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Separation and analysis of glycyrrhizin, 18β-glycyrrhetic acid and 18α-glycyrrhetic acid in liquorice roots by means of capillary zone electrophoresis

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Abstract

Glycyrrhizin is the main active compound of *Glycyrrhiza glabra* root extracts; according to recent studies, glycyrrhizin and its aglycon, glycyrrhetic acid, have interesting therapeutic properties. A new capillary electrophoretic method has been developed for the separation and quantification of glycyrrhizin, β-glycyrrhetic acid and its isomer α-glycyrrhetic acid. Separation of the analytes was achieved in less than 3 min on a fused silica capillary, by injecting the samples at the short end of the capillary (effective length: 8.5 cm). The background electrolyte was composed of pH 10.0 carbonate buffer, methanol and ethylene glycol (80/10/10) and contained 0.4% β-cyclodextrin; indomethacin was used as the internal standard. Diode array detection was used, with quantitative assays carried out at 254 nm. Linearity was found over the 5–200 and 2.5–100 μg mL^{-1} concentration ranges for glycyrrhizin and glycyrrhetic acid, respectively. This method has been applied to the determination of the analytes in different matrices (liquorice roots and commercial confectionery products), and to the purity control of β-glycyrrhetic acid obtained from the hydrolysis of glycyrrhizin. When analysing β-glycyrrhetic acid and its epimer in roots, the samples were purified by means of a suitable solid-phase extraction (SPE) procedure with Oasis HLB cartridges, which granted good selectivity, eliminating matrix interference.

Keywords: Glycyrrhizin; 18β-Glycyrrhetic acid; 18α-Glycyrrhetic acid; Liquorice root; Capillary electrophoresis

1. Introduction

Glycirrhiza glabra (liquorice) roots and rhizomes are extensively used in herbal medicines for their emollient, antitussive, anti-inflammatory, antiviral and gastroprotective properties.

The product called "liquorice" in confectionery manufacturing (i.e. flakes or pastilles of pure liquorice) is obtained by treating dried *G. glabra* roots with boiling water, which is then evaporated to obtain a semi-solid extract. Liquorice extract is largely used in confectionery, as well as a masking agent or taste corrective in several pharmaceutical formulations (e.g. in preparations containing cascara, ammonium chloride and quinine) and in food production (e.g. to improve the taste of beer).

The main active compound of *G. glabra* is glycyrrhizin or glycyrrhizic acid (G, 3-*O*-(2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl)-3β-hydroxy-11-oxo-18β, 20β-olean-12-en-29-oic acid). This molecule is present in the root as potassium and calcium salts at percentages of between 2 and 15% (w/w) depending on plant species, geographic and climatic conditions [1–2], and consists of an aglycon (a pentacyclic triterpenic structure) bound to two glucuronic

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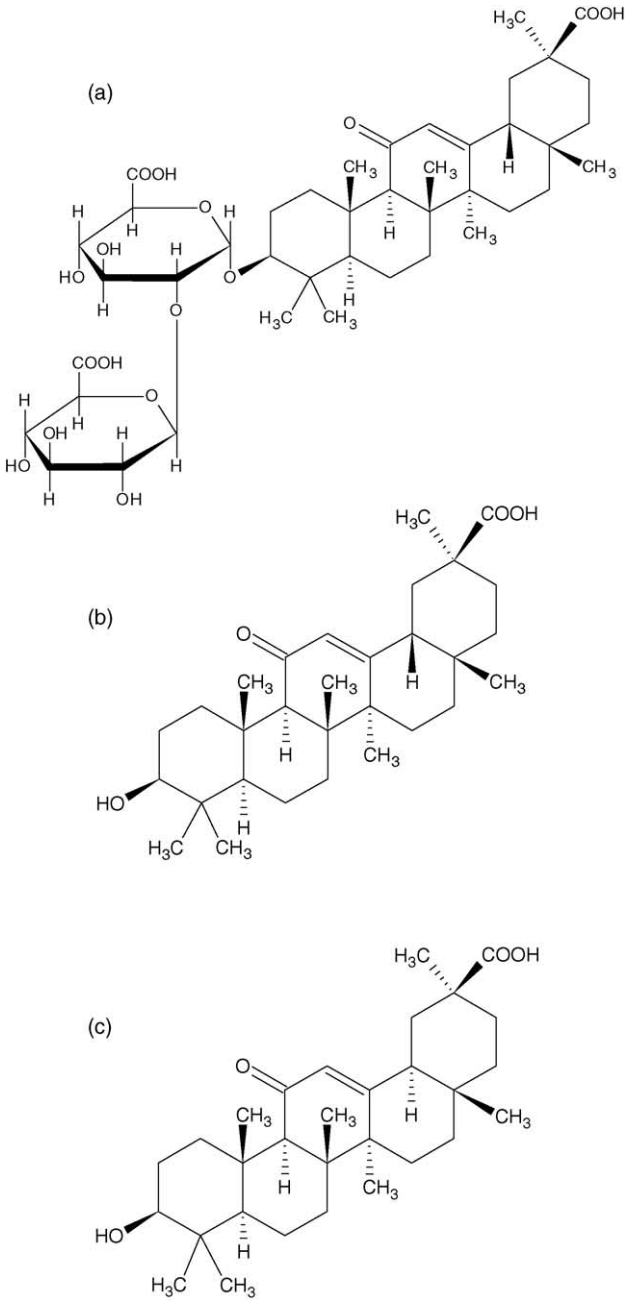

