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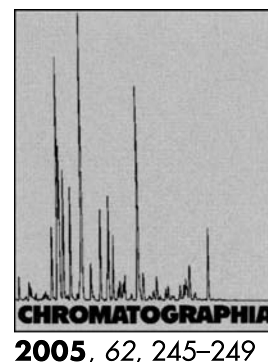
Simultaneous LC-DAD and LC-MS Determination of Ellagitannins, Flavonoid Glycosides, and Acyl-Glycosyl Flavonoids in *Cistus salvifolius* L. Leaves



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Abstract

A rapid and inexpensive HPLC method has been developed for simultaneous separation of the three main classes of polyphenol in the leaves of *Cistus salvifolius* L. Time devoted to extraction of polyphenols, which was performed using small volume of solvent, did not exceed 120 min. We identified three ellagitannins (punicalagin and related compounds), a total of ten glycosyl derivatives of quercetin and myricetin, and two coumaroyl glucosyl kaempferols by use of both diode-array detection (DAD) and mass spectrometry. The polyphenol composition of C. salvifolius leaves, which may contribute to the metabolic plasticity of the species, may explain its distribution in infertile soils of the Mediterranean area, and may also indicate this shrub is an important source of metabolites of potential use in human health care.

Keywords

Column liquid chromatography
Ellagitannins
Punicalagin
Myricetin and quercetin glycosides
Coumaroyl glucosyl kaempferols

Introduction

Species of the genus *Cistus* are mostly distributed in Mediterranean-type ecosystems and are therefore exposed to a wide-range of environmental injury, particularly during the dry and warm summer season [1–3]. As a consequence, the evolutionary adaptation of *Cistus* species to such extreme ecosystems has largely depended on efficient secondary metabolism, particularly polyphenol metabolism. In fact, polyphenols have been widely reported to protect plants effectively against environmental stress of both abiotic and biotic origin [4–6]. Remarkably, polyphenols may also affect soil nitrogen dynamics by reducing nitrogen mobility and hence serve a key function in the adaptation of most slow-growing Mediterranean shrubs, for example *Cistus* species, to unfertile (nitrogen-deficient) soils [7, 8].

Identification and quantification of polyphenols, particularly flavonoids, in the genus *Cistus* have mainly focussed on exudates or secreted materials from the dense covering of leaf trichomes [9–11]. Although *Cistus* species have usually been studied because of their physiological responses to environmental agents [12–14], and because of their use in folk medicine [15, 16], analysis of their internal 'soluble' polyphenols (excluding wall-bound polyphenols, which are commonly released after alkaline hydrolysis) has received little attention [17]. The whole-leaf polyphenol content is, however, usually used to effectively counter the detrimental effects of different environmental conditions [18–20] and, at the same time, to confer potential therapeutic value to tissue extracts [21, 22].

In our opinion, there is an urgent need both for rapid and small-scale analytical methods for identification and quantification of polyphenols for routine analysis of plant biology. There is an increasing body of evidence that polyphenol biosynthesis is up-regulated by, and counters, oxidative damage, and hence, plant physiology and biochemistry may be strictly linked to human healthcare [23–25].

In the experiment reported here we analysed, for the first time, the whole spectrum of polyphenols in the leaf blade of *C. salvifolius*, a facultative-evergreen shrub widely distributed on the seashore dunes of Italian coasts. We used a simple and rapid method to extract and purify small samples (less than 100 mg fresh leaf) and performed preliminary identification of the very complex polyphenol pool using both HPLC–DAD and HPLC–MS analysis.

