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Analysis of catechins in extracts of *Cistus* species by microemulsion electrokinetic chromatography

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Abstract

A microemulsion electrokinetic chromatographic (MEEKC) method was developed for the separation of six catechins, specific marker phytochemicals of *Cistus* species. The MEEKC method involved the use of sodium dodecyl sulfate (SDS) as surfactant, heptane as organic solvent and butan-1-ol as co-solvent. In order to have a better stability of the studied catechins, the separation was performed under acidic conditions (pH 2.5 phosphate buffer). The effects of SDS concentration and of the amount of organic solvent and co-solvent on the analyte resolution were evaluated. The optimized conditions (heptane 1.36% (w/v), SDS 2.31% (w/v), butan-1-ol 9.72% (w/v) and 50 m*M* sodium phosphate buffer (pH 2.5) 86.61% (w/v)) allowed a useful and reproducible separation of the studied analytes to be achieved. These conditions provided a different separation profile compared to that obtained under conventional micellar electrokinetic chromatography (MECK) using SDS. The method was validated and applied to the determination of catechin and gallocatechin in lyophilized extracts of *Cistus incanus* and *Cistus monspeliensis*.

Keywords: *Cistus* species; Microemulsion electrokinetic chromatography; Plant materials; Catechins; Gallocatechins

1. Introduction

The genus *Cistus* (Cistaceae family) includes many typical species of Mediterranean flora. *Cistus* species are used as general remedies in folk medicine for treatment of various skin diseases and as anti-inflammatory agents. Phytochemical studies on different *Cistus* species have revealed the presence of several flavonoid compounds [1,2] with an anti-oxidant activity [3].

The antifungal activity of *Cistus incanus* extract was attributed to the presence of condensed tannins and the aqueous extract from *Cistus incanus* was found to have a gastroprotective effect. The main component of *Cistus* species are polyphenolic compounds, commonly known as catechins, which represent a group of compounds belonging to the flavonoid family. These compounds have shown several biological activities including anti-inflammatory, antiallergic, antiplatelet, antiviral and antitumoral. Epidemiological studies have shown a correlation between a higher content of bioflavonoids (catechins) in the diet and a lower risk of cancer and cardiovascular diseases, due to their ability to protect against the damaging action of free radicals [3].

The analysis of catechins in plant extracts has traditionally been carried out by reversed-phase liquid chromatography (HPLC) with UV detection [4–7].

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Recently, capillary electrophoresis (CE), due to its high-resolution separation and versatility, has become an effective alternative to HPLC for the separation of charged analytes and the potential of this technique in the field of natural product analysis is well documented [8–13]. For the determination of catechins, capillary zone electrophoresis (CZE) [14,15] and micellar electrokinetic chromatography (MEKC) [16–22] with UV detection are the most applied approaches.

In all instances, uncoated fused-silica capillaries have been used, and in general, the MEKC methods provide better separation, resolution and quantitation for a larger number of catechins than do the CZE methods.

Microemulsion electrokinetic chromatography (MEEKC) [23–26] is a relatively new variant of CE. The MEEKC separation is based on the partitioning of neutral or charged analytes into moving oil droplets, negatively charged by a surfactant (SDS) coating. The microemulsion stability is usually improved by adding a co-solvent such as an alcohol (e.g. 1-butanol). These MEEKC systems are characterized by UV transparence, allowing detection at low UV wavelength to be performed.

In the present study, a MEEKC system was developed for the separation of (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-gallocatechin, (−)-epigallocatechin gallate and (−)-epicatechin gallate, specific marker phytochemicals of *Cistus* species (Fig. 1). The MEEKC method involved the use of SDS as surfactant, heptane as organic solvent and butan-1-ol as co-solvent. In order to have a better stability of the studied catechins, the separation was performed under acidic conditions (pH 2.5 phosphate buffer 50 m*M*) and reverse polarity. The effects of SDS concentration, amount of organic solvent and co-solvent on the analyte resolution were evaluated. The optimised conditions (heptane 1.36% (w/v), SDS 2.31% (w/v), butan-1-ol 9.72% (w/v) and 50 m*M* sodium phosphate buffer (pH 2.5) 86.61% (w/v)) allowed a useful and reproducible separation of the studied analytes to be achieved and provided a different separation profile compared to that obtained under the conventional MEKC using SDS. The method was validated and applied to the determination of catechin and gallocatechin in lyophilized extracts of *Cistus incanus* and *Cistus monspeliensis*.