Fig. 1. Chemical structures of: (a) glycyrrhizin, (b) 18β-glycyrrhetic acid and (c) 18α-glycyrrhetic acid.

acid molecules (Fig. 1a). The best-known organoleptic property of G is its sweet taste; it is 170 times sweeter than sucrose [3] ("Glycyrrhiza" means "sweet root" in ancient Greek), and this explains the wide use of G as a sweetener and masking agent in pharmaceutical products.

The aglycon of G is glycyrrhetic acid (GA) which exists as two isomers, the trans form (αGA, Fig. 1b) and the cis form (βGA, Fig. 1c) [4].

G has anti-inflammatory, antiallergenic, antihepatotoxic, antiulcer and antiviral properties [4–7]. Furthermore, G is one of the leading natural compounds for clinical trials in recent studies on chronic viral hepatitis and human immunodeficiency virus (HIV) infections [5–7]. Chronic consumption of G prevents the development of hepatic carcinoma from C hepatitis [8] and the antiviral activity of G against SARS-associated corona virus has been demonstrated in vitro last year [9]. G finds application also in inhibiting unwanted effects of contraceptive formulations, such as alterations in blood coagulation and thrombosis [10].

It has been noted that G sometimes produces side effects such as: cardiac dysfunctions, edema, weight gain and hypertension. However, recent clinical studies, supported by pharmacological studies, have demonstrated that these side effects only arise in predisposed subjects or those receiving very high doses of pure G. Moreover, the unwanted effects are less frequent and less severe in subjects receiving liquorice extracts [11], because the bioavailability of G considerably decreases when it is administered as liquorice extract, as opposed to the pure compound [12].

The amount of βGA in liquorice root is reported to be in the 0.1–1.6% range [13,14], depending on species and growing region, while the amount of αGA in Asian root is usually lower than 0.7% [14]. Some authors have investigated the pharmacological effects of βGA and αGA, demonstrating that their activity is qualitatively very similar, but with different intensity: for instance, the antihepatotoxic activity of βGA is higher than that of αGA, while its anti-inflammatory activity is considerably lower [15,16]. Differences were also observed in the activity of cis and trans isomers in inhibiting the mutagenicity in *Salmonella typhymurium* [17]. Other authors have described the metabolism of G to GA, by observing the formation of the two isomers in animal species [4]. GA can be commercially produced by hydrolysis of G; this procedure yields mainly βGA isomers, but αGA may also be present [18].

The aglycon has been found to possess antitumor [19], antiviral [7] and antiulcer [20] properties. Moreover, GA has demonstrated a "simil-estrogenic" property, and for this reason can be used in the substitutive treatment of menopausal dysfunctions [21].

The Italian pharmacopoeia [22] reports an analytical procedure for the quality control of liquorice root, in which the analysis of G is carried out by means of reversed-phase liquid chromatography (RP-HPLC) with UV detection; no official method is reported for the quantification of βGA or αGA.