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Table 1. The linear solvent gradient used for HPLC–DAD and HPLC–MS analysis of polyphenols in *Cistus salvifolius* leaves. Compounds were separated on a 4.6 mm × 250 mm Polaris E, RP_{18} (5 μm) column at 27 ± 0.5 °C; the mobile phase flow rate was 1.0 mL min^{-1} and the analysis time 53 min

H_2O/H^+, pH 3.2 (%)	CH_3CN (%)	Time (min)
95	5	0.1
85	15	20
85	15	25
75	25	35
75	25	43
0	100	53

Experimental

Sample Preparation and Extraction of Polyphenols

Plant Material

Fully-expanded leaves were sampled from adult plants of *Cistus salvifolius* L. growing on sea-shore dunes of Southern Tuscany (42 ° 46′ N, 10 ° 53′ E) at monthly intervals from May until August. Leaves were collected from both the apical and basal portions of the current-year shoot at midday and combined in an individual sample. Petioles were removed and leaf lamina were rapidly frozen in liquid nitrogen and stored at -80 °C until analysis.

Standards

Authentic standards of quercetin 3-*O*-glucoside (isoquercetin), quercetin 3-*O*-galactoside (hyperoside), and myricetin 3-*O*-rhamnoside (myricitrin) were purchased from Extrasynthèse (Lyon, Nord-Genay, France), and ellagic acid from Fluka (Sigma-Aldrich, Milano, Italy). Natural compound standards punicalagin, myricetin 3-*O*-galactoside, myricetin 3-*O*-(6″-*O*-galloylgalactoside), quercetin 3-*O*-(6″-*O*-galloylgalactoside), and quercetin 3-*O*-glucuronide were isolated by semi-preparative HPLC–DAD as described below.

Solvents

All solvents were of HPLC-grade purity (BDH, Bristol, UK).

Sample Preparation

Lyophilised leaf tissue (30–40 mg) was extracted, at room temperature, over a 45-min period, with 3 × 5 mL 70% EtOH adjusted to pH 3.5 with formic acid. The raw ethanol extract was then defatted with 2 × 5 mL *n*-hexane, dried at room temperature under reduced pressure by use of a Savant Speedvac Plus SC210A equipped with a refrigerated vapour trap (Thermo Quest, Holbrook, NY, USA), and rinsed with 70% EtOH adjusted to pH 3.5 with formic acid, to a final volume of 1.0 mL. Separation and identification of individual polyphenols were conducted on 20-μL volumes by use of both HPLC–DAD and HPLC–MS.

Flavonoid glycosides and quercetin 3-*O*-glucuronide were isolated from leaf tissues collected from myrtle and grape, respectively [26–28]. Punicalagin and other ellagitannins were isolated from 25 g fresh leaf material from *C. salvifolius*, which was extracted with 3 × 150 mL 70% EtOH, partitioned with 3 × 150 mL *n*-hexane, and reduced to a 10 mL under reduced pressure at room temperature by use of a 144R Rotavapor (Buchi, Flawil, Switzerland). The aqueous solution was then applied into a Sephadex LH-20 column (40 cm × 3.8 cm i.d., Amersham Biosciences, Uppsala, Sweden) and fractionated by consecutive elution with water, 30% and 50% MeOH, and 10%, 30%, 50% and 70% Me_2CO, in accordance with previous work [29].

Analytical Techniques and Equipment

HPLC–DAD analysis was performed with an HP 1100L liquid chromatograph equipped with a diode-array detector and managed by an HP Chemstation (all from Agilent Technologies, California, USA). Polyphenols were separated on a 4.6 mm × 250 mm Polaris E RP_{18} (5 μm) column (Varian, Germany) at 27 ± 0.5 C. The mobile phase was a four-step linear gradient prepared from water (adjusted to pH 3.2 with formic acid) and CH_3CN; the composition was changed from 95% H_2O to 100% CH_3CN in 53 min (Table 1). The flow rate was 1.0 mL min^{-1}.

HPLC–MS analysis was performed using the same analytical conditions as for HPLC–DAD analysis. In detail, the HPLC–DAD was interfaced with an HP 1100 MSD API-electrospray (Agilent Technologies) operating in the negative-ion mode with nitrogen gas temperature 350 °C, nitrogen flow rate 10 L min^{-1}, nebulizer pressure 30 psi, quadrupole temperature 30 °C, and capillary voltage 3500 V. The mass spectrometer fragmentor potential was 140 eV and 300 eV for flavonoids and ellagitannins, respectively.

