzene (pyrogallol)



4-hydroxyacetophenone

Fig. 2. Structures of two compounds identified for the first time from *Posidonia oceanica*.

were identified for the first time in this phanerogam (Fig. 2): pyrogallol (1,2,3-trihydroxybenzene) and 4-hydroxyacetophenone. The number of phenolics and phenylpropenoids detected in *Posidonia oceanica* is much higher than that observed in other marine phanerogams of the Mediterranean (two in *Zostera noltii* Horneman, [11]; eight in *Zostera marina* L., [12, 13, 14]; three in *Cymodocea nodosa* (Ucria) Ascherson, [15]. Only rosmarinic acid was not found in *Posidonia oceanica*, despite the fact that this compound is present in *Zostera marina* [10, 11]. Previous studies on *Posidonia oceanica* have also revealed that there exists great differences in the relative abundances of the different phenolic compounds with a high concentration of both ferulic acid and acetosyringone [10]. These authors also noted a high concentration of *p*-anisic acid in the winter (13% on average) and a low concentration at the end of summer (0.3%). Values observed in the present study (spring) are intermediate, which is consistent with the suggested seasonal fluctuation of this compound.

Several phenolic compounds are not present in all the tissues and/or at all the sites examined (Table 1). The number of identified compounds was the highest at Tonnara (21) and the lowest at Figari (17). In addition, intermediate leaves presented the greatest number of phenolic compounds, while the lowest numbers were recorded in the blades of adult leaves (Table 1). Only two phenolic compounds are present at all five sites and in all three tissue types: acetosyringone and acetovanillone. This variability in the distribution of phenolic compounds has already been observed in other aquatic phanerogams [16].

The concentration of phenolic compounds varied

Table 1. Mean abundances (in mg g^{-1} dry weight) of identified phenolic compounds according to sampling site and tissue examined (compounds listed according to their elution sequence in HPLC)

