Simultaneous Determination of Catechins, Rutin, and Gallic Acid in *Cistus* Species Extracts by HPLC with Diode Array Detection

Natale Alfredo Santagati[1,*], Loredana Salerno[1], Giuseppina Attaguile[2], Francesca Savoca[3], and **Giuseppe Ronsisvalle[1]**

[1]Dipartimento di Scienze Farmaceutiche, Università di Catania. Viale A. Doria 6, 95125 Catania, Italy; [2]Dipartimento di Farmacologia Clinica Sperimentale, Università di Catania. Viale A. Doria 6, 95125 Catania, Italy; and [3]Dipartimento di Botanica, Università di Catania. Via A. Longo 19, 95100 Catania, Italy

Abstract

A simple high-performance liquid chromatography method using a diode array detector (DAD) is developed for the simultaneous analysis of five major catechins: (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GCT), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), and the phenolic plant metabolites gallic acid (GA) and rutin (RT) in lyophilized extracts of *Cistus* species. The optimal analytical conditions are investigated to obtain the best resolution and the highest UV sensitivity for the quantitative detection of catechins. The optimized conditions (acetonitrile–phosphate buffer 50mM, pH 2.5, gradient elution system on a C_{18} reversed-phase column with a flow rate of 1 mL/min and UV absorbance at 210 nm) allowed a specific and repeatable separation of the studied analytes to be achieved. All compounds are successfully separated within 32 min. Calibration curves are linear in the 2–50 µg/mL range for GCT, C, and EGCG and in the 5–50 µg/mL range for GA, EGC, EC, and RT. The limit of detection values ranged from 0.24 to 0.74 µg/mL. The limit of quantitation limit values ranged from 0.77 to 1.94 µg/mL. The validated method is applied to the determination of the specific phytochemical markers GA, GCT, C, and RT in *Cistus incanus* and *Cistus monspeliensis* lyophilised extracts. The recovery values ranged between 78.7% and 98.2%. The described HPLC method appears suitable for the differentiation and determination of the most common catechins together with the glycoside rutin and the phenolic compound gallic acid and can be considered an effective and alternative procedure for the analyses of this important class of natural compounds.

Introduction

Cistus incanus and *Cistus monspeliensis* are two species of genus Cistus (Cistaceae) widely present in the Mediterranean area (1). Cistus species are used in folk medicine as a general remedy for treatment of various diseases (2). Studies led by F. Petereit et al. (3) indicate the principal components of Cistus species were polyphenolic compounds, particularly monomeric flavonoids belonging to the flavan-3-ols or catechins family such as catechin, gallocatechin, gallocatechin-3-gallate together with oligomeric procyanidin B1 and B3. Another phytochemical study on Cistus leaves revealed the presence of five different flavonoid aglycones and glycosides compounds belonging to the flavonols family (4). Moreover, proanthocyanidins and biogenetically related dihydroflavonols (5), as well as shikimic acid, epicatechin-(4→6)-catechin, dimeric prodelphinidins (6), and further polyphenols (7,8) were also found in different Cistus species.

Polyphenols are known to have beneficial effects on the human health (9). The antioxidant activity of several polyhydroxylated flavonoids (10–12) and their antitumor, antimicrobial, antiviral, and enzyme inhibiting activities have been demonstrated (13). The inhibition of low-density lipoprotein (LDL) (14) and the possible protection against coronary heart diseases and strokes have been also described (15,16). Several studies carried out on different Cistus species demonstrated gastroprotective effects for *Cistus incanus* (17) and Cistus laurifolius (18), anti-inflammatory activity for *Cistus incanus* (19,20), protective effect on DNA cleavage and dose-dependent free-radical scavenging capacity for *Cistus incanus* and *Cistus monspeliensis* (21), cytotoxic activity against several human leukemic cell lines in vitro for Cistus creticus (22) and *Cistus monspeliensis* (23) and antibacterial and antifungal activities for *Cistus incanus* and *Cistus monspeliensis* leaf extracts (24).

Traditionally, the analysis of catechins in tea or plant extracts has been undertaken by high-performance liquid chromatography (HPLC) with UV detection, and the gradient elution is the most frequently used method (25–31).

Other studies used a post-column derivatization by reaction with a cinnamaldehyde derivative and detection at absorbance of 640 nm (32) or by using an electrochemical detector (33,34).

Isocratic elution HPLC methods to separate catechins and gallic acid in tea infusions were described (35,36) but some less polar catechins showed broadening and tailing of peaks.

Moreover, new techniques such as liquid chromatography coupled with mass spectrometry (LC–MS) or capillary electrophoresis have been introduced in this field. LC–MS has been applied to the analysis of catechins and other polyphenols (37–39). Capillary zone electrophoresis has been used for anal-

* Author to whom correspondence should be addressed: email: santagat@unict.it.

ysis of catechins in tea samples (40,41). Several micellar electrokinetic chromatographic methods with UV detection have been developed for the separation of catechins and other phenolic compounds (42–49). Besides a new variant of MEKC, the microemulsion electrokinetic chromatography (MEEKC) has been successful applied to the separation of catechins in extracts of Cistus species (50).

Recently, the analyses of catechins together with compounds contained in Japanese green tea (51), in black tea, green tea or oolong tea aqueous infusions (52) and in green tea leaves and bottle green tea (53) were investigated. Presently, there are not any HPLC-diode array detection (DAD) methods to analyse the catechins in *Cistus incanus* and *Cistus monspeliensis* extracts.