The ellagitannins fractions obtained from the Sephadex column were separated with a Perkin–Elmer LC-410 liquid chromatograph equipped with an ABI 159-A UV–visible spectrophotometer (Applied Biosystems, 759A) operating at 380 nm. The column was a 250 mm × 10 mm Polaris E RP_{18} (5 ± m) (Varian); the column temperature was 27 ± 0.5 °C. The mobile phase gradient was identical with that used for HPLC–DAD analysis and the flow rate was 5.0 mL min^{-1}.

Results and Discussion

The extraction and purification procedures (total time 120 min) tested in this work enabled rapid analysis of the three main classes of polyphenol (Fig. 1) in *C. salvifolius* leaves. Samples were, furthermore, ready for HPLC analysis within one day's work. In addition, the very low concentrations of both chlorophyll and carotenoids (chlorophyll on a dry weight basis averaged 1.88 mg g^{-1}) and waxes in *C. salvifolius* enabled the use of small volumes of *n*-hexane to remove lypophilic compounds from the EtOH–H_2O extract. Our method thus seems particularly suitable for plant biology experiments, in which several small samples (often an individual leaf) must be analysed in a short time. Nevertheless, our method enabled elucidation of the very complex polyphenol composition of *C. salvifolius* leaves in only one HPLC analysis.

We detected three main classes of polyphenol in the leaves of *C. salvifolius*—ellagitannins, flavonol glycosides, and acetyl glycosyl flavonols (Fig. 1). Six ellagitannins (compounds 1–6) were detected in the leaves of *C. salvifolius* and their identification was attempted by use of both UV–visible and mass spectral data. The UV–visible spectra reported in Figs. 2a and 2c all contained the very peculiar peak at approximately 380 nm, which led to conclusion that ellagic acid was present [30]. Peaks 1 and 4 (Fig. 1) were consistent with the presence of the α and β anomers of punicalagin [30, 31]. The fragmentation pattern reported in Fig. 2b consisted of the molecular ion at