Several papers report analytical methods for the determination of G and/or GA with different techniques such as HPLC [18,23–31], capillary electrophoresis (CE) [32–36], gas chromatography (GC) [14], and high-performance thin-layer chromatography (HPTLC) [4].

Only a few papers regard procedures to separate βGA and αGA, using HPLC [18,31], GC [14] or HPTLC [4]; no one, however, reports the separation of these analytes by means of CE.

Thus, the aim of this paper is the development of a feasible and rapid CE procedure for the simultaneous analysis of G, βGA and αGA. In fact, HPLC techniques require very

expensive chiral stationary phases for the separation of isomers, while HPTLC requires high manual ability and it is not a widespread technique.

2. Experimental

2.1. Chemicals

Glycyrrhizin [3-*O*-(2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl)-3β-hydroxy-11-oxo-18β,20β-olean-12-en-29-oic acid], α- and β-glycyrrhetic acid (3β-hydroxy-11-oxo-18β,20β-olean-12-en-29-oic acid), indomethacin used as the internal standard (for the control of migration times) and β-cyclodextrin hydrate (β-CD) were purchased from Sigma (St. Louis, MO, USA). Ethanol (96%), orthophosphoric acid (85%), sodium hydroxide, diethylene glycol and methanol were of analytical grade from Carlo Erba (Milan, Italy), and *G. glabra* root was from Saila (Silvi Marina, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore (Milford, MA, USA) Milli-Q apparatus.

2.2. Solutions

Stock solutions of glycyrrhizin, glycyrrhetic acid and the internal standard (indomethacin) 1 mg mL^{-1} were made by dissolving 20 mg of compound in 20 mL of methanol and were stable for at least five months when stored at −20 °C. Working solutions were prepared every day by diluting the stock solutions with a mixture (named Sol. A) of 2.5 mM, pH 10.0 carbonate buffer added of 1% of methanol.

The background electrolyte (BGE) was prepared by dissolving 20 mg of β-CD in 4 mL of carbonate buffer (25 mM, pH 10.0) and mixing this solution with 0.5 mL of methanol and 0.5 mL of diethylene glycol. The carbonate buffer was prepared by dissolving 26.5 mg of sodium carbonate in about 5 mL of water; the solution was brought to pH 10.0 with 0.1 M HCl and then diluted to 10 mL with water.

The BGE was filtered through a cellulose acetate syringe filter (0.20 μm, Albet-Jacs-020-25) prior to use.

2.3. Apparatus and electrophoretic conditions

CE experiments were carried out with a ^{3D}CE apparatus (Agilent Technologies, Palo Alto, CA, USA). An uncoated, fused silica capillary (50 μm i.d., 375 μm o.d., 48.5 cm total length, 8.5 cm effective length) from Composite Metal (Hallow, UK) was used.

Analysis was performed using the BGE above described. Injection was carried out by pressure at the anodic end at 50 mbar for 30 s. The separation voltage was of −25 kV, temperature 25.0 °C, detector wavelength 254 nm.

Before use, the new capillary was purged with deionised water, with 1.0 M sodium hydroxide, water, and then with the BGE for 10 min each. Before each run, the capillary was rinsed with the BGE for 2 min. After each run the capillary was rinsed as follows: 1 min with water, 1 min with hydrochloric acid 0.1 M, 1 min with water, 1 min with 1 M sodium hydroxide and 2 min with water, all at 5 bar. For storage overnight, the capillary was washed with water for 5 min, with 1 M sodium hydroxide for 5 min and water again for 10 min, all at 5 bar.

2.4. Method validation

2.4.1. Calibration curves

Standard solution, in the 5–200 μg mL^{-1} range for glycyrrhizin and in the 2.5–100 μg mL^{-1} range for α-and β-glycyrrhetic acid, were prepared and injected into the CE system (internal standard was maintained at the concentration of 20 μg mL^{-1}).

The analyte peak area values were plotted against the corresponding concentrations of the analytes (expressed as μg mL^{-1}) and the calibration curves constructed by means of the least-square method.

2.4.2. Precision

The assays to evaluate intermediate precision (interday) and repeatability (intraday) were performed injecting solutions at the same concentration six times over different days and six times in the same day, respectively.