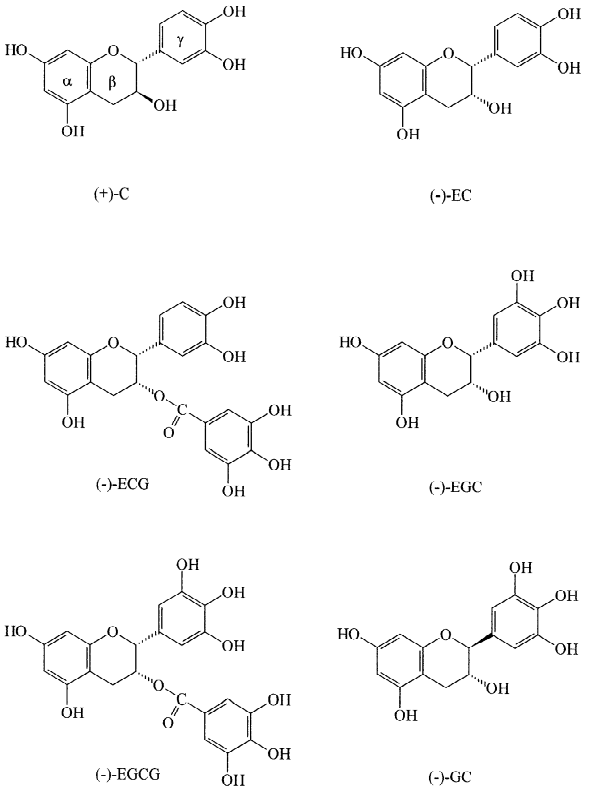

Fig. 1. Structures of the examined catechins.

2. Experimental

2.1. Plant materials

Cistus incanus L. ssp. *Incanus* and *Cistus monspeliensis* L. were harvested from a wild study area near Caltagirone (Catania, Italy) in May, 1998 and in May, 2001.

The plants were identified by Professor C. Barbagallo Furnari of the Department of Botany, University of Catania, Italy.

Aerial parts of *C. incanus* and *C. 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 three times with boiling water (3×150 ml). The combined extracts were filtered through a Buchner sintered-glass filter funnel and lyophilized. 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 for the analysis.

2.2. Chemicals

(+)-Catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-gallocatechin (GC), (−)-epigallocatechin gallate (EGCG) and (−)-epicatechin gallate (ECG) were obtained from Sigma (St. Louis, MO, USA); SDS was from Fluka (Buchs, Switzerland). Heptane and butan-1-ol were purchased from Aldrich (Milwaukee, WI, USA). Phosphoric acid, methanol, sodium hydroxide and all the other chemicals were purchased from Carlo Erba (Milan, Italy). The water used for preparation of the solutions and running buffers was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

2.3. Capillary electrophoresis apparatus and conditions

Electrophoretic analyses were carried out using a Biofocus 2000 system (Bio-Rad, Hercules, CA, USA). A ^{3D}CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector, was also used to acquire on line the UV spectra. The data were collected on a personal computer using a Biofocus System Integration Software Version 5.2. An untreated, fused-silica capillary of total length 24 cm (effective length 19.5 cm)×50 μm I.D. was used for separation. All separations were carried out at 40 °C with an optimized voltage maintained at −10 kV (reverse polarity); hydrodynamic injections were performed at 1 p.s.i. for 1 s (1 p.s.i.=6894.76 Pa) and the detection wavelength was 200 nm.

Prior to first use, the capillary was conditioned by flushing sequentially 1 *M* sodium hydroxide, 0.1 *M* sodium hydroxide and finally water (10 min each). The capillary was equilibrated (10 min) at the beginning of the day with the running buffer. The repeatability of migration times was found to be strongly dependent on the rinsing procedure; the highest reproducibility of the migration times was obtained by flushing the capillary between the runs as follows: 1 min with methanol, 1 min with 0.1 *M* sodium hydroxide, 1 min with water and 2 min with background electrolyte (BGE). Vials of BGE were replaced every injection to keep the same reservoir level of the buffer and to avoid changes of EOF due to the electrolysis of the solutions. The microemulsions were prepared by weighing 1.36% (w/v) of heptane, 9.72% (w/v) of butan-1-ol, 2.31% (w/v) of SDS and 86.61% (w/v) of 50 m*M* sodium phosphate buffer. This was then sonicated until all the SDS was dissolved. After this time, an optically transparent microemulsion had formed which was stable for a long time. The microemulsion was filtered through a 0.2 μm filter (GyroDisc, Orange Scientific, Waterloo, Belgium) to remove particulate matter.

