Phytochemical Analysis

Simultaneous HPLC Analysis, with Isocratic Elution, of Glycyrrhizin and Glycyrrhetic Acid in Liquorice Roots and Confectionery Products

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Glycyrrhizin (1), the main active principle of *Glycyrrhiza glabra* (liquorice) roots, is extensively used in herbal medicines, in pharmaceutical preparations and confectionery products. A feasible and reliable method which allows the simultaneous analysis of 1 and its aglycone, 18β-glycyrrhetic acid (2), by means of an isocratic HPLC procedure is described. The system uses a C_8 column as the stationary phase, and a mixture of acetonitrile, methanol, water and glacial acetic acid as the mobile phase. Good linearity was found in the concentration ranges 1–50 and 0.05–2.50 µg/mL for 1 and 2, respectively. A simple and rapid sample pre-treatment, based on the extraction of the two analytes with a mixture of water and ethanol, was developed for the examination of liquorice confectionery products and root samples. The HPLC method was shown to be appropriate, in terms of precision and feasibility, for the quality control of the analytes in these matrices. Copyright © 2005 John Wiley & Sons, Ltd.

INTRODUCTION

Glycyrrhiza glabra (liquorice) roots and rhizomes are extensively used in herbal medicines for their emollient, anti-tussive, anti-inflammatory, anti-viral and gastro-protective properties.

The product referred to as 'liquorice' in the manufacture of confectionery (i.e. chips or cylinders of pure liquorice) is obtained by treating dried roots of *G. glabra* with boiling water, which is then evaporated to obtain a semi-solid extract that may be subjected to further treatment to obtain different commercial products. Liquorice extract is also used 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 {**1**; glycyrrhizic acid, [3-*O*-(2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl)-3β-hydroxy-11-oxo-18β, 20β-olean-12-en-29-oic acid]}, a saponin with a pentacyclic triterpenic structure bound to two glucuronic acid molecules. The content of **1** (as potassium and calcium salt) in liquorice roots is between 2 and 15% by weight of the drug depending on the species and the geographic and climatic conditions

Glycyrrhizin (1)

18β-Glycyrrhetic acid (2)
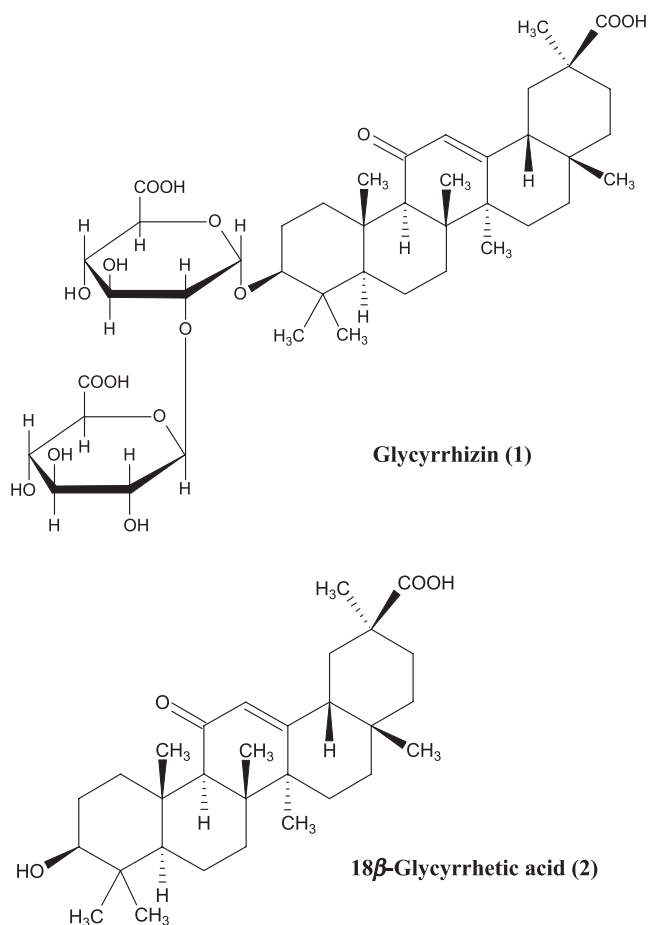
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(Fenwick *et al.*, 1990; Spinks and Fenwick, 1990). The most readily appreciable characteristic of **1** is its sweet taste, from which it derives its name 'Glycyrrhiza' which means 'sweet root' in ancient Greek. In fact, **1** is 170 times sweeter than sucrose (Mizutani *et al.*, 1994), and this is the basis of its use in the food industry and in pharmaceutical products.