		Tonnara			Livourne			Nice			Marseille			Figari		
Ref.	Compound (trivial name)	S[a]	I[b]	A[c]	S	I	A	S	I	A	S	I	A	S	I	A
R1	1,3,5-trihydroxybenzene (Phloroglucinol)													1.1	0.8	0.9
R2	1,2,3-trihydroxybenzene (Pyrogallol)	1.3	1.0	0.4	1.5	0.7	0.3		0.6			1.1			0.8	1.0
R3	3,4,5-trihydroxybenzoic acid (Gallic acid)	0.2	0.3													
R4	3,4-dihydroxybenzoic acid (Protocatechuic acid)	1.0	0.4	2.1	1.1	3.3		1.1	0.7	2.3	1.2	0.8	0.6			
R5	1,2-dihydroxybenzene (Pyrocatechol)					0.3	0.4							1.8	0.8	0.8
R6	3,4-dihydroxybenzaldehyde (Protocatechualdehyde)		1.2	0.8	*t*[d]	0.8	1.0	2.4	0.8	0.8	1.2	0.9	1.2	0.7	0.7	0.9
R7	4-hydroxybenzoic acid (4-hydroxybenzoic acid)	2.6	0.8		2.1	0.1	0.7	0.2			1.8	*t*				
R8	Phenol		0.1		1.3	2.9			0.3	0.2						
R9	4-hydroxybenzaldehyde	0.4	0.1	0.1	1.0	*t*		1.2				*t*				
R10	4-hydroxy-3-methoxybenzoic acid (Vanillic acid)	1.1	1.1	1.4	1.4	1.2	1.7	1.5	0.8	1.0	2.4	0.9		0.9	1.5	1.9
R11	3,4-dihydroxycinnamic acid (Caffeic acid)	0.1	0.5	0.6		0.5	0.5			0.1	0.6	0.7				
R12	3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid)	0.8	0.5	0.3	0.7	0.5	0.5	0.7	0.6	0.6	0.9	0.7		0.7	0.5	0.5
R13	4-hydroxyacetophenone (Piccol)	0.2	0.4	0.6	0.2	0.4	0.4	0.7	0.6	0.5	0.9	0.4	0.8		0.5	0.6
R14	4-hydroxy-3-methoxybenzaldehyde (Vanillin)	0.5	0.6	0.4	0.4	0.5	0.4	0.7	0.5	0.6		0.4		0.7	0.4	0.9
R15	4-hydroxycinnamic acid (4-coumaric acid)	0.7	2.1	1.2	0.6	2.8	1.6	0.7	1.5	0.7	0.9	0.4	0.5	0.7		
R16	3,5-dimethoxy-4-hydroxybenzaldehyde (Syringaldehyde)	0.5	0.5	0.5	0.3	0.5	0.5	0.8	0.5	0.3	0.7	0.5	0.3	3.9	6.0	8.0
R17	4-hydroxy-3-methoxyacetophenone (Acetovanillone)	0.7	0.4	6.6	0.8	0.5	0.5	0.4	0.5	0.5	0.9	0.5		0.5	0.8	0.6
R18	4-hydroxy-3-methoxycinnamic acid (Ferulic acid)	4.5	8.1	3.8	3.7	7.5	7.4	0.8	0.5	0.7	1.1	2.6	1.1	0.7	29	29
R19	3,5-dimethoxy-4-hydroxyacetophenone (Acetosyringone)	0.9	39	15	0.8	35	27	3.3	7.0	7.7	3.1	11	9.0	1.1	0.4	
R20	Benzoic acid	1.0	0.5	0.5	1.1	0.6	0.6	*t*	0.7	0.7	1.3	0.7	0.6	1.0		
R21	3,5-dimethoxy-4-hydroxycinnamic acid (Sinapic acid)	0.7	*t*	0.6								*t*		0.5		0.6
R22	4-methoxybenzoic acid (*p*-anisic acid)		*t*	0.9		0.8	0.8		0.9	*t*		6.7	2.4		0.9	1.2
R23	Cinnamic acid	0.8	0.5	0.6	0.6	0.4	0.5		0.5	0.5	0.6	0.5	0.6	0.5	0.5	0.5
	Total number of compounds		21			20			19			19			17	

[a] S: adult leaf sheaths.
[b] I: intermediate leaf blade.
[c] A: adult leaf blade.
[d] t: trace.

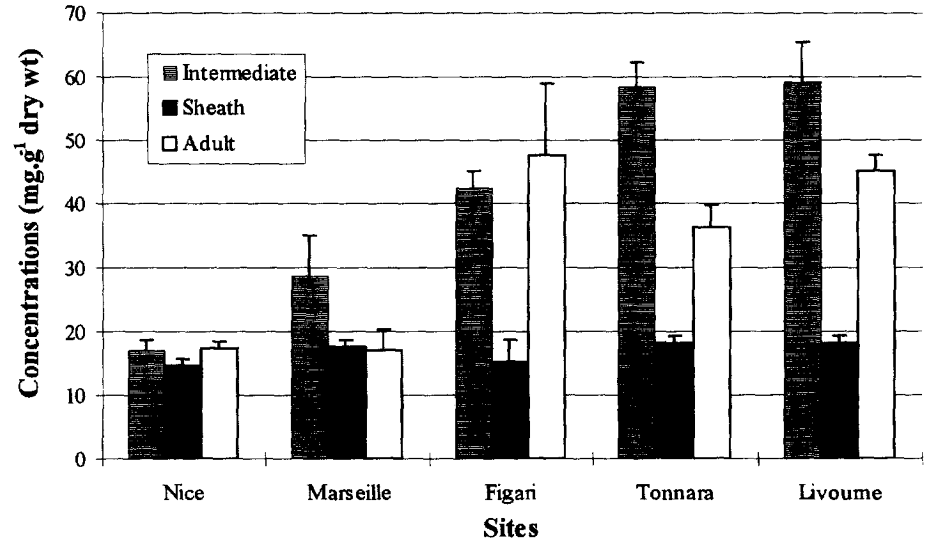


Fig. 3. Total content of phenolic compounds (in mg g^{-1} dry weight) using HPLC (±95% confidence interval).