Each assay was carried out at three different concentrations of the analytes and in particular: 5, 100 and 200 μg mL^{-1} for G, and 2.5, 50 and 100 μg mL^{-1} for α- and β-GA. The percentage relative standard deviations (RSDs) of the data obtained were calculated.

2.5. Sample analysis

2.5.1. Liquorice roots and confectionery products

At first, the liquorice root or confectionery products were finely ground to a powder. Then, a known amount of the I.S. (50 μL) was added to 1 g of powder. To this mixture, 10 mL of a solution of methanol and water 1:1 were added and transferred into a 25 mL round-bottom flask. The mixture was thermostatted at 60 °C for 25 min under stirring, and then centrifuged for 10 min at 3000 rpm. The supernatant was filtered through a cellulose acetate syringe filter.

The analysis of G was performed directly injecting the extract into the CE after 1:100 dilution with Sol. A.

To analyse αGA and βGA, a pre-treatment was carried out by means of a solid-phase extraction (SPE) procedure using Oasis HLB (hydrophilic–lipophilic balance) cartridges (60 mg, 3 mL) from Waters (Milford, MA, USA). The cartridges were conditioned and equilibrated by passing through them 3 mL of methanol and 3 mL of deionised water.

To 250 μL of extract, 50 μL of I.S. (in order to obtain a final concentration of 20 μg mL^{-1}) were added, the mixture was dried at rotary evaporator under vacuum at 60 °C, and redissolved with 1 mL of Sol. A by stirring and sonication. The resulting solution was loaded onto the cartridge. After

washing twice with 2 × 1 mL of water and with 1 mL of a water/methanol mixture (80/20, v/v) the cartridge was dried under vacuum (40 kPa) for 30 s. The analytes were then eluted with 2 mL of methanol which was dried by means of a rotary evaporator and the residue redissolved, by stirring and sonication, in 1 mL of Sol. A.

The solution was filtered through a cellulose acetate syringe filter (0.20 μm, Albet-Jacs-020-25) and injected into CE.

2.5.2. Purified GA

Samples of GA, obtained by hydrolysis of G extracted from liquorice root and subsequent purification, were dissolved in methanol at a nominal concentration of 1000 μg mL^{-1}.

After suitable dilution with Sol. A, the sample was injected into CE to determinate the purity grade of the extract and to evaluate the amount of the α and β isomers.

3. Results and discussion

3.1. CE separation of G and GA

In the last few years some papers report CE procedures for the analysis of G [32–36], however only some of them simultaneously determine both GA and G by means of CE [32,36]. The aim of this study is the development of a rapid and feasible CZE procedure for the separation and simultaneous analysis of G and the two isomers αGA and βGA.

This work was planned in two steps: firstly, the best electrophoretic conditions to separate G from GA were studied, and secondly, the BGE composition was modified to obtain the separation of the α and β isomers of GA.

A basic BGE buffer was used to obtain a strong electroosmotic flow (EOF) and to negatively charge the analytes (G pK_{a1} = 2.75 ± 0.70[1]; GA pK_a = 4.70 ± 0.2[1]). It should be noted that G possesses three acidic hydrogen atoms, however a basic pH value assures that at least one negative charge is always present on each G molecule.

The strong electroosmotic flow favours the cathodic migration of the analytes while the negative charge, with strong difference of charge/mass ratio between G and GA, allows the separation of the two compounds.

Thus, in order to have a strong EOF, a pH 10.0, 25 mM carbonate buffer was selected for BGE preparation. The electrophoretic run was carried out using the short section of the capillary to obtain brief run times and to decrease the broadening of the electrophoretic peaks. Since the solubility of GA in water is very low, also at high pH, methanol was added to the BGE to increase its solubility.