2.4. Calibration graphs

Stock solutions (1 mg/ml) of (+)-catechin and (−)-gallocatechin were prepared in water and then diluted 1:10 to give the working standard solutions.

The linearity of the response was evaluated analysing standard solutions of (+)-catechin (2–6 μg/ml) and (−)-gallocatechin (15–20 μg/ml), containing siringic acid (the internal standard) at the fixed concentration of 15 μg/ml. Triplicate injections were made for each standard solution and the ratios of the corrected peak area (area/migration time) of drug to internal standard were plotted against the drug concentration to obtain the calibration graphs.

2.5. Sample analysis

The developed MEEKC method was applied to the analysis of sample solutions of: (a) *Cistus incanus* from plants collected in the 1998 and 2001 years, (b) *Cistus monspeliensis* from plants collected in 1998 and in 2001. All the sample solutions were prepared in water from lyophilized extracts which were completely soluble in this solvent. The sample solutions (5 mg/ml) were subjected to the MEEKC analysis and the content of (+)-catechin and (−)-gallocatechin was determined by comparison with an appropriate standard solution: (+)-catechin 6 μg/ml and (−)-gallocatechin 20 μg/ml.

3. Results and discussion

3.1. Method development

Previous papers report the application of CZE [14,15] and MEKC [16–22] to the separation of catechins, but most of these methods show long analysis times or poor resolution which are generally

considered unsuitable for routine analysis. Applications of the MEEKC approach have been reported for other analytes [23–33], but applications to the catechins are not available to our knowledge. Thus, the first aim of the present study was to provide a relatively rapid and reproducible MEEKC method for the resolution of the principal catechins. Therefore, a short (19.5 cm effective length) and narrow bore (50 μm) capillary and a high temperature (40 °C) with a voltage of −10 kV were used to reduce analysis time and limit the generation of excessive operating current (about −80 μA). Most papers describing MEEKC methods use the standard conditions (0.81% (w/v) of heptane or octane, 6.61% (w/v) of butan-1-ol, 3.31% (w/v) of SDS and 89.27% (w/v) of 10 m*M* sodium tetraborate buffer, pH 9–10) to obtain the microemulsion [26,31–33]. These conditions sometimes lead to poor reproducibility of the migration time and alkaline pH is not always compatible with the studied analytes; actually polyphenols have pK_a values between 8 and 10. The catechins are chemically unstable in an alkaline environment and this may cause the peak distortion. In this study, using an anodic outlet, we achieved best results by conditioning the capillary as described in Section 2.3; moreover the catechins' stability was improved using an acidic pH buffer (pH 2.5 after optimisation). Subsequently, the best SDS concentration value was investigated as well as the amounts of both organic solvent (heptane) and co-solvent (butan-1-ol). Finally, a comparison between MEEKC and MEKC in the same conditions (SDS 2.31% (w/v), but without heptane and butan-1-ol, and pH 2.5, 50 m*M*, sodium phosphate buffer 97.69% (w/v)) was carried out to confirm the usefulness of MEEKC.

3.2. Method optimisation

The optimisation of the separation of the chosen catechins was performed with the aim of developing a MEEKC method of general applicability; particular attention was nevertheless focused on the specific separation of (+)-catechin and (−)-gallocatechin, the marker phytochemicals in the *Cistus incanus* and *Cistus monspeliensis* extracts. In order to develop a method able to meet the previous requirements, the effects of the pH buffer value (range 2.5–4.5), SDS concentration (2.02–3.31% (w/v) or 70–115 m*M*) and amount of heptane and butan-1-ol (range 0.81–2.04% (w/v) and 6.61–12.96% (w/v), respectively) on the migration times of the studied analytes were evaluated.