with both the tissue type and the site considered (Kruskal–Wallis test, $p < 0.05$; Fig. 3). The concentration was the highest in the blades of intermediate leaves (45.4 ± 6.4 mg g^{-1}) followed by the blades of adult leaves (32.0 ± 4.6 mg g^{-1}), and, the lowest values were recorded in the leaf sheaths of these last leaves (16.8 ± 0.9 mg g^{-1}). This distribution of phenolic compounds in relation to the different *Posidonia oceanica* tissues confirms the results obtained by [17] for chicoric acid (dicaffeyl-tartaric acid). Harrison [12] demonstrated a similar pattern for the concentration of caffeic, gentisic and protocatechic acid, in leaves of *Zostera marina*, with a maximal concentration in young growing leaves and a minimal value in older leaves.

The sites seem to characterize three distinct groups (multiple Student-Newman-Keuls comparison test): (i) Nice and Marseille are characterized by low phenol levels of 16.2 and 21.1 mg g^{-1}, respectively; (ii) Tonnara and Livourne exhibit high concentrations in the order of 37.6 and 40.9 mg g^{-1} respectively, and (iii) Figari appears to be an intermediate site with levels of 35.1 mg g^{-1} (Fig. 3).

The low concentrations recorded for Nice, which is a site subjected to high interspecific competition with the tropical algae *Caulerpa taxifolia*, seem to confirm the preliminary results of Cuny [10], who showed no significant difference between the area colonized by *Caulerpa taxifolia* and the non-colonized area, for either winter (February) and late summer (September). Hence the increased number of tannin cells observed in *Posidonia oceanica* tissues in areas colonized by *Caulerpa taxifolia* [9] does not appear to bring about an increase in the total abundance of phenolic compounds. Future studies should include an examination of chicoric acid levels as this compound has been found to be present at high concentrations in tannin cells [7].

The low phenolic compound levels recorded at Marseille (site characterized by high grazing by herbivores) can be considered paradoxical. Indeed, high grazing pressure has been observed to be associated to an increase in phenol compound production in the kelp *Alaria marginata* [18]. However, the tissues analyzed in our study correspond to the lower part of adult blades (which is rarely eaten). It is precisely in these lower portions of the foliar blades that Cariello and Zanetti [7] observed the lowest phenolic compound levels.

Deterioration of environmental conditions lead to stress and are expected to result in an increase in the production of phenolic compounds in both the terrestrial [2, 3, 5, 6, 19] and marine environments [15, 18]. Yet, no clear evidence of such effects are available for marine phanerogams subject to intensive intraspecific competition. It is in the seagrass meadows of high density, however, (484 leaf shoots per m^2 at Tonnara, and 500 shoots per m^2 at Livourne) that the highest phenolic compound concentrations are recorded. At the Livourne site, the input of massive levels of metals (mercury, lead and zinc, Petrilli *et al.* [20], Maserti *et al.* [21]), which originate from an industrial waste outlet, could be responsible for the observed regression of this seagrass meadow [22]. It is, therefore, possible to hypothesize that this stress could be the reason behind the high production of phenolic compounds.

The concentrations of the main phenolic compounds recorded for the adult leaf sheaths were relatively similar. Conversely, only a few compounds contributed significantly to total abundances in the blades of intermediate and adult leaves (syringaldehyde, acetovanillone, ferulic acid, acetosyringone, *p*-anisic acid; Table 2). Indeed, two compounds (ferulic acid and acetosyringone) represented over 81% of the total phenolic content of the intermediate blades at

Table 2. The main phenolic compounds and their concentrations (in mg g^{-1} dry weight) according to sampling site and tissue type