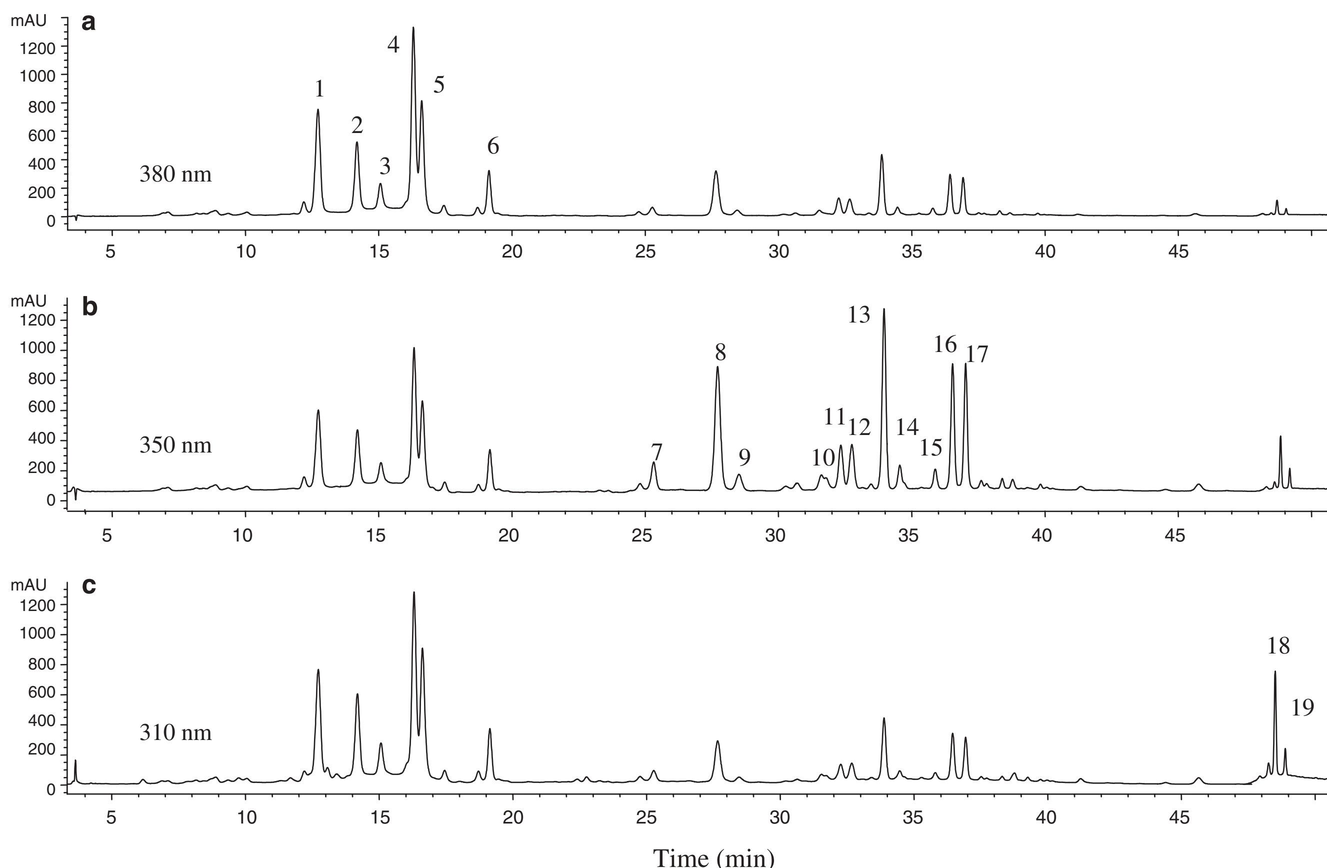

Fig. 1. Typical chromatograms obtained by HPLC–DAD from a 20-μL injection of an ethanol extract of *C. salvifolius* leaves. Chromatograms were acquired at the wavelengths of maximum absorbance of the ellagitannins (**a**, 380 nm), quercetin and myricetin derivatives (**b**, 350 nm), and coumaroyl glucosyl kaempferols (**c**, 310 nm). Analytical conditions are reported in Table 1. Phenol peaks: **1–6** = ellagitannins, **7** = myricetin 3-*O*-(6″-*O*-galloylgalactoside), **8** = myricetin 3-*O*-galactoside, **9** = quercetin xylosil glucoside, **10** = quercetin 3-*O*-(6″-*O*-galloylgalactoside), **11**, **12** = myricetin xyloside, **13** = quercetin 3-*O*-galactoside, **14** = quercetin 3-*O*-glucuronide, **15–17** = quercetin xylosides, **18** = mono-coumaroyl kaempferol glucoside, **19** = di-coumaroyl kaempferol glucoside

m/*z* 1083, the fragment after loss of ellagic acid at *m*/*z* 781, the fragment corresponding to gallagic acid at *m*/*z* 601 (which originated from the loss of both ellagic acid and glucose from punicalagin), and that of ellagic acid at *m*/*z* 301. The mixture of compounds 1 and 4, isolated by use of semi-preparative HPLC, was fully identified by comparison of their ^{1}H and ^{13}C NMR data with those reported in the literature for α,β-punicalagin [31]. The mass spectra of ellagitannins 2 and 5 and ellagitannins 3 and 6 (Fig. 2d), both with a molecular ion at *m*/*z* 1251, were consistent with the α and anomers of a punicalagin-like compound with addition of gallic acid. The carboxyl group of gallic acid did not actually bind to punicalagin, because the fragments at *m*/*z* 1207 and *m*/*z* 1082 correspond to loss of a carboxyl group and gallic acid itself, respectively.