Owing to its known pharmacological properties as an anti-tumour (Ukiya *et al.*, 2002), anti-viral (Baltina, 2003) and anti-ulcer agent (Doll *et al.*, 1962), much research interest has been shown in the aglycone of **1**, 18β-glycyrrhetic acid (**2**), that is readily formed by hydrolysis or metabolism of **1** and may also be present in very small amounts in the plant. Glycyrrhizin is one of the leading natural compounds to be taken to clinical trials with regard to chronic active viral hepatitis and human immunodeficiency virus (HIV) infections. It has been suggested that the demonstrated anti-viral activity of **2** is due to its antioxidant properties (Baltina, 2003; Fujioka *et al.*, 2003; Liu *et al.*, 2003). Studies have shown that long-term treatment of **1** prevents the development of hepatic carcinoma from C hepatitis (Van Rossum *et al.*, 1998), and recently it has been found that **1** has *in vitro* anti-viral activity against SARS-associated coronavirus (Cinatl *et al.*, 2003). Compound **1** may also be co-administered with pharmaceutical formulations containing oestrogen derivatives to inhibit unwanted effects such as alterations in blood coagulation and thrombosis (Francischetti *et al.*, 1997); on the other hand, a 'similoestrogenic' activity of **2** has been demonstrated and its use in the substitutive treatment of menopausal dysfunctions has been proposed (Sharaf *et al.*, 1975).

Recent clinical and pharmacological studies have verified the high level of safety of **1**. In fact, side effects such as cardiac dysfunctions, oedema, weight gain and hypertension have only been found in predisposed subjects or in those receiving very high doses of pure **1**. These effects, however, were shown to be less frequent and less severe in subjects receiving liquorice extract containing the same amount of **1** (Bernardi *et al.*, 1994). Pharmacological studies on both humans and rats have reported a significant decrease in the bioavailability of **1** when it is administered as liquorice extract, as opposed to the pure compound (Cantelli Forti *et al.*, 1994a).

From a consideration of the above, there is clearly a need for a simple analytical method by which to determine **1** and **2** in plant roots, pharmaceutical formulations, confectionery products and plasma samples. Several papers report analytical methods for the determination of **1** and **2** in different matrices including commercial products and biological fluids (Hurst *et al.*, 1983; Ichikawa *et al.*, 1984; Collinge *et al.*, 1985; Hermesse *et al.*, 1986; De Groot *et al.*, 1988; Spinks and Fenwick, 1990; Yamamura *et al.*, 1991; Raggi *et al.*, 1994a; Okamura *et al.*, 2001). Such procedures are mostly based on reversed phase HPLC with UV (Hurst *et al.*, 1983; De Groot *et al.*, 1988; Raggi *et al.*, 1994b; Collinge *et al.*, 1985; Okamura *et al.*, 2001) or fluorometric detection (Yamamura *et al.*, 1991). Most of these methods analyse **1** and **2** separately, and only two of them simultaneously determine both analytes using HPLC with gradient elution (Okamura *et al.*, 2001) or column switching (De Groot *et al.*, 1988).

To the best of our knowledge, no method has been reported that permits the determination of both **1** and **2** by isocratic liquid chromatography. The aim of this paper was to develop a rapid, facile and economic HPLC method for the simultaneous analysis of **1** and **2** in confectionery products and in roots of *G. glabra*.