In the present study, a gradient reversed-phase HPLC method with DAD was developed for the analysis of five catechins: (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GCT), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), and other components such as rutin (RT) and gallic acid (GA) potentially contained in the examined Cistus species. Figure 1 depicts the structures of the analyzed compounds. The optimum condition was investigated for the best resolution and highest sensitivity of UV detection of all studied compounds. The described HPLC method appears useful for the differentiation and determination of the most common flavan-3-ols together with the glycoside rutin and the phenolic compound gallic acid. The method was applied to the determination of C, GCT, RT, and GA in *Cistus incanus* and *Cistus monspeliensis* lyophilised extracts.

Experimental

Plant materials and extraction procedure

Cistus incanus L. ssp. incanus and *Cistus monspeliensis* L. were harvested in May 2003 from a wild study area located near Catania, Italy.

The plants were identified by Dott. F. Savoca of the Department of Botany, University of Catania, Italy.

Aerial parts of *Cistus incanus* and *Cistus monspeliensis* (20.0 g each) were air dried at 40°C and powdered using a pulverizing mill. A known amount of material (4 g each) was extracted 3 times with boiling water (3 × 150 mL). The combined extracts were filtered through a Buckner sintered-glass filter funnel and lyophilised. The final yields were in the 13.4%–14.5% range.

The brown solid lyophilized residues were stored at –20°C and dissolved in water-methanol (4:1) for the analysis. The solutions were filtered through 0.45-µm Nylon membrane filter before the use.

Chemicals

C, EC, EGC, GCT, EGCG, and GA were purchased from Sigma (St. Louis, MO). RT was obtained from Extrasynthèse (Lyon, France). Acetonitrile, methanol, and water were HPLC-grade and were obtained from Carlo Erba (Milano, Italy). All other chemicals and reagents were purchased from Carlo Erba Reagenti (Milan, Italy) and were analytical-reagent grade. Distilled and deionized water was used for the preparation of all samples and solutions and they were used after filtration through HA filters (0.45 µm Millipore, Bedford, MA).

HPLC apparatus and chromatographic conditions

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT) Series 200 pump equipped with a LC-235C Diode Array Detector (DAD), auto-sampler, and column oven. Chromatographic data were processed with Turbochrom Workstation software, version 6.1.2. Separation was accomplished on a 5 µm Hypersil ODS RP-18 column (150 × 4.6 mm; Supelco, Bellefonte, PA) fitted with a guard column (Hypersil ODS RP-18, 5 µm particles; 10 × 4.6 mm; Supelco, Bellefonte, PA) and eluted with acetonitrile–phosphate buffer 50mM adjusted to pH 2.5. The gradient elution was performed by varying the proportion of solvent A (phosphate buffer) to solvent B (acetonitrile) with a flow rate of 1 mL/min. The mobile phase composition started at 100% solvent A for 2 min, followed by a gradual increase (the type of gradient curve is convex with degree of curvature of –3) of solvent B to 40% in 32 min. The column was then re-equilibrated with the initial conditions for 3 min before the next run. The total analysis time was 35 min per injection, which included a 3 min allowance for column equilibration prior to the next injection. The mobile phase was prepared daily and filtered through HA filters (0.45 µm). The detection wavelength was 210 nm, and the

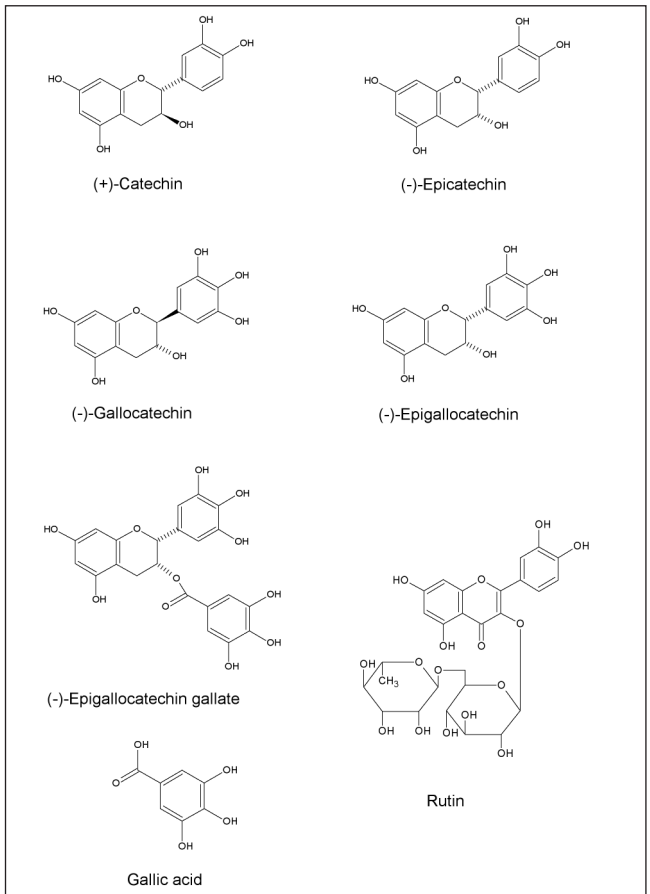

Figure 1. depicts the structures of the five catechin compounds analyzed: (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GCT), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG) and other components such as rutin (RT) and gallic acid (GA) potentially contained in examined of Cistus species.

reference wavelength was set at 380 nm. The DAD acquisition wavelength was set in the range of 200-400 nm. Analog output channel A at wavelength 210 and analog output channel B at wavelength 280 nm both with bandwidth 20 nm were fixed. The injection volume was 10 µL. All the samples were filtered through 0.45-µm membrane filter and degassed by an ultrasonic bath prior the injection. The elution order of analytes was GA, GCT, EGC, C, EC, EGCG, and RT. The oven temperature was maintained at 30°C.