	Intermediate		Adult sheaths		Adult blades	
Sites	Compounds	Concentrations	Compounds	Concentrations	Compounds	Concentrations
Nice	Acetosyringone	7	Acetosyringone	3.3	Acetosyringone	7.7
Marseille	Acetosyringone	11	Acetosyringone	3.1	Acetosyringone	9
	p-Anisic acid	6.7				
	Ferulic acid	2.6				
Figari	Ferulic acid	29	Syringaldehyde	3.9	Ferulic acid	29
	Syringaldehyde	6			Syringaldehyde	8
Tonnara	Acetosyringone	39	Ferulic acid	4.5	Acetosyringone	15
	Ferulic acid	8.1	4-hydroxybenzoic acid	2.6	Acetovanillone	6.6
					Ferulic acid	3.8
Livourne	Acetosyringone	3.5	Ferulic acid	3.7	Acetosyringone	27
	Ferulic acid	7.5			Ferulic acid	7.4
	Protocatechuic acid	3.3				
	Phenol	2.9				
	4-coumaric acid	2.8				

Tonnara, while a value of 77% was observed for adult leaf blades at Livourne. The relative importance and the respective concentrations of the main compounds were similar in the blades of adult and intermediate leaves, while, differences are observed for the adult leaf sheaths. The abundances of the main compounds is quite similar for a given site and this regardless of the tissue considered.

In *Posidonia oceanica*, the abundance of the main phenolic compounds seems to differ from that observed in terrestrial plants. Indeed, acetosyringone, which was found to be the most abundant phenolic compound in *Posidonia oceanica*, is very rarely observed in terrestrial plants [23] and seems to be species specific. Conversely, sinapic acid, which is prevalent in terrestrial habitats, is never observed as being a part of the main phenolic compounds in the phanerogam of the present study (Table 2).

EXPERIMENTAL

Plant material

One hundred *Posidonia oceanica* leaf shoots were sampled by SCUBA divers from five sites of the western Mediterranean in May 1996, at a depth of 10 m (Fig. 4): Tonnara (southern Corsica) a relatively pristine site where the density of the seagrass meadow is high (484 shoots per m^2); Livourne (Italy) a site situated in the proximity of an important industrial waste outlet [20]; Nice Cap Martin (Maritime Alpes) a site with a high level of competition between the meadow and the tropical alga *Caulerpa taxifolia* [24]; Marseille (Bouches du Rhône) a site characterized by heavy grazing pressure by the herbivors *Sarpa salpa* and *Paracentrotus lividus* [25]; Figari (southern Corsica) a site located under aquaculture facilities (cages).

Using the classification system outlined by [26], the leaves of *Posidonia oceanica* were separated into three parts based on leaf age and tissue type: (i) intermediate leaf blades (leaves longer than 50 mm and lacking a leaf sheath); (ii) adult leaf blades (leaves longer than 50 mm, but possessing a leaf sheath); (iii) leaf sheaths of adult leaves. The epiphytes present on the foliar tissues were removed by scraping with a razor blade after which the tissues were lyophilized.

Extraction

The extraction method adopted here is derived from that used in the study of terrestrial plants by [27] and adapted to marine phanerogams by [10]: the *Posidonia oceanica* leaves were homogenized in 50% aqueous EtOH in the absence of light. The homogenate was filtered, and the filtrate acidified with a few drops of 2 N HCl. After evaporation of the ethanol under vacuum using a Rotavapor, the aqueous residue was extracted with EtOAc. Finally, the organic phase was dried using anhydrous Na_2SO_4, filtered and then evaporated under vacuum using a Rotavapor. The dry residue was then dissolved in 1 ml of MeOH, ready for analysis by HPLC.