EXPERIMENTAL

Reagents and samples

Methanol, acetonitrile (HPLC-grade), glacial acetic acid and 96% ethanol (pure for analysis) were from Carlo Erba (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained using a Millipore (Milford, MA, USA) MilliQ apparatus. Glycyrrhizin ammonium salt (ca. 75% pure), glycyrrhetic acid (ca. 95% pure) and indomethacine (**3**; internal standard) were purchased from Sigma (St. Louis, MO, USA). Stock solutions of **1**, **2** and **3** were prepared at concentrations of 1 mg/mL in methanol, and were stable for at least 5 months when stored at –20°C. Working solutions were prepared every day by diluting the stock solutions with the HPLC mobile phase.

The different liquorice-based confectionery products analysed were purchased from stores in Bologna (Italy). Pure extracts, 'Oronero' chips and cylinders (Sirea, Reggio Emilia, Italy) and 'Saila liquirizia purissima extraforte' (Saila, Silvi Marina, TE, Italy) contained pure liquorice extract and natural flavours. 'Tabù' candies (Perfetti S.p.A., Lainate, MI, Italy) contained pure liquorice flavoured with mint. Roots of *Glycyrrhiza glabra* were of commercial quality from Saila and Sirea.

Chromatographic protocols

Isocratic system. The HPLC apparatus consisted of an Agilent (Waldbronn, Germany) 1100 series isocratic pump and an Agilent 1100 series photodiode array detector (PAD). The data system consisted of an Hewlett Packard CORE Chemstation LC 3D (Waldbronn, Germany). Isocratic separation was achieved using an Agilent Zorbax Eclipse XDB C$_8$ reversed-phase column (150 × 4.6 mm i.d.; 5 μm) and a Phenomenex (Torrance, CA, USA) SecurityGuard C$_8$ pre-column (4.0 × 3.0 mm i.d.; 5 μm). The mobile phase, consisting of methanol: acetonitrile:water:glacial

Time (min)	Composition[a] of mobile phase (%)	
	A	B
0	99	1
10	95	5
11	90	10
13	80	20
14	75	25
15	65	35
17	60	40
18	50	50
45	50	50

[a] Solvent A, acetonitrile:water:glacial acetic acid (35:64:1, v/v/v); solvent B, methanol.

acetic acid (35:35:30:1, by volume), was filtered through Millipore nylon filters (47 mm diameter; 0.2 µm pore size) and degassed by sonication (Transsonic T-310 instrument; Elma, Singen, Germany) prior to use. Separations were carried out at room temperature with a flow rate of 1 mL/min and an injection loop of 20 µL. The PAD detector was operated in the range 200–320 nm, and quantitative analysis was performed at 254 nm.

Gradient system. For the purposes of validation, a gradient system was also employed to analyse the same samples. The HPLC apparatus consisted of a Jasco (Tokyo, Japan) PU-1580 gradient pump and a Jasco UV-970 spectrophotometric detector set at 254 nm. Separations were achieved on an Agilent Zorbax Eclipse XDB C_8 reversed-phase column (150 × 4.6 mm i.d.; 5 µm) and a Phenomenex SecurityGuard C_8 pre-column (4.0 × 3.0 mm i.d.; 5 µm). The mobile phase was composed of acetonitrile:water:glacial acetic acid (35:64:1, by volume; solvent A) and methanol (solvent B). Components of the mobile phase were filtered through Millipore nylon filters (47 mm diameter; 0.2 µm pore size) and degassed by sonication prior to use. The composition of the mobile phase was varied during the run according to the nonlinear gradient scheme shown in Table 1. Separations were carried out at room temperature with a flow rate of 1 mL/min and an injection loop of 20 µL. After the end of each chromatographic run, the system was allowed to equilibrate under initial conditions for 30 min.