3.2.1. Running buffer pH

Usually, the buffer pH is a very important parameter controlling the EOF and the ionization degree of each analyte. Due to their pK_a values (8.0–10), the catechins were completely undissociated and more stable [17,21] under the full studied pH range (2.5–4.5). The EOF was almost absent at pH 2.5, with a weak increment from pH 2.5 to 3.5 and, finally, when pH 4.5 was reached, the measured EOF remained constant (5×10^{-5} cm^2 s^{-1} V^{-1}). For each pH value, within the studied range, all analytes showed anodic migration due to their strong interaction with SDS micelles, but all the catechins exhibited a progressive increase of migration with higher pH values (Fig. 2) as a result of the increased EOF; pH values greater than 4.5 led to high EOF with loss of peak symmetry and excessive increase of migration times. Therefore, within the studied pH range, the best results were at pH 2.5 which allowed a good compromise between resolution and analysis time.

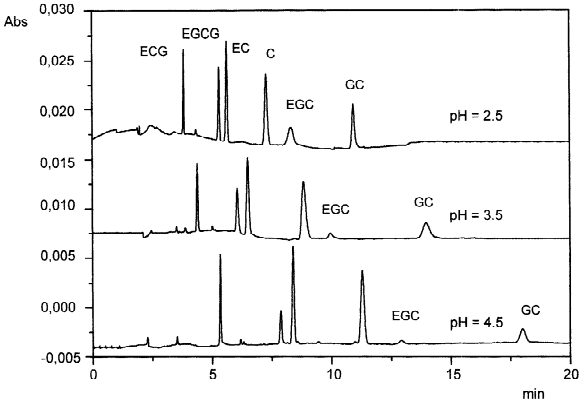

Fig. 2. Effect of the pH value on the catechins separation. Electrophoretic conditions: 0.81% (w/v) of heptane, 6.61% (w/v) of butan-1-ol, 3.31% (w/v) of SDS and 89.27% (w/v) of 50 m*M* sodium phosphate buffer. Other conditions: fused-silica capillary, total length 24 cm (effective length 19.5 cm)×50 μm I.D.; injection of 5 p.s.i. for 1 s; voltage, −10 kV; temperature, 40 °C; detection wavelength, 200 nm.

3.2.2. SDS concentration

The surfactant concentration is an important parameter controlling the analysis selectivity. During the optimisation of the method, a crucial step was the separation between (−)-epigallocatechin gallate and (−)-epicatechin. The effect of SDS concentration was evaluated over the 2.02–3.31% (w/v) concentration range (70–115 m*M*), using 0.81% (w/v) of heptane, 6.61% (w/v) of butan-1-ol and 89.27–90.56% (w/v) of 50 m*M* sodium phosphate buffer (pH 2.5). Actually, at the standard concentrations of SDS for MEEKC (3.31%, w/v), the separation between (−)-epigallocatechin gallate and (−)-epicatechin was very poor and the peak shapes were asymmetric. Decreasing SDS concentration, the peak shapes gradually improved with an optimum around 2.31% (w/v) of SDS concentration. These effects of SDS are illustrated in Fig. 3. For the routine analyses, 2.31% w/v SDS was chosen.

3.2.3. Heptane and butan-1-ol concentration

The role played by organic solvent and co-solvent, heptane and butan-1-ol, respectively, is fundamental in MEEKC [34]. In the present application, at the beginning the catechin separation was carried out under standard conditions of heptane and butan-1-ol concentration (0.81% and 6.61% (w/v), respectively), but large and asymmetric peaks were obtained

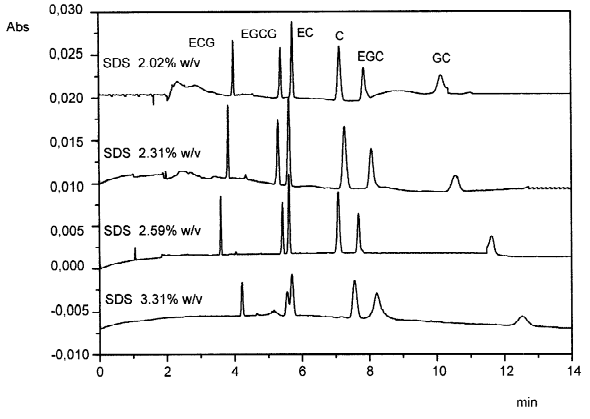

Fig. 3. Effect of the sodium dodecyl sulfate (SDS) concentration. Electrophoretic conditions: 0.81% (w/v) of heptane, 6.61% (w/v) of butan-1-ol and 89.27% (w/v) of 50 m*M* sodium phosphate buffer (pH 2.5). Other conditions as in Fig. 2.