Standard solutions

Stock solutions (1 mg/mL) of analytes C, EC, EGC, GCT, EGCG, GA, and RT were prepared from pure compounds by dissolving each compound in water–methanol (4:1). Working standard solutions were prepared daily from stock solution by dilution with the appropriate volume of mobile phase (80% of solvent A and 20% of solvent B). All solutions were stored in a refrigerator at 4°C and used within five days.

Precision

The precision of the method was assessed by multiple injections of a single solution of all analytes at two different levels. The first level was the intra-day precision or repeatability and was determined within the same day, in a single analysis on a single instrument. The second level was the inter-day precision or intermediate precision and was determined over three days, on the same instrument. The obtained results were expressed as relative standard deviations (RSDs, $n = 5$) at concentration level of 30 µg/mL.

Calibration curves

The linearity of the response was evaluated for the principal potential components of *Cistus* extracts by analysis of standard solutions of GCT, C, and EGCG in the 2–50 µg/mL range and of GA, EGC, EC, and RT in the 5–50 µg/mL range. Triplicate injections were made for each standard solution. The calibration curves, constructed by means least-squares linear regression analyses, were obtained by plotting the peak area ratios of each analyte versus its concentration.

Limit of detection and limit of quantitation

The baseline noise was calculated by Perkin-Elmer Turbochrom software and signal-to-noise (S/N) ratio values were determined dividing each peak height by the noise. The values of limit of detection (LOD) and quantitation (LOQ) were determined with an injected volume of 10 µL from five runs, using progressively lower concentrations of analytes for a S/N ratio of approximately 3 and of approximately 10, respectively.

Sample analysis

The developed HPLC method was applied to the analysis of sample solutions of *Cistus incanus* and *Cistus monspeliensis* from plants collected in a wild study area located near Catania, Italy.

All the sample solutions were prepared in water–methanol (4:1) from lyophilised extracts (5 mg/mL), were left to stand for 2 min in an ultrasonic bath and were filtered through 0.45 µm membrane filter. The obtained solutions were then diluted with appropriate volume of mobile phase (80% of solvent A and 20% of solvent B), and subjected to the HPLC analysis. The content of (+)-catechin (C), (–)-gallocatechin (GCT), gallic acid (GA) and rutin (RT) were determined by comparison with the appropriate standard solutions. The external standard method was used for the quantitation of analytes. The found values are reported.

Identification of analytes

The peaks of C, GCT, GA, and RT were identified comparing the retention times against the standards and through addiction of small amount of the standards substances to the sample. Moreover, the evaluation of the peak purity was made. To check the peak purity, the eluates were monitored with DAD (λ = 200–400 nm). The three spectra corresponding to the upslope, apex, and downslope of each peak were computer normalized and superimposed. Peak were considered pure when there was exact coincidence between the three spectra (match factor ≥ 99.0).

Recovery studies

The recovery efficiency was evaluated by adding measured amounts of pure standards GA, GCT, C, and RT to *Cistus incanus* and GA, GCT, and C to *Cistus monspeliensis* lyophilised extracts sample solutions and, after the described treatment (Sample analysis section), by analyzing the fortified sample solutions with the proposed HPLC method. The recoveries were determined by subtracting the values obtained for the control matrix from those of the samples prepared with the added standards. Each lyophilized extract was added of 30 µg/mL of the suitable pure analyte and was injected three times. The calculated amount recovered and the mean values with standard deviations are reported.

Results and Discussion

Chromatographic performance and sensitivity

The optimized separation of the principal potential catechins of *Cistus* extracts was performed with the first aim to develop a relatively rapid and reproducible HPLC method for the resolution of the principal catechins. Particular attention was, nevertheless, focused on the specific separation of C and GCT, that are the marker phytochemicals in the *Cistus incanus* and *Cistus monspeliensis* extracts (50). In order to develop a method able to meet the previous requirements, the effects of binary gradient elution on the performance of separation and sensitivity of the studied analytes were evaluated.

Although the method was mainly designed for the separation and quantitation of the major catechins present in *Cistus* species, others potential components such as RT and GA were separated and quantified by using the appropriate standards. For optimization purposes the following seven analytes were chosen: C, EC, EGC, GCT, EGCG, GA, and RT. Both isocratic and gradient elution program with methanol–acetate buffer, methanol–phosphate buffer, and acetonitrile–phosphate buffer phases were tested. By isocratic elution, all studied catechins could not be well separated. Particularly, it was not possible to separate

completely EGC from C and EC from EGCG. Moreover, the separation time was over 60 min and the last component (RT) was broadened. A gradient elution program using acetonitrile–phosphoric acid–water (pH 2.5) as solvent was finally chosen. Figure 2 represents a chromatogram of a standard mixture of the five catechins and GA and RT. The catechins are chemically unstable in alkaline environment, and the catechins stability was improved by using an acidic pH buffer. Thus, the presence of acid in the mobile phase is essential to both complete resolution of the components present in the mixture and efficient chromatography of analytes, specifically, the elimination of peak tailing. All studied compounds were successfully separated within 32 min. The total analysis time was 35 min per injection, which included a 3 min allowance for column equilibration prior the next injection.