Method validation

Calibration curves. Standard solutions in the concentration range 1–50 µg/mL for **1** and 0.05–

20 µg/mL for **2**, were prepared and injected into the HPLC system: the internal standard **3** was maintained at a concentration of 1 µg/mL. The analyte peak area values were plotted against the corresponding concentrations of the analytes (expressed in µg/mL) and the calibration curves constructed by means of the least-squares method.

Extraction procedure. The confectionery products or roots were ground to a fine powder and a sample (200 mg) transferred to a 50 mL round bottom flask together with 1 mL of the standard solution of **3** and 19 mL of ethanol:water (1:1, v/v). The mixture was maintained by thermostat at 60°C for 25 min with stirring and then centrifuged for 10 min at 3000 rpm. The supernatant was filtered through a paper filter (55 mm diameter; Whatman, Maidstone, UK) and an aliquot of the filtrate, suitably diluted with mobile phase, was subjected to HPLC analysis.

Precision. In order to evaluate intermediate precision (inter-day precision) and repeatability (intra-day precision), assays were performed by extracting and injecting the same sample six times on the same day and six times over different days. Each assay was carried out at three different concentrations of **1** (1, 10 and 20 µg/mL) and of **2** (0.05, 0.10 and 2.50 µg/mL). The percentage relative standard deviations (RSD%) of the data thus obtained were calculated.

Accuracy. Accuracy was evaluated by means of recovery assays carried out by adding standard solutions of the analytes to the samples. The amounts of analytes added corresponded to concentrations of 1, 10 and 20 µg/mL for **1** and 0.05, 0.10 and 2.50 µg/mL for **2**. The mean recoveries of the added analytes were then calculated.

RESULTS AND DISCUSSION

HPLC method

From our previous experience concerning the analysis of glycyrrhizin (**1**) and glycyrrhetic acid (**2**) in different matrices (Raggi *et al.*, 1994a, b), it was known that an acid mixture of acetonitrile and water and an acid mixture of methanol and water worked well as a mobile phase for the chromatographic analysis of **1** and **2**, respectively; however, none of these mixtures were suitable for the simultaneous determination of both analytes. For this reason, a mobile phase containing different ratios of acetonitrile, methanol, water and glacial acetic acid was employed. Table 2 shows the retention times of the analytes obtained with different compositions of mobile phase. The results show that it is necessary to balance the amounts of the organic

Table 2 Chromatographic behaviour of glycyrrhizin (1) and glycyrrhetic acid (2) with isocratic elution with different HPLC mobile phases

Composition of mobile phase (%)				Retention time (min)	
Water	Methanol	Acetonitrile	Acetic acid	Glycyrrhizin	Glycyrrhetic acid
64.5	15	20	0.5	25	>60
54	16.5	29	0.5	10	>60
50	25	25	1	5.1	33.6
29	35	35	1	2.9	16
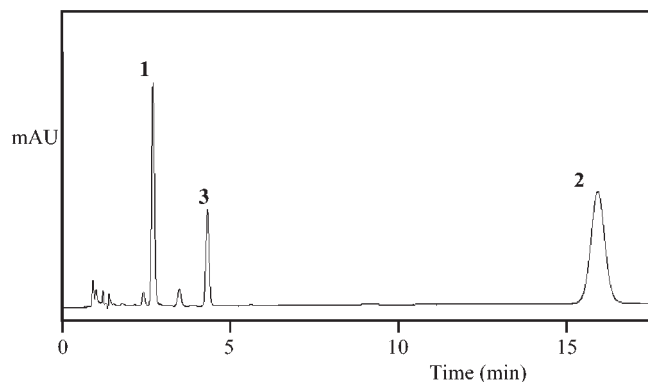
Figure 1 HPLC chromatogram of a standard solution of glycyrrhizin (**1**) and glycyrrhetic acid (**2**) (10 µg/mL) and indomethacine (**3**) (1 µg/mL) obtained following isocratic elution with a mobile phase containing acetonitrile: methanol:water:glacial acetic acid (35:35:29:1, by volume) at a flow rate of 1 mL/min. (For chromatographic protocol see the Experimental section.)