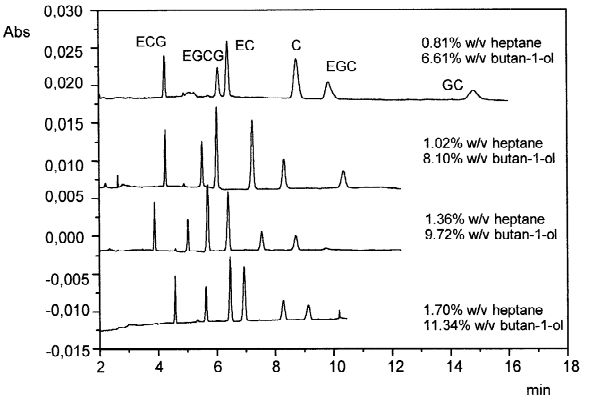

Fig. 4. Effect of the heptane and butan-1-ol concentration. Electrophoretic conditions: 2.31% (w/v) of SDS in 50 m*M* sodium phosphate buffer (pH 2.5). Other conditions as in Fig. 2.

with high migration time, especially for (−)-epigallocatechin and (−)-gallocatechin (Fig. 4).

The effect of organic solvent and co-solvent concentration on the MEEKC analysis was then evaluated and was found to be of interest, because an increased resolution between (−)-epigallocatechin gallate and (−)-epicatechin with a general reduction of the migration times was obtained after optimisation. From these observations, an active role on the separation by organic solvent and co-solvent can be confirmed regarding both the migration times and the peak shape. As shown in Fig. 4, concentrations of 1.36% (w/v) and 9.72% (w/v), for heptane and butan-1-ol, respectively, were chosen as the optimum conditions in terms of resolution, analysis times and peak shape.

3.3. Comparison between MEEKC and MEKC

In order to confirm the usefulness of the proposed MEEKC method, the studied catechins were subjected to an MEKC analysis, using the same conditions of BGE (2.31% (w/v) of SDS in 50 m*M* phosphate buffer, pH 2.5). As shown in Fig. 5, under MEKC conditions, there was a general loss of resolution and the peak shapes were worse than in MEEKC conditions. Interestingly, the MEKC and the MEEKC approaches provided a different selectivity.

Actually in MEEKC there were two inversions of migration times: between (−)-epicatechin and (−)-epigallocatechin gallate and between (−)-epigal-

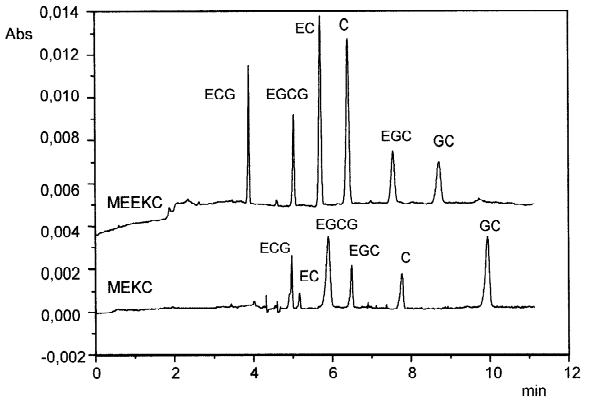

Fig. 5. Comparison between MEEKC and MEKC methods for the catechins separation. MEEKC conditions: heptane 1.36% (w/v), SDS 2.31% (w/v), butan-1-ol 9.72% (w/v) and 50 m*M* sodium phosphate buffer (pH 2.5) 86.61% (w/v). MEKC conditions: SDS 2.31% (w/v) in 50 m*M* sodium phosphate buffer (pH 2.5). Other conditions as in Fig. 2.

locatechin and (+)-catechin. In the developed MEEKC method, due to lack of EOF, the detection was anodic, thus the lipophilic analytes migrated faster than the hydrophilic analytes. Microemulsion droplets are formed from anionic surfactant (SDS), water-immiscible organic solvent (heptane) and co-solvent (butan-1-ol) in aqueous buffer solution. The microemulsion has a core of minute droplets of organic solvent with the surfactant and co-solvent located on the outside to stabilise the oil droplet. Among the studied catechins, the differences in lipophilic properties were marked by the MEEKC method.