In HPLC analysis of catechins, different detection methods have been tried. Several authors performed HPLC analysis of catechins by using a conventional UV detector of varying wavelength. It has been reported that an optimal detection sensitivity for different catechins could be obtained with absorbance at 280 nm (31,52,54). Another study demonstrated a higher sensitivity at UV 205 nm (55). Dalluge et al. (27), Bronner et al. (30), and Mizukami et al. (53) mentioned the use of 210 nm, while Goto et al. (25) used 231 nm. In the proposed study, the UV spectra of all studied compounds were reviewed using DAD. The DAD acquisition wavelength was set in the range 200–400 nm. The results demonstrated that an higher sensitivity was obtained in the range 205–210 nm with the use of acetonitrile instead of methanol as the eluent. Under the described conditions (HPLC apparatus and chromatographic conditions section), we achieved best results at wavelength of 210 nm.

Table I Intra- and Inter-Day Precision of the Retention Times and Peak Areas (n = 5) for the Studied Analytes (Concentration: 30 mg/mL)*

	Intra-day Precision		Inter-day precision	
Analyte	t_r (min) (RSD %)	Peak area (RSD %)	t_r (min) (RSD %)	Peak area (RSD %)
GA	4.16 (0.98)	84209 (1.12)	4.19 (1.04)	88062 (2.12)
GCT	8.98 (1.39)	104587 (2.49)	8.93 (1.33)	107197 (2.91)
EGC	19.43 (0.94)	104437 (1.44)	19.44 (1.10)	105837 (1.62)
C	20.80 (1.45)	108336 (2.75)	20.77 (1.42)	107934 (2.96)
EC	26.71 (1.28)	109015 (2.26)	26.88 (0.95)	107013 (2.03)
EGCG	27.70 (1.44)	126334 (2.68)	27.82 (1.38)	123314 (2.37)
RT	31.40 (1.02)	123089 (1.77)	31.01 (1.49)	136010 (2.71)

* Experimental conditions were described in the HPLC apparatus and chromatographic conditions section.

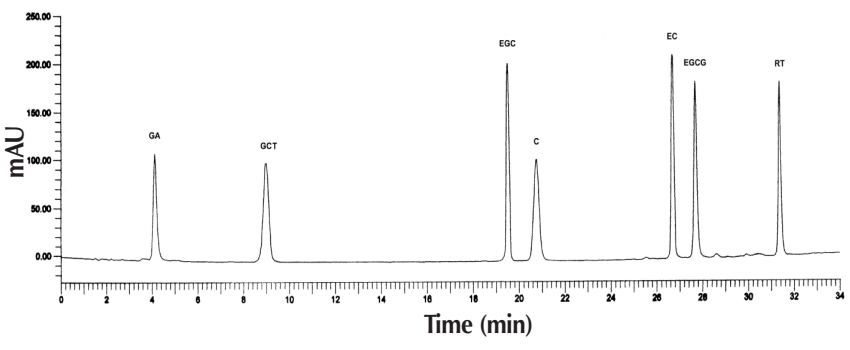

Figure 2. Representative chromatogram of a standard mixture of the studied catechins, gallic acid and rutin (30 µg/mL of each analyte) at 210 nm. Peaks: gallic acid (GA), gallocatechin (GCT), epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG), and rutin (RT).

Method validation

The developed HPLC method was validated under the optimized experimental conditions (acetonitrile–phosphate buffer 50mM, pH 2.5, gradient elution system on a C_{18} reversed-phase column with a flow rate of 1 mL/min and UV absorbance at 210 nm). In order to evaluate the precision of the method five replicate analyses of all compounds were performed on the single standard solution (30 µg/mL for each compound). The results (Table I) show that RSD values obtained for intra-day precision were in the same order of magnitude as those obtained for inter-day precision. In all cases, the values of the RSD were less than 1.5% for the retention times, and less than 3.0% for the quantitation. For quantitative applications, the response linearity was verified for all studied potential components of *Cistus* species extracts. The linearity of the method was investigated over a concentration range of 2–50 µg/mL for GCT, C and EGCG and 5–50 µg/mL for GA, EGC and RT. The correlation coefficients of the standard curves linear regression were greater than 0.996. The obtained linearity data are shown in Table II. The LOD corresponding to a S/N of approximately 3 was evaluated for the same analytes by progressive dilution. The LOD values ranged from 0.24 to 0.74 µg/mL. The LOQ corresponding to a S/N of approximately 10 was also evaluated for the same analytes. The LOQ values ranged from 0.77 to 1.94 µg/mL (Table II). These data support the suitability of the proposed HPLC method for its application to real samples.