solvents in order to obtain good retention times for both **1** and **2**, which are two chemically very different substances, the former being highly hydrophilic (due to the two glucuronic acid groups), whilst the latter is lipophilic. A mixture of acetonitrile, methanol, water and acetic acid (35:35:29:1) was found to be optimal for this purpose. Quantitative analyses were carried at a wavelength of 254 nm, where both analytes showed a relative absorbance maximum, and PAD analysis allowed unambiguous peak identification (by means of on-line spectra) and analysis of peak purity. Indomethacine was selected as internal standard and was also used for retention time control. Figure 1 shows the chromatogram of a standard solution containing **1**, **2** (10 µg/mL of each) and **3** (1 µg/mL); all peaks are symmetrical and well separated with retention times of 3.0 min for **1**, 4.5 min for **3**, and 16.0 min for **2**.

Different linearity ranges were studied for the two analytes, according to the amount of each substance expected to be present in the matrix, that is, **1** is usually >4% in liquorice root and 3–5% in ethanolic extract (European Pharmacopoeia, 2005), whilst **2** is typically present only in trace amounts. Good linearity

was obtained with standard solutions over the concentration range 1–50 µg/mL for **1** and 0.05–2.50 µg/mL for **2**. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to recommendations of the United States Pharmacopeia (2005) and were, respectively, 4 and 10 ng/mL for **1** and 8 and 20 ng/mL for **2**. The precision of the chromatographic method was evaluated and acceptable RSD% values were obtained: the repeatability data were within the ranges 0.6–1.4% for **1** and 0.6–1.8% for **2**, and the intermediate precision data varied from 1.2 to 1.6% for **1** and from 1.0 to 2.1% for **2**. The full set of validation parameters is reported in Table 3.

Extraction procedure

The chemical characteristic of the analytes should be carefully considered when developing an extraction procedure for **1** and **2** owing to their rather different chemical–physical properties. Compound **1** is very soluble in water, whereas **2** is almost insoluble in water but soluble in alcohol. Different solvents, previously employed by other authors, were tested for the extraction of **1** and **2** from confectionery and liquorice roots including water: ethanol (80:20; Lauren *et al.*, 2001), 2 M ammonium hydroxide (Frattini *et al.*, 1977; Okamura *et al.*, 2001), and water (Ong and Len, 2003). The amounts of analytes obtained when extracting the same sample with the three solutions are reported in Table 4. Extraction with water (carried out for the industrial production of liquorice sweets) gave the lowest recovery, especially for **2**. Extraction with 2 M ammonia was tested to exploit the acidic properties of the analytes; however, the extraction yields were not satisfactory. It was found that a mixture of water and ethanol was the best extraction medium but required continuous infusion with hot ethanol:water (20:80, v/v) for 4 h. In order to reduce the length of the extraction procedure, the percentage of ethanol was increased to 50%, obtaining a quantitative extraction in 25 min. Thus, this latter procedure was chosen for subsequent assays.

In order to confirm that the extraction procedure with this medium was quantitative, a sample of confectionery

Table 3 Validation parameters for the developed isocratic HPLC method

Parameter	Glycyrrhizin			Glycyrrhetic acid		
Linearity range (µg/mL)	1–50			0.05–2.5		
Regression equation[a]	$y = 0.20445x + 0.04414$			$y = 0.57554x - 0.01823$		
Limit of detection						
Standard solutions (µg/mL)	4×10^{-3}			8×10^{-3}		
Confectionery products (%)[b]	0.002			0.004		
Limit of quantitation						
Standard solutions (µg/mL)	10×10^{-3}			20×10^{-3}		
Confectionery products (%)[b]	0.005			0.010		
Analyte concentration (µg/mL)	1	10.0	25.0	0.05	1	2.5
Repeatability (RSD%)[c]	1.4	0.8	0.6	1.8	1.4	0.6
Intermediate precision (RSD%)[c]	1.5	1.4	1.2	2.1	1.9	1.0