The γ-ring hydroxylation (Fig. 1) influences catechin migration times that increase with an increasing number of hydroxy groups; so (+)-catechin migrates faster than (−)-epigallocatechin. Besides, substitution at the hydroxy group on the β-ring, such as galloylation, increases affinity to the SDS micelles leading to a decrease in migration time; thus (−)-epicatechin migrates slower than (−)-epigallocatechin gallate.

3.4. Method validation

The developed MEEKC method was validated under the optimised experimental conditions (heptane 1.36% (w/v), SDS 2.31% (w/v), butan-1-ol 9.72% (w/v) and 50 m*M* sodium phosphate buffer (pH 2.5) 86.61% (w/v)). The selectivity of the method was verified by analysing mixtures of pure and commercially available standard catechins. The peak identity for the analysed samples was confirmed by the migration time values and the on-line recorded UV spectra (diode array detection, DAD). Multiple injections inter-day and intra-day of a single solution of all catechins were performed to verify the repeatability of the migration times and the corrected peak area (area/migration time). The RSDs obtained at the level of 15 μg/ml for all the analytes are summarised in Table 1.

For quantitative applications, the response linearity was verified for the principal potential components of *Cistus* species extracts: (+)-catechin and (−)-gallocatechin using siringic acid as the internal standard and measuring the absorbance at 200 nm.

Table 1
Intra-day and inter-day precision of the migration time and peak area (RSD, $n=5$) for the studied catechins (concentration: 15 μg/ml)[a]

Analyte	Intra-day precision		Inter-day precision	
	t_m (min) (RSD, %)	Corrected peak area (RSD, %)	t_m (min) (RSD, %)	Corrected peak area (RSD, %)
ECG	4.39 (1.15)	28 836 (1.50)	4.40 (1.01)	28 712 (2.12)
EGCG	5.62 (1.25)	17 481 (2.18)	5.53 (1.00)	17 879 (2.71)
EC	6.38 (1.48)	37 377 (1.98)	6.21 (1.10)	37 453 (2.62)
C	7.25 (1.52)	34 362 (2.85)	7.01 (0.994)	34 196 (2.01)
EGC	8.61 (1.66)	16 980 (2.62)	8.55 (0.847)	17 017 (3.03)
GC	10.01 (1.55)	23 304 (3.08)	9.82 (1.02)	23 413 (3.37)

[a] Experimental conditions: heptane 1.36% (w/v), SDS 2.31% (w/v), butan-1-ol 9.72% (w/v) and 50 m*M* sodium phosphate buffer (pH 2.5) 86.61% (w/v). Fused-silica capillary (19.5 cm effective length) thermostated at 40 °C. Hydrodynamic injection (5 p.s.i. for 1 s). UV detection at 200 nm. Voltage −10 kV.

The corrected peak area (analyte to internal standard) ratios were plotted against the corresponding analyte concentrations and the linear regression data are reported in Table 2. The limit of detection (LOD) corresponding to a signal-to-noise ratio (S/N) of ~3, was evaluated for (+)-catechin and (−)-gallocatechin by progressive dilution. The limit of quantification (LOQ) corresponding to an S/N of ~10 was also evaluated for the same analytes (Table 2). These data support the suitability of the proposed MEEKC method for its application to real samples.

3.5. Applications to Cistus species extracts

The developed MEEKC method was applied to the identification and quantification of (+)-catechin and (−)-gallocatechin in lyophilized extracts of: (a) *Cistus incanus* from plants collected in the years 1998 and 2001, (b) *Cistus monspeliensis* from plants collected in the years 1998 and 2001. Representative electropherograms obtained from the analysed samples are reported in Fig. 6. The identity of the peaks in the electropherograms from samples was confirmed on the basis of the migration times and on the corresponding UV spectra, which were found to be overimposable to those from standard. In all samples, two catechins were found: (+)-catechin and (−)-gallocatechin. The identity of the analytes was also confirmed by spiking experiments using both the MEKC and MEEKC methods which, offering different selectivity, enhance the performance and the versatility of the electrophoretic approach. The other peaks at higher migration times can be ascribed to myricetin derivatives as myricetin 3-*O*-galactoside and myricetin 3-*O*-rhamnoside [2].