Applications to Cistus species extracts

Samples of lyophilized extracts were used to prepare solutions as described in the Sample analysis section. The developed HPLC–DAD method was applied to the identification and quantitation of C, GCT, GA, and RT in lyophilized extracts of *Cistus incanus* and *Cistus monspeliensis*. Representative chromatograms obtained from the analyzed samples of *Cistus incanus* and *Cistus monspeliensis* are reported in Figures 3 and 4, respectively. The peak identity for the found compounds in the plant samples was confirmed by the retention time values, by the standard addition method, and on the basis of the on-line recorded UV spectra (DAD), which were found to be overimposable to those from standards. Only the retention times obtained for the standard C and for the same compound

in real samples were lightly different; this could be due to matrix effects. In addition, a peak purity assessment was conducted by processing every peak through Turbochrom Workstation software. In both samples gallic acid, C and GCT were found. The presence of rutin only in *Cistus incanus* was assessed. As shown in Table III, different levels of C and GCT were found in analyzed samples. In fact, C and GCT were found at higher concentration in the *Cistus monspeliensis* species than in the *Cistus incanus* species, whereas an higher concentration of GA in the *Cistus incanus* samples was observed. The levels of the found catechins were rather similar to previous investigation (50), considering the usual compositional variations of vegetal samples.

In order to evaluate the accuracy of the method, a known amount of the pure compounds found in the studied *Cistus* species were added (within the linearity range method) to lyophilised extracts. Then, the fortified samples were analysed with the proposed HPLC method. The recoveries of the added standard compounds given in Table IV ranged from 78.7% to 98.2%, with RSD within approximately 5%. Therefore, interferences by other matrix components are not significant, and the HPLC conditions are suitable to obtain adequate method accuracy.

Conclusion

The developed chromatographic method showed to be able to provide an efficient and repeatability separation of an important class of natural compounds, such as the catechins, together with gallic acid and rutin. The method was found to be suitable for the determination of specific catechins: (+)-catechin, (–)-gallocatechin and secondary metabolites rutin and gallic acid in complex matrices such as lyophilised samples obtained from *Cistus incanus* and *Cistus monspeliensis* species. The described technique represent a useful contribution in the field of phytochem-

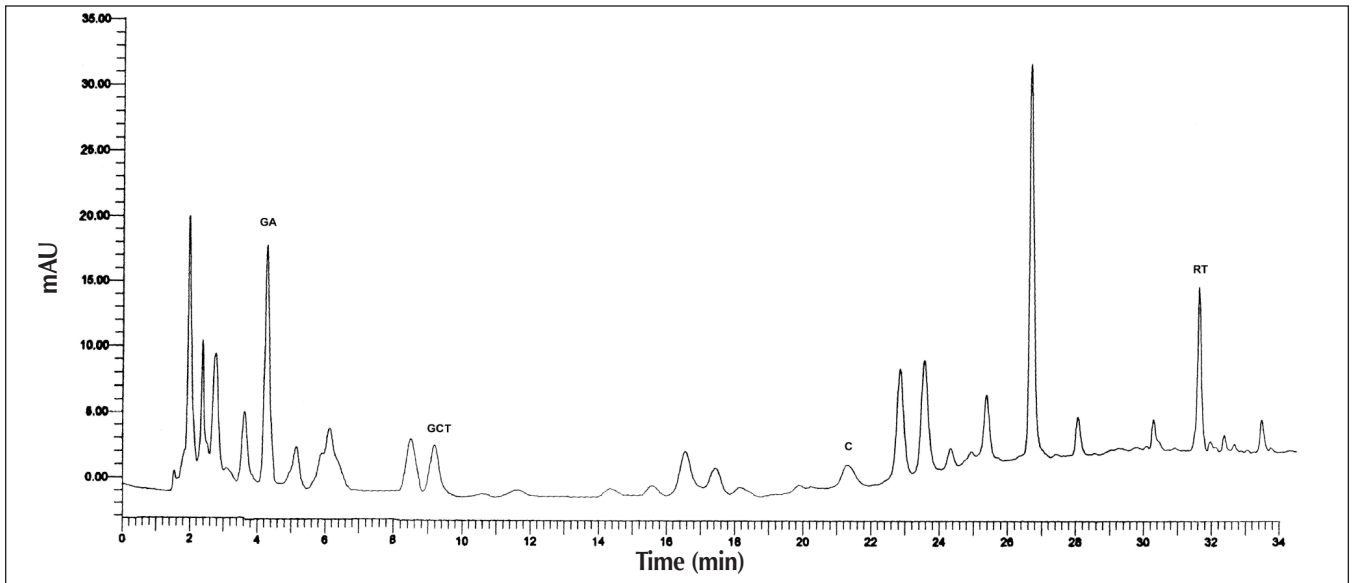

Figure 3. Typical chromatogram of a lyophilized extract of Cistus incanus at 210 nm. Peaks: gallic acid (GA), gallocatechin (GCT), catechin (C), and rutin (RT).