In Fig. 2, the effective and apparent mobilities of G and GA are plotted against the percentage of methanol in the BGE. As

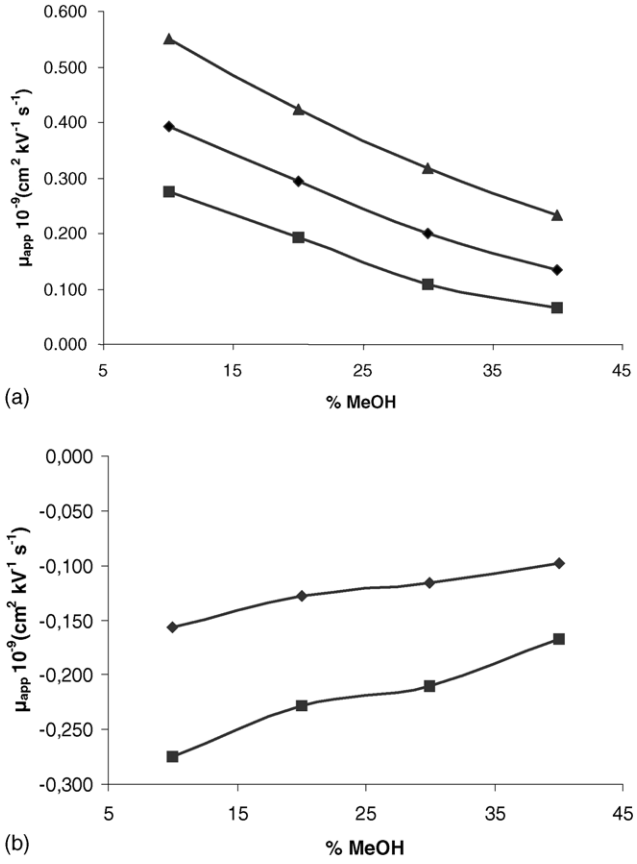

Fig. 2. (a) Apparent mobilities of: (♦) G, (■) GA and (▲) EOF at different percentages of methanol in the BGE; and (b) effective mobilities of (♦) G and (■) GA as a function of the methanol percentage in the BGE. The BGE was composed of a pH 10.0, 25 mM carbonate buffer, to which different amounts of methanol were added.

one can see, the addition of a methanol percentage higher than 10% decreases the apparent and effective mobilities of the analytes, as well as the differences in mobility, thus reducing peak resolution. Therefore, an amount of 10% of methanol was chosen to prepare the BGE.

Fig. 3 shows an electropherogram obtained from the simultaneous analysis of a standard solution of G, αGA and βGA. Indomethacin was used as the internal standard [29].

As one can see, the analytes are well separated and the run time is less than 1.2 min, but as expected there is no separation between the two isomers.

3.2. CE separation of αGA and βGA

The second step in the development of the CE procedure was to find a BGE that allowed the separation of the α and β isomers of GA.

Assays were carried out adding various amounts of β-CD, in the 0.17–0.40% range, to the BGE. A partial separation is already present at 0.17% β-CD, but even the highest percentage of β-CD (0.4%) did not allow the baseline separation of the analytes. To improve the separation, the addition of diethylene glycol was investigated, which increases the vis-

[1] Calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V 4.67 (© 1994-2004 ACD/Labs).

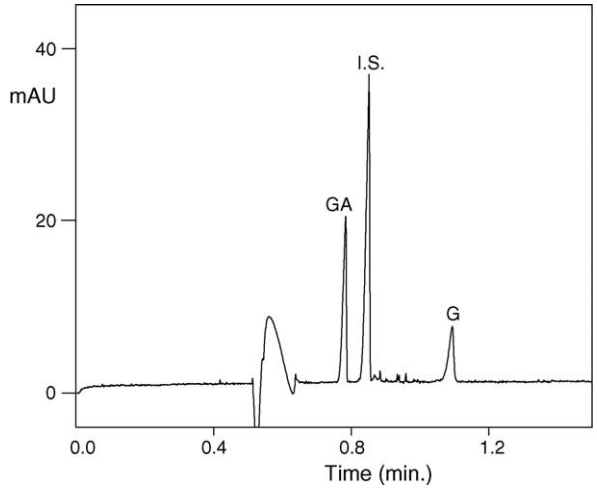

Fig. 3. Electropherogram of a standard solution containing 20 μg mL^{-1} of αGA, βGA, G and I.S. Electrophoretic conditions: fused silica capillary: 50 μm i.d., 48.5 cm total length, 8.5 cm effective length; BGE: 90% of 25 mM, pH 10.0 carbonate buffer, 10% of methanol; injection: by pressure, anodic end, 50 mbar × 30 s; voltage: −25 kV; temperature 25.0 °C; detector wavelength 254 nm.

cosity of the BGE. Using the BGE containing 0.4% β-CD, an amount of 10% of diethylene glycol gave good results, as one can see from Fig. 4, which reports an electropherogram of a 20 μg mL^{-1} standard solution of αGA, βGA, G and I.S. (used for the control of migration times). The two isomer peaks are baseline separated, the migration time of the analytes are 1.1, 1.25, 1.5 and 2.1 min for αGA, βGA, I.S. and G respectively, with an electrophoretic run shorter than 2.5 min.