The presence of water-soluble ellagitannins, which may also be released from the leaf trichomes of *Cistus* spp. [8], may effectively increase the availability of nitrogen in nitrogen-deficient soils, where *C. salvifolius* is widely distributed [3]. We also note that ellagitannins probably contribute substantially to the antioxidant properties of crude aqueous leaf extracts of *C. incanus* and *C. monspeliensis* [22] (the leaves of both species have contain ellagitannins; Saracini, unpublished results) although some authors have (erroneously) associated antioxidant activity exclusively with the flavonoid content.

The flavonoid pool in *C. salvifolius* leaves also seemed of particular interest, because of the simultaneous presence of three flavonol classes with different OH substitution in the B-ring of the flavonoid skeleton, from kaempferol to myricetin derivatives (Fig. 1b). Identification of individual metabolites was performed by comparison of retention times, UV–visible, and MS data with those of authentic standards (hyperoside) or isolated compounds. The 3-*O*-galactosides and 3-*O*-(6″-*O*-galloylgalactosides) of both myricetin (peaks 7, 8) and quercetin (peaks 10, 13) have previously been identified and quantified in the leaf tissues of *Myrtus communis* [26, 27], and quercetin 3-*O*-glucuronide (peak 14) has been identified and quantified in grape pomace [28]. Partial identification of other flavonoid glycosides, xylosides of both myricetin (peaks 11, 12) and quercetin (peaks 9, 15–17), was achieved by LC–MS analysis.

The kaempferol derivatives in *C. salvifolius* leaves, identified as coumaroyl glucosyl derivatives, also seemed of particular interest. First, UV spectra of peaks 18 and 19 (Fig. 3a) overlapped those of coumaric acid (maximum molar extinction coefficient, ε, at 310 nm) and kaempferol (maximum ε in the UV-A region at 367 nm). The mass spectrum in Fig. 3b seemed consistent with that of a mono-coumaroyl glucoside of kaempf-