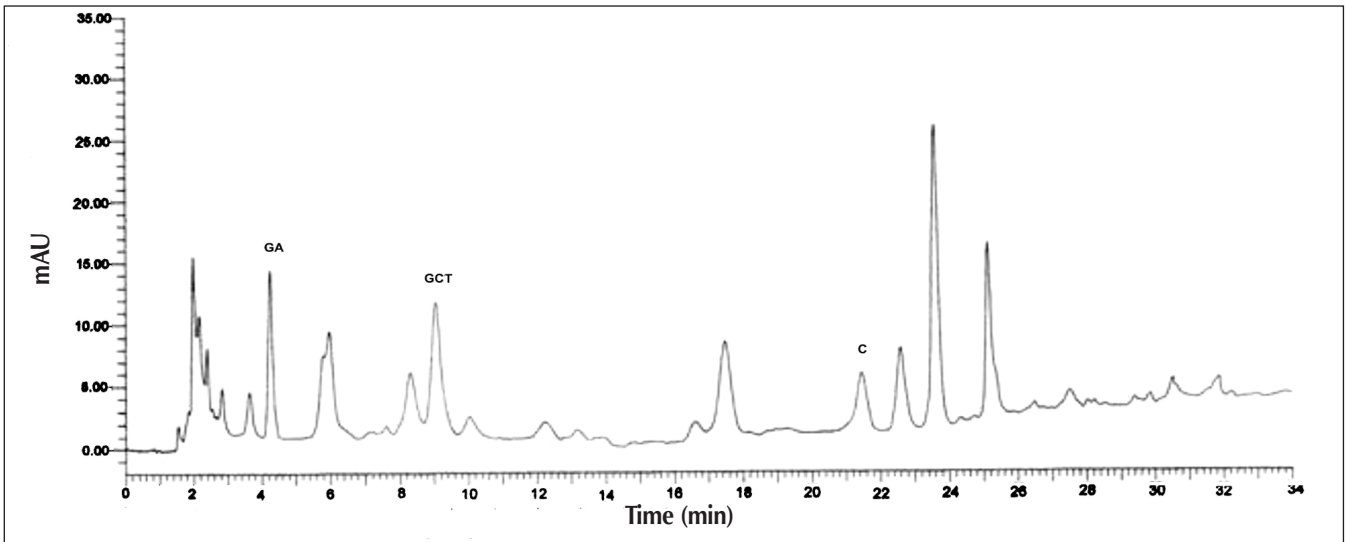

Figure 4. Typical chromatogram of a lyophilized extract of Cistus monspeliensis at 210 nm. Peaks: gallic acid (GA), gallocatechin (GCT), catechin (C).

Table II. Linearity Data*, LOD, and LOQ Values for the Studied Analytes

Analyte	Concentration range (µg/mL)	a	b	R^2	LOD† (µg/mL)	LOQ‡ (µg/mL)
GA	5–50	2.920 · 103 (± 102.6)	–35.935 (± 23.53)	0.9981	0.41	1.26
GCT	2–50	3.617 · 103 (± 141.4)	–360.276 (± 32.33)	0.9977	0.24	0.77
EGC	5–50	8.315 · 103 (± 299.2)	–2310.139 (± 68.56)	0.9980	0.53	1.66
C	2–50	3.670 · 103 (± 125.1)	–73.337 (± 28.61)	0.9982	0.27	0.85
EC	5–50	1.970 · 104 (± 505.2)	2554.362 (± 115.77)	0.9968	0.74	1.94
EGCG	2–50	1.242 · 104 (± 532.2)	1735.751 (± 121.71)	0.9988	0.59	1.78
RT	5–50	3.005 · 103 (± 122.3)	635.308 (± 28.04)	0.9975	0.31	0.97

* Regression curve data for five calibration points. y = ax + b, where y is the peak area, x is the concentration (µg/mL), a is the slope, b is the intercept and R^2 is the correlation coefficient.
† Limit of Detection, as ~ 3 S/N.
‡ Limit of Quantitation, as ~ 10 S/N.

Table III. Determination of GA, GCT, C, and RT in Different *Cistus* Specie Samples (*n* = 5)

Analyte	*Cistus incanus* (µg/g) (RSD %)	*Cistus monspeliensis* (µg/g) (RSD %)
GA	11.94 (3.45)	8.14 (2.10)
GC	3.10 (2.54)	10.36 (3.09)
C	1.58 (4.04)	4.07 (4.88)
RT	6.04 (2.75)	N.D.*

* N.D. = not detected.

Table IV Recoveries (%) for GA, GCT, C, and RT from *Cistus* species lyophilised extracts (*n* = 3)

Analyte	*Cistus incanus* Mean recovery (RSD %)	*Cistus monspeliensis* Mean recovery (RSD %)
GA	98.2 (2.67)	89.2 (3.48)
GCT	78.7 (5.02)	90.6 (4.83)
C	84.8 (4.49)	92.5 (3.52)
RT	91.1 (2.36)	*N.A.

* Not Added.

icals analysis and can be considered of general applicability in the analysis of *Cistus* species or other plant species containing these compounds.

Acknowledgment

This work was supported by "Ministero dell'Università e della Ricerca (MUR)", Rome, Italy.