3.3. Method validation

This methodology was applied to the analysis of standard working solutions in the 2.5–100 μg mL^{-1} concentration range for αGA and βGA, and 5–200 μg mL^{-1} for G, prepared as described in Section 2. A standard calibration curve was established by plotting the area of the analytes against the analyte concentrations. The regression equations of αGA, βGA and G (obtained by means of the least square method) were $y = -0.39 + 0.40x$, $y = -0.13 + 0.49x$, and $y = 0.25 + 0.23x$, respectively, where y is the area of analyte peak, and x is the concentration expressed as μg mL^{-1}; the linear correlation coefficients were 0.9987 for αGA, 0.9992 βGA and 0.9987 for G.

Precision expressed by the RSD values ranged from 1.0 to 2.1% for repeatability and from 2.5 to 4.5% for intermediate precision. The limit of detection (LOD) was 1 μg mL^{-1} for αGA and βGA and 2.5 μg mL^{-1} for G, while the limit of quantification (LOQ) was of 2.5 μg mL^{-1} for αGA and βGA and 5 μg mL^{-1} for G. Detection and quantification limits were calculated according to the USP guidelines [37].

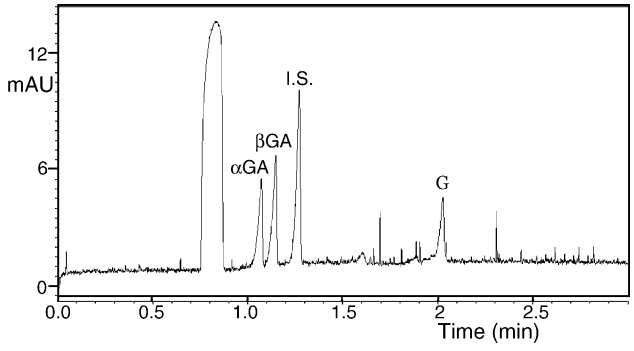

Fig. 4. Electropherogram of a standard solution containing 20 μg mL^{-1} of αGA, βGA, G and I.S. Electrophoretic conditions: as in Fig. 3 except BGE: 80% of 25 mM, pH 10.0 carbonate buffer, 10% of methanol, 10% of diethylene glycol containing 0.4% β-CD.

3.4. Analysis of G in roots and confectionery products

The extraction of αGA, βGA and G from liquorice root and confectionery products was developed starting from the procedure reported in our previous paper describing the analysis of GA and G by means of HPLC [13]. However, it is necessary to have more concentrated samples, due to CE-diode array detection (DAD) having shorter optical path and thus lower sensitivity with respect to HPLC-DAD. In order to avoid possible precipitation of GA, a certain amount of methanol was included both in the BGE and in the solutions used for sample redissolution and dilution.

Assays were carried out to find the best conditions of extraction and the procedure is reported in Section 2; the extracts of confectionery products and roots are injected directly into CE after filtration and dilution 100 times to determinate G. As an example, in Fig. 5 the electropherogram of an extract of root is reported. The G concentration in the root sample, obtained by interpolation of the peak area on the appropriate calibration curve, was found to be 52 μg mL^{-1}, corresponding to 5.2% (w/w) in dried root.