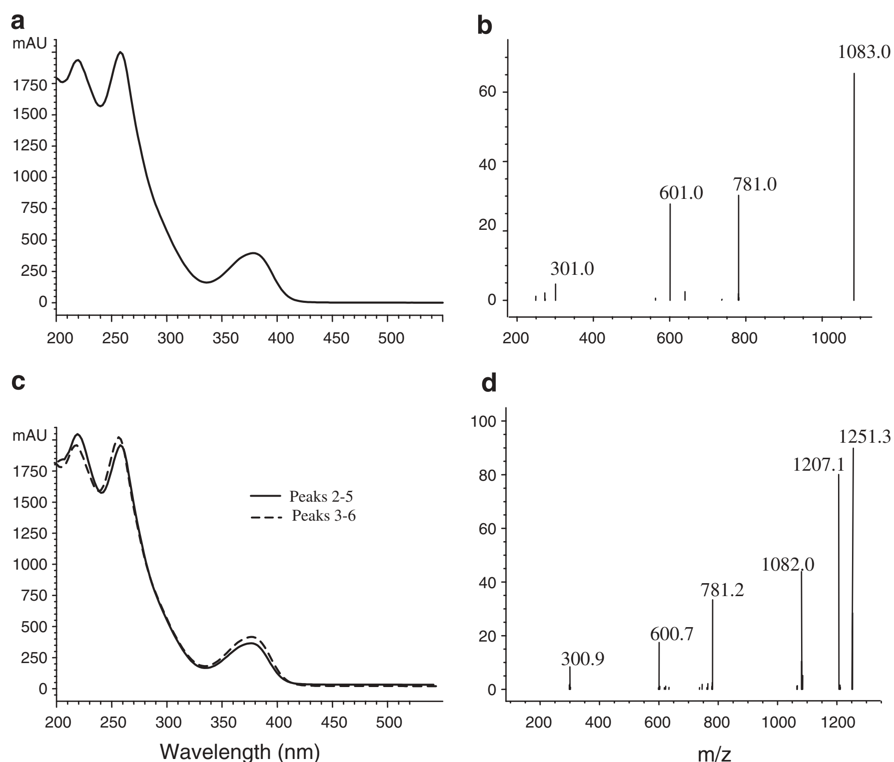

Fig. 2. UV–visible (a, c) and MS (b, d) spectral data of ellagitannins detected in *C. salvifolius* leaves. Figures a and b are the UV spectrum and MS fragmentation pattern of the α and β anomers of punicalagin (peaks 1 and 4). In Figs c and d spectral data for peaks 2 and 5 and peaks 3 and 6 are reported; these are indicative of galloyl derivatives of punicalagin. HPLC–MS operating conditions: nitrogen gas temperature 350 °C, nitrogen flow rate 10.0 L min^{-1}, nebulizer pressure 30 psig, quadrupole temperature 30 °C, and capillary voltage 3500 V. The fragmentor potential was 300 eV

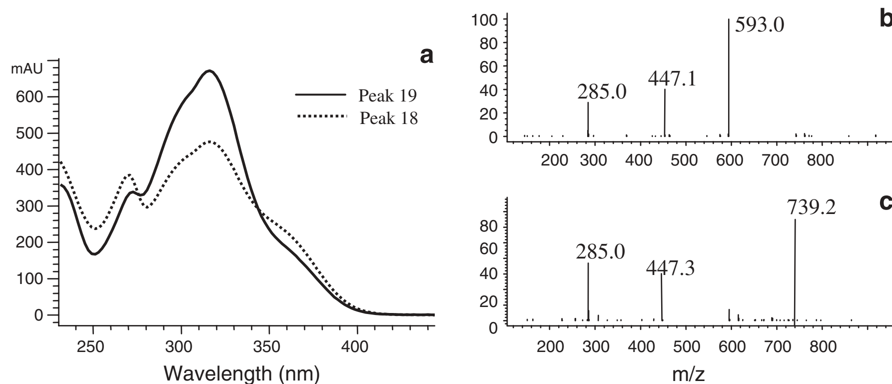

Fig. 3. UV–visible (a) and MS spectral data for both a mono (b, peak 18) and a di-coumaroyl (c, peak 19) kaempferol glucoside. HPLC–MS conditions were as reported in Fig. 2. The fragmentor potential was 140 eV

erol (*m*/*z* 593), with the fragments at *m*/*z* 447 and *m*/*z* 285 corresponding to successive loss of coumaric acid and glucose, respectively, from the quasi-molecular ion. UV–visible and MS data of peak 19 were indicative of the occurrence of a di-coumaroyl glucosyl kaempferol (see ε at 310 nm compared with peaks 18 and 19). In fact, in Fig. 3c the quasi-molecular ion fragment at *m*/*z* 739 seemed to have originated from compound 18 after addition of a coumaroyl moiety. The di-acylated kaempferol 3-*O*-glucosides, with 3″,6″ substitution of the glucose ring, have been reported to occur in Scots pine needles [32, 33].

The ability to synthesize a wide range of secondary metabolites with potentially different functional roles has been termed "metabolic plasticity" [34], and may be a key biochemical feature of a plant's strategy enabling adaptation to sunny and dry ecosystems [25]. In fact, the soft leaves of *C. salvifolius* may suffer from high solar radiation-induced oxidative damage to a much greater extent than those experienced by other Mediterranean species, the morpho-anatomical features of which are more effective at screening out highly damaging UV radiation [24, 25]. Nevertheless the simultaneous presence of ellagitannins, which are efficient free-radical quenchers [35, 36], and flavonoid glycosides, which may be less effective in capturing free electrons but quite efficient at inhibiting the formation of free radicals [37, 38], may actually preserve sensitive targets in *C. salvifolius* leaves from photochemical damage. As a consequence, Mediterranean shrubs like *Cistus salvifolius* not only serve a key ecological function in the stabilization of highly-disturbed ecosystems, but may be a very interesting, and still not fully explored, source of metabolites with potential use in human healthcare.

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