References

1. P.G. Gulz, T. Herrmann, and K. Hangst. Leaf trichomes in the genus *Cistus*. *Flora*. **191:** 85 (1996).
2. R. Hegnauer. Chemotaxonomie der Pflanzen, III, Birkhäuser Verlag, Basel, Stuttgart, 1964.
3. F. Petereit, H. Kolodziej, and A. Nahrstedt. Flavan-3-ols and proanthocyanidins from Cistus incanus. *Phytochem.* **30:** 981 (1991).
4. C. Demetzos, S. Mitaku, F. Hotellier, and A. Harvala. Heterosides polyphenoliques des feuilles de Cistus creticus L. *Ann. Pharm. Fr.* **47:** 314 (1989).
5. F. Petereit, H. Kolodziej, and A. Nahrstedt. Proanthocyanidins and biogenetically related dihydroflavonols from Cistus incanus L. B*asic Life Sci.* **59:** 729 (1992).
6. A. Danne, F. Petereit, and A. Nahrstedt. Proanthocyanidins from Cistus incanus. *Phytochem.* **34:** 1129 (1993).
7. A. Danne, F. Petereit, and A. Nahrstedt. Flavan-3-ols, prodelphinidins and further polyphenols from Cistus salvifolius. *Phytochem.* **37:** 533 (1994).
8. E. Saracini, M. Tattini, M.L. Traversi, F.F. Vincieri, and P. Pinelli. Simultaneous LC-DAD and LC-MS determination of ellagitannins, flavonoid glycosides, and acyl-glycosyl flavonoids in Cistus salvifolius L. leaves. *Chromatographia.* **62:** 245 (2005).
9. M.T. Huang, C.T. Ho, C. Lee, editors, in: Phenolic Compounds in Food and Their Effects on Health, II: Antioxidants and Cancer Prevention, American Chemical Society, Washington DC, 1992.
10. Y.T. Chen, R.L. Zheng, Z.J. Jia, and Y. Ju. Flavonoids as superoxide scavengers and antioxidants. Free Rad. Biol. Med. 9: 19 (1990).
11. J. Robak, R.J. Gryglewski. Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **37:** 837 (1988).
12. G. Sichel, C. Corsaro, M. Scalia, A.J. Di Bilio, and R.P. Bonomo. In vitro scavenger activity of some flavonoids and melanins against O2-(.). *Free Rad. Biol. Med.* **11:** 1 (1991).
13. D. Pathak, K. Pathak, and A.K. Singla. Flavonoids as medicinal agents – recent advances. *Fitoterapia.* **5:** 371 (1991).
14. C.V. de Whalley, S.M. Rankin, R.S. Hoult, W. Jessup, and D.S. Leake. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem. Pharmacol.* **39:** 1743 (1990).
15. M.G. Hertog, E.J. Feskens, P.C. Hollman, M.B. Katan, and D. Kromout. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly study. *Lancet.* **342:** 1007 (1993).
16. M.G. Hertog, D. Kromhout, and C. Aravanis. Flavonoids intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med.* **155:** 381 (1995).
17. G. Attaguile, A. Caruso, G. Pennisi, and F. Savoca. Gastroprotective effect of aqueous extract of Cistus incanus L. in rats. *Pharmacol. Res.* **31:** 29 (1995).
18. E. Yesilada, I. Gurbuz, and E. Ergun. Effects of Cistus laurifolius L. flowers on gastric and duodenal lesions. *J. Ethnopharmacol.* **55:** 201 (1997).
19. F. Petereit, A. Nahrstedt, B. Innerlich, N.P. Lupke. N.L. Theissen, F.H Kemper, and H. Winterhoff. Antiinflammatory activity of the traditionally used herb of Cistus incanus. *Planta Medica*. **55:** 650 (1989).
20. C. Demetzos, K. Dimas, S. Hatziantoniou, T. Anastasaki, and D. Angelopoulou. Cytotoxic and anti-inflammatory activity of labdane and cis-clerodane type diterpenes. *Planta Medica*. **67:** 614 (2001).
21. G. Attaguile, A. Russo, A. Campisi, F. Savoca, R. Acquaviva, N. Ragusa, and A. Vanella. Antioxidant activity and protective effect on DNA cleavage of extracts from Cistus incanus L. and Cistus monspeliensis L. *Cell Biology Toxicol.* **16:** 83 (2000).
22. K. Dimas, C. Demetzos, M. Marsellos, R. Sotiriadou, M. Malamas, and D. Kokkinopoulos. Cytotoxic activity of labdane type diterpenes against human leukemic cell lines in vitro. *Planta Medica*. **64:** 208 (1998).
23. K. Dimas, C. Demetzos, D. Angelopoulou, A. Kolokouris, and