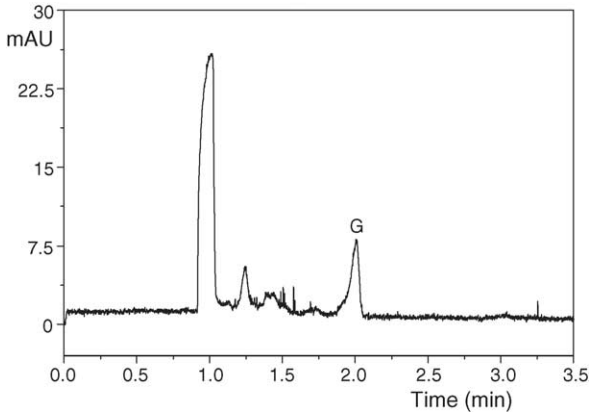

Fig. 5. Electropherogram of an extract of liquorice root diluted 100 times. Electrophoretic conditions: fused silica capillary: 50 μm i.d., 48.5 cm total length, 8.5 cm effective length; BGE: 80% of 25 mM pH 10.0 carbonate buffer, 10% of methanol, 10% of diethylene glycol containing 0.4% β-CD; injection: by pressure, anodic end, 50 mbar × 30 s; voltage: −25 kV; temperature 25.0 °C; detector wavelength 254 nm.

As one can see, it is not possible to identify the peaks of the GA isomers due to the strong matrix signal in the range of migration times from 1.0 to 1.5 min and the low levels of the analytes in the matrix; thus, an SPE procedure was developed.

3.5. Analysis of GA in roots after SPE pre-treatment

Different stationary phases were tested to find the one that allowed good matrix purification and a high extraction yield. Preliminary studies, loading the analytes in basic aqueous solution, were performed using cartridges with C2, C8, C18 and HLB (hydrophilic–lipophilic balance) sorbents.

The HLB cartridges gave the best performances, in fact C2, C8 and C18 sorbents did not give good extraction yields as well as strong interference from the matrix. Therefore, Oasis HLB cartridges were chosen for subsequent assays. The evaluation of recovery was calculated on αGA and βGA concentrations because the extraction yield of G is not important since it is possible to analyse G in roots by direct CE injection of the extract after suitable dilution (see Fig. 5).

To minimize the interference of the matrix, the washing steps were investigated using mixtures of water and methanol at concentrations between 10 and 50%. The best conditions were found to be a washing with 1 mL of water/methanol (80:20, v/v).

The analytes were eluted with 2 mL of methanol, then the eluate was dried and redissolved in Sol. A. As an example, the electropherograms of a root extract sample purified by means of the SPE procedure described and of the same sample spiked with a 20 μg mL^{-1} standard solution of αGA, βGA and the I.S. are reported in Fig. 6.

In Fig. 6a, which corresponds to the analysis of a root sample, the signals of both αGA and βGA are too low to be quantified. Since the LOD is 1 μg mL^{-1}, the amount of each epimer in the examined root sample resulted to be lower than 0.4%. These results were confirmed by means of HPLC analysis [13], where the sum of αGA and βGA in the root was equal to 0.13%. A peak corresponding to G is also present in this electropherogram, however its quantification is not important because G can be determined by direct injection. The peaks of αGA and βGA are well defined in Fig. 6b, corresponding to the same root sample which was spiked with the analytes and subjected to the SPE procedure. The extraction yield values obtained were satisfactory, being 77% for αGA and βGA and 80% for the I.S.

3.6. Analysis of purified GA

A procedure for the purification of βGA is being studied in our laboratory. The aim of this study is to obtain pure GA from liquorice root extract; the crude extract of G is hydrolysed to obtain GA and purification steps are carried out to obtain pure βGA.

The CE procedure described above was applied to check the hydrolysis of G to GA and the subsequent purification of