As shown in Table 3, (+)-catechin and (−)-gallocatechin were found at higher concentration in the *Incanus* species than in the *Monspeliensis* species. A comparable concentration of the analytes was observed in the *Incanus* species samples for the years 1998 and 2001, whereas significantly different levels of (+)-catechin and (−)-gallocatechin were found in the *Monspeliensis* samples collected in the years 1998 and 2001. These differences may result from environmental variation as well as variation in plant parts used and in preservation after harvest. The accuracy of the method can be considered essentially depending on the method selectivity which was high and able to avoid interference, because the lyophilised samples were completely soluble in water and extractive steps were not involved.

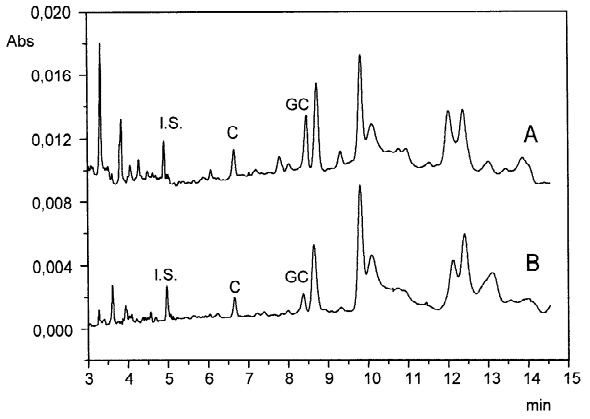

Fig. 6. Representative electropherograms obtained from: (A) lyophilized extract of *Cistus incanus* collected in 1998; (B) lyophilized extract of *Cistus monspeliensis* collected in 1998. MEEKC conditions: heptane 1.36% (w/v), SDS 2.31% (w/v), butan-1-ol 9.72% (w/v) and 50 m*M* sodium phosphate buffer (pH 2.5) 86.61% (w/v). Other conditions as in Fig. 2.

Table 2
Regression curve data[a] and LOD and LOQ values for the studied analytes

Analyte	Conc. range (μg/ml)	*a*	*b*	r^2	LOD[b] (μg/ml)	LOQ[c] (μg/ml) (RSD, %)
C	2–6	0.2536 (±0.0025)	−0.18158 (±0.0109)	0.999	0.391	1.170 (2.13)
GC	15–19	0.1357 (±0.0016)	−1.10931 (±0.0270)	0.999	0.781	2.344 (1.65)

[a] Regression curve data for five calibration points. $y = ax + b$, where y is the corrected peak area (area/migration time), x is the concentration (μg/ml), a is the slope, b is the intercept and r^2 is the correlation coefficient. Experimental conditions as in Table 1.
[b] Limit of detection, as 3 S/N.
[c] Limit of quantification, as 10 S/N.

Table 3
Determination of (+)-catechin (C) and (−)-gallocatechin (GC) (RSD, $n=5$) in four different *Cistus* species samples[a] collected in the years 1998 and 2001

Analyte	*Cistus incanus*		*Cistus monspeliensis*	
	1998 (μg/g) (RSD, %)	2001 (μg/g) (RSD, %)	1998 (μg/g) (RSD, %)	2001 (μg/g) (RSD, %)
C	1.26 (2.40)	1.31 (3.54)	1.11 (4.27)	0.54 (3.43)
GC	6.93 (1.23)	8.32 (3.01)	1.69 (3.28)	4.16 (3.57)

[a] Experimental conditions as in Table 1.

4. Conclusion

The developed microemulsion electrokinetic chromatographic (MEEKC) method proved to be able to provide a rapid separation of the principal catechins, offering a different selectivity with respect to a micellar approach (MEKC) using SDS under the same acidic conditions. The method was found to be suitable for the determination of specific catechins: (+)-catechin and (−)-gallocatechin, in complex matrices such as lyophilised samples obtained from *Cistus* species plants. Therefore, the MEEKC methodology can be considered an effective, alternative approach to the MEKC and HPLC methods for the analyses of an important class of natural compounds such as the catechins.

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