[a] y = peak area; x = concentration (µg/mL).
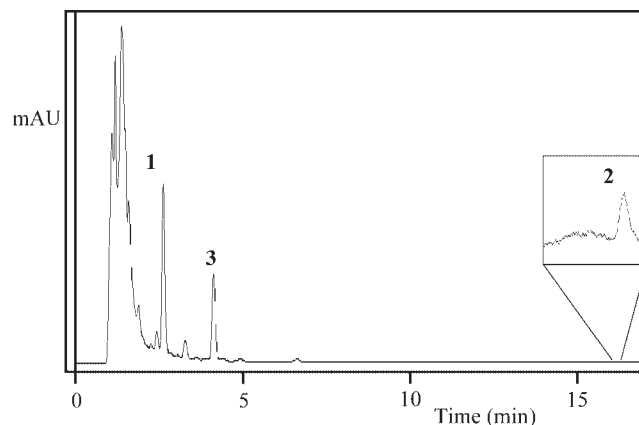[b] Percentage in confectionery products corresponding to the reported LOD and LOQ values.
[c] $n = 6$.

Table 4 Comparison of the efficiency of extraction of glycyrrhizin (1) and glycyrrhetic acid (2) from the same sample using different extraction solvents

| Analyte extracted (amount, µg/mL)	Extraction solvent		
	Water:ethanol (80:20, v/v)	Ammonium hydroxide (2 M)	Water
Glycyrrhizin	11.7	10.6	9.3
Glycyrrhetic acid	0.27	0.25	0.17

Figure 2 HPLC chromatogram of a sample of Sirea Oronero chips following extraction with 20 mL of a mixture of water: ethanol (1:1) and dilution (1:50) with the mobile phase. (For a key to peak identity see the legend to Fig. 1; for chromatographic protocol see the Experimental section).

product and a sample of root were extracted twice with the extraction medium, and the solutions thus obtained were injected separately into the HPLC. No detectable analyte peak was found in the solutions obtained from the second extraction step.

Application to commercial confectionery products and liquorice roots

Following validation of the extraction procedure and the chromatographic method, six different commercially available liquorice products were analysed. The chromatogram of a confectionery product (Sirea Oronero chips) after the extraction procedure and dilution with mobile phase (50 times), is shown in Fig. 2. No interference with the resolution of **1**, **2** or **3** was observed, and the retention times remained the same as for the respective standard materials. The analyte concentrations in the sample, obtained by interpolation on the appropriate calibration curves, were found to be 10.6 µg/mL of **1** and 260 ng/mL of **2**. The amounts of analytes found in the examined products are reported in Table 5. The amounts of **1** were very similar for the four different confectionery materials, and were

between 4.5 and 5.4% w/w, while **2** was present in only trace levels. Moreover, the amount of **1** in roots was lower than in the confectionery materials.

The precision of the method (extraction and chromatography) was then evaluated: intermediate precision values were obtained by repeating the extraction of the same sample (Sirea Oronero chips containing 5.3% of **1** and 0.13% of **2**) six times over different days; the RSD% thus calculated was 3.0% for **1** and 3.5% for **2**. Accuracy was assessed using powdered commercial product (Sirea Oronero chips) spiked with three different levels of each analyte. Figure 3 shows the chromatogram of the sample to which 10 µg/mL of **1** and 2 µg/mL of **2** had been added. The recovery values were between 95 and 102% for **1** and between 99 and 108% for **2**, thus indicating the satisfactory accuracy of the method.

Table 5 Amounts of glycyrrhizin (1) and glycyrrhetic acid (2) in commercial liquorice confectionery samples and roots

Amount found (%)[a]	Sirea Oronero chips	Sirea Oronero cylinders	Tabu cylinders	Saila cylinders	Saila roots	Sirea roots
Glycyrrhizin	5.31	4.50	5.37	4.91	2.96	4.03
Glycyrrhetic acid	0.13	0.04	n.d.[b]	0.03	0.25	n.d.