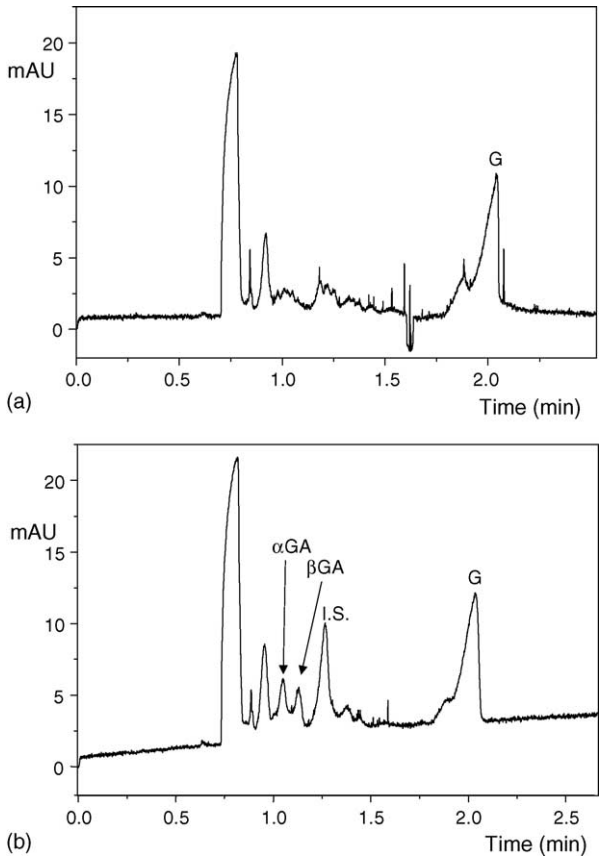

Fig. 6. Electropherograms of: (a) an extract of liquorice root after the SPE procedure, and (b) the same solution spiked with 20 μg mL^{-1} of αGA and βGA. Electrophoretic conditions: as in Fig. 5.

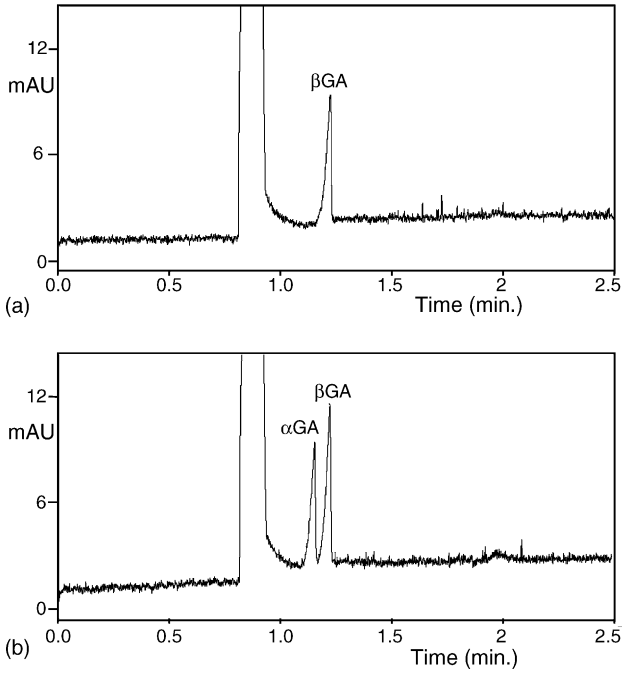

Fig. 7. Electropherograms of: (a) a solution of βGA at a nominal concentration of 20 μg mL^{-1} obtained from the hydrolysis and purification of liquorice root extract, and (b) the same solution spiked with a 20 μg mL^{-1} standard solution of αGA. Electrophoretic conditions: as in Fig. 5.

GA; thus, the method allows to determine the purity grade of βGA and the possible amount of αGA obtained. Two electropherograms are reported in Fig. 7: the first one (a) corresponds to a solution of purified βGA with a nominal concentration of 20 μg mL^{-1}, while the second (b) corresponds to the same solution spiked with 20 μg mL^{-1} of αGA.

As one can see, no trace of αGA was detected in the first electropherogram and the matrix did not give signals; the βGA concentration found, obtained by interpolation of the peak area on the appropriate calibration curve, was 18 μg mL^{-1}, thus the purity of the βGA sample resulted to be 90%. A neat and well-separated peak, corresponding to αGA (added by spiking), was detected in the second electropherogram, confirming that αGA concentration is under the LOD (1 μg mL^{-1}) in the purified βGA.

4. Conclusion

An original CE procedure for the analysis of G and the cis and trans isomers of GA has been developed for the first time in our laboratory and applied to real samples of different natural and commercial products.

This procedure demonstrated to be faster than the other methods reported, which applied HPLC, HPTLC and GC; it is also cheaper (in fact, the additive used to obtain the separation of isomers is simple native β-CD, while HPLC requires chiral columns), and uses minimal amounts of organic solvents.

The CE method was successfully applied to the direct analysis of G in different samples, such as roots and confectionery products. Moreover, a simple and reliable SPE procedure was developed for the analysis of αGA and βGA in root.

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