T. Mavromoustakos. Biological activity of myricetin and its derivatives against human leukemic cell lines in vitro. *Pharmacol. Res.* **42:** 475 (2000).
24. H. Bouamama, J. Villard, A. Benharref, and M. Jana. Antibacterial and antifungal activities of Cistus incanus and Cistus monspeliensis leaf extracts. *Therapie.* **54:** 731 (1999).
25. T. Goto, Y. Yoshida, M. Kiso, and H. Nagashima. Simultaneous analysis of individual catechin and caffeine in green tea. *J. Chromatogr. A* **749:** 295 (1996).
26. A.C. Hoefler and P. Coggon. Reversed-phase high-performance liquid chromatography of tea constituents. *J. Chromatogr.* **129:** 460 (1976).
27. J.J. Dalluge, B.C. Nelson, J.B. Thomas, and L.C. Sander. Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. *J. Chromatogr. A* **793:** 265 (1998).
28. R. Saijo and Y. Takeda. HPLC analysis of catechins in various kinds of green teas produced in Japan and abroad. *Nippon Shokuhin Kagaku Kogaku Kaishi.* **46:** 138 (1999).
29. F. Hashimoto, G. Nonaka, and I. Nishioka. Tannins and related compounds. LXXVII. Novel chalcan-flavan dimers, assamicains A, B and C, and a new flavan-3-ol and proanthocyanidins from the fresh leaves of Camellia sinensis L. var. assamica Kitamura. *Chem. Pharm. Bull.* **37:** 77 (1989).
30. W.E. Bronner and G.R. Beecher. Method for determining the content of catechins in tea infusion by high-performance liquid chromatography. *J. Chromatogr. A* **805:** 137 (1998).
31. Y. Zuo, H. Chen, and Y. Deng. Simultaneous determination of catechins, caffeine and gallic acids in green, oolong, black and pu-erh teas using HPLC with photodiode array detector. *Talanta.* **57:** 307 (2002).
32. D. Treutter. Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. *J. Chromatogr.* **467:** 185 (1989).
33. K. Umegaki, T. Esashi, M. Tezuka, A. Ono, M. Sano, and I. Tomita. Determination of tea catechins in food by HPLC with an electrochemical detector. *J. Food Hyg. Soc. Jpn.* **37:** 77 (1996).
34. K.O. Chu, C.C.C. Wang, Y. Chu, M.S Rogers, K.W. Choy, and C.P. Pang. Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction. *J. Chromatogr. B* **810:** 187 (2004).
35. H. Wang, K. Helliwell, and X. You. Isocratic elution system for the determination of catechins, caffeine and gallic acid in green tea using HPLC. *Food Chem.* **68:** 115 (2000).
36. Y.K. Lin, C.L. Lin, Y.C. Liang, S.Y. Lin-Shiau, and I.M. Jaun. Survey of catechins, gallic acid and methylxanthines in green, oolong, pu-erh, and black teas. *J. Agric. Food Chem.* **46:** 3536 (1998).
37. Y.Y. Lin, K.J. Ng, and S. Yang. Characterization of flavonoids by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **629:** 389 (1993).
38. A. Kiehne and U.H. Engelhardt. Thermospray-LC-MS analysis of various groups of polyphenols in tea: I. Catechins, flavonol O-glycosides and flavone C-glycosides. *Z. Lebensm. Unters. Forsch.* **202:** 48 (1996).
39. J.J. Dalluge, B.C. Nelson, J.B.Thomas, M.J. Welch, and L.C. Sander. Capillary liquid chromatography/electrospray mass spectrometry for the separation and detection of catechins in green tea and human plasma. *Rapid Communic. Mass Spectrom.* **11:** 1753 (1997).
40. H. Horie, T. Mukai, and K. Kohata. Simultaneous determination of qualitatively important components in green tea infusions using capillary electrophoresis. *J. Chromatogr. A* **758:** 332 (1997).
41. L. Arce, A. Rios, and M. Valcarcel. Determination of anti-carcinogenic polyphenols present in green tea using capillary electrophoresis coupled to a flow injection system. *J. Chromatogr. A* **827:** 113 (1998).
42. B.C. Nelson, J.B. Thomas, S.A. Wise, and J.J. Dalluge. The separation of green tea catechins by micellar electrokinetic chromatography. *J. Microcol. Sep.* **10:** 671 (1998).
43. M.B. Barroso and G. van de Werken. Determination of green and black tea composition by capillary electrophophoresis. *J. High Resolut. Chromatogr.* **22:** 225 (1999).
44. P.J. Larger, A.D. Jones, and C. Dacombe. Separation of tea polyphenols using micellar electrokinetic chromatography with diode array detection. *J. Chromatogr. A* **799:** 309 (1998).
45. T. Watanabe, R. Nishiyama, A. Yamamoto, S. Nagai, and S. Terabe. Simultaneous analysis of individual catechins, caffeine, and ascorbic acid in commercial canned green and black teas by micellar electrokinetic chromatography. *Anal. Sci.* **14:** 435 (1998).
46. H. Horie and K. Kohata. Application of capillary electrophoresis to tea quality estimation. *J. Chromatogr. A* **802:** 219 (1998).
47. C.C.T. Worth, M. Wiessler, and O.J. Schmitz. Analysis of catechins and caffeine in tea extracts by micellar electrokinetic chromatography. *Electrophoresis.* **21:** 3634 (2000).
48. D. Stach and O.J. Schmitz. Decrease in concentration of free catechins in tea over time determined by micellar electrokinetic chromatography. *J. Chromatogr. A* **924:** 519 (2001).
49. J.P. Aucamp, Y. Hara, and Z. Apostolides. Simultaneous analysis of tea catechins, caffeine, gallic acid, theanine and ascorbic acid by micellar electrokinetic capillary chromatography. *J. Chromatogr. A* **876:** 235 (2000).
50. R. Pomponio, R. Gotti, N.A. Santagati, and V. Cavrini. Analysis of catechins in extract of Cistus species by microemulsion electrokinetic chromatography. *J. Chromatogr. A* **990:** 215 (2003).
51. T. Yoshida and R.E. Majors. High-speed analyses using rapid resolution liquid chromatography on 1,8-µm porous particles. *J. Sep. Sci.* **29:** 2421 (2006).
52. A.P. Neilson, R.J. Green, K.V. Wood, and M.G. Ferruzzi. High-throughput analysis of catechins and theaflavins by high performance liquid chromatography with diode array detection. *J. Chromatogr. A* **1132:** 132 (2006).
53. Y. Mizukami, Y. Sawai, and Y. Yamaguchi. Simultaneous analysis of catechins, gallic acid, strictinin, and purine alkaloids in green tea by using catechol as an internal standard. *J. Agr. Food Chem.* **55:** 4957 (2007).
54. J. Harms and G. Schwedt. Application of capillary electrophoresis in element speciation analysis of plant and food extracts. *Fresenius J. Anal. Chem.* **350:** 93 (1994).
55. B.L. Lee and B.N. Ong. Comparative analysis of tea catechins and theaflavins by high-performance liquid chromatography and capillary electrophoresis. *J. Chromatogr. A* **881:** 439 (2000).

Manuscript received July 9, 2007;
revision received October 23, 2007.