HPLC analysis

The liquid chromatograph was equipped with a steel column (250 mm × 4 mm, Hibar RT Lichrosorb) prepacked with Lichrosorb RP 18, and a pre-column (30 mm × 4 mm, Lichrocart). Elution is carried with a

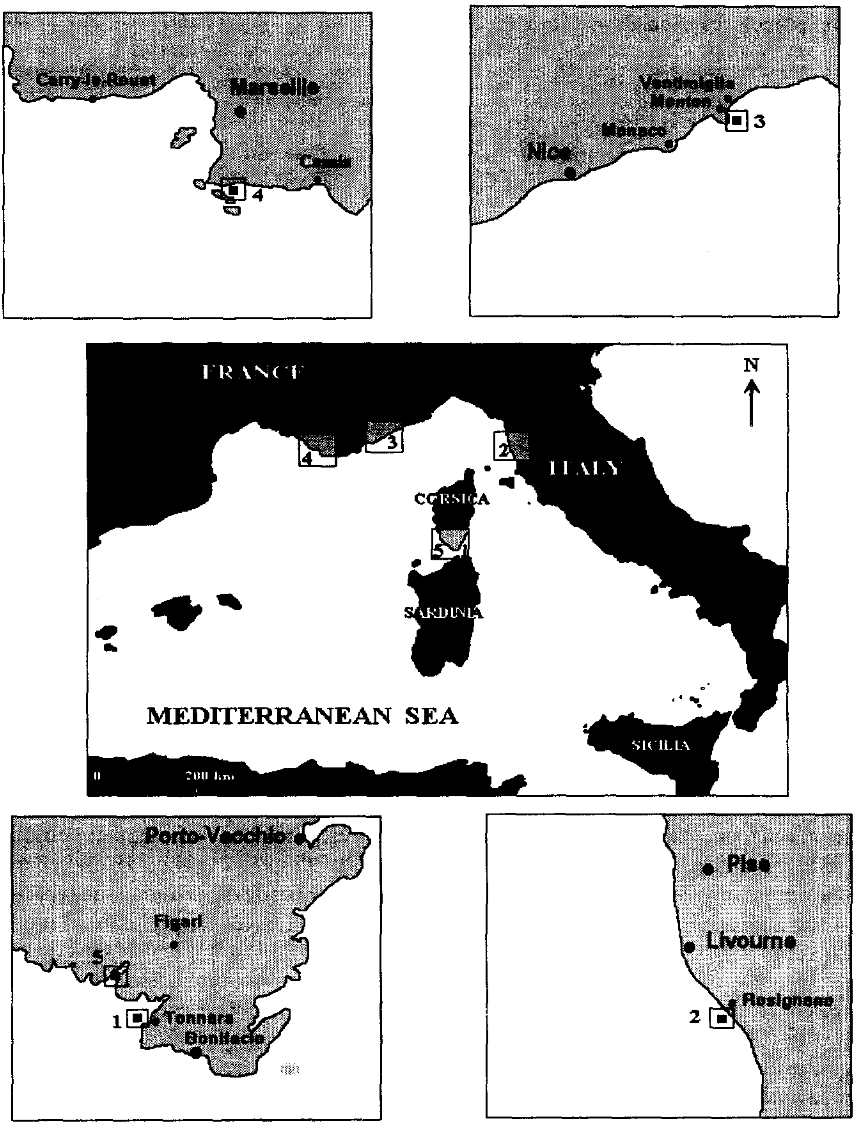


Fig. 4. Localization of the *Posidonia oceanica* sampling sites.

binary gradient and is obtained by the mixing of two eluents of differing polarity, running from 100% of solvent A: H_2O—HOAc—CH_3CN (97.5:2:0.5) to 100% of solvent B: H_2O—HOAc—CH_3CN (73:2:25). Into 130 min the elution flow rate is set at 1 ml min^{-1}.

Phenol determination

The detection of phenolic compounds was performed by UV spectrometry (LC-85 Detector Perkin–Elmer) at a wavelength of 275 nm. The identification of each phenolic compound was determined by comparing its retention time with that of reference compounds (Table 1). For each extract, the percentage abundance of the different phenolic compounds was obtained. This percentage was calculated from the area under the identified peaks of the chromatogram. The quantitative analysis of the phenolic compounds was obtained by external standardization. For each of the reference phenolic compounds, a standardization curve was determined.

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