[a] Expressed on a w/w basis.
[b] n.d. = not detectable (<LOD).
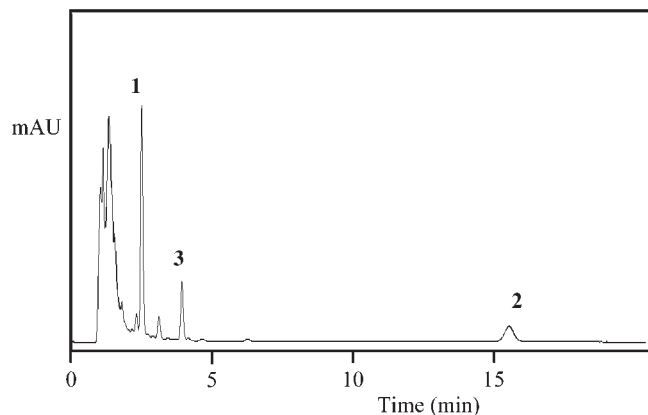
Figure 3 HPLC chromatogram of the same sample depicted in Fig. 2 spiked with 10 μg/mL of glycyrrhizin (1) and 2 μg/mL of glycyrrhetic acid (2). (For a key to peak identity see the legend to Fig. 1; for chromatographic protocol see the Experimental section).
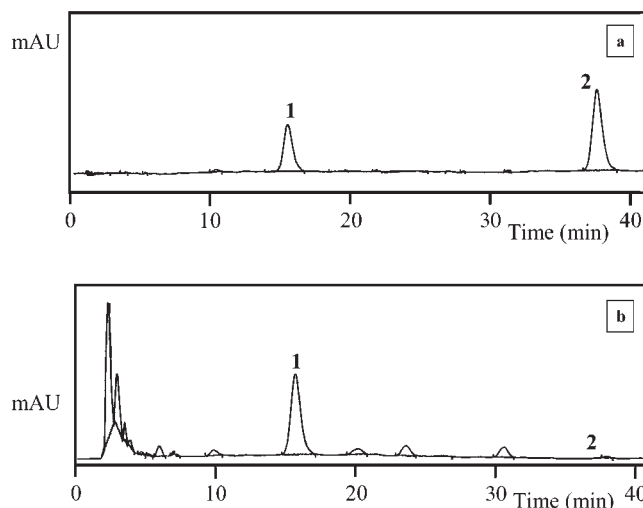
Figure 4 HPLC chromatograms obtained using a gradient elution system (for chromatographic protocol see Experimental section) of: (a) a standard solution containing 10 μg/mL each of glycyrrhizin (1) and glycyrrhetic acid (2), and (b) a liquorice root sample.

Comparison of the developed isocratic method with a gradient method

While isocratic elution is less expensive and less time-consuming than gradient elution, co-elution with other components of the matrix can occur, thus lowering the selectivity of the method. For this reason, the same liquorice extracts and confectionery products analysed by the proposed method were analysed using a gradient method. The representative chromatograms of a standard solution containing 1 and 2, and a liquorice root sample are reported in Fig. 4(a and b), respectively. As can be seen, the gradient method shows greater resolution than the isocratic method but the quantitative results derived from the gradient and the isocratic method were in very good agreement. For this reason, it can be concluded that none of the compounds separated with the former method interfered with 1 or 2 when employing the latter method. Furthermore, the peak purities of 1 and 2 in confectionery materials and root samples, as evaluated by the Chemstation software, were always well within the prescribed limits with both methods.

The proposed HPLC method with UV detection, coupled to a simple and rapid extraction procedure, seems to be suitable, in terms of selectivity and sensitivity, for the analysis of 1 and 2 in liquorice roots and confectionery products. The method also has the advantage of allowing for the simultaneous analysis of 1 and 2 with a single isocratic system. Compared with other methods presented in the literature that employ gradient systems or column switching (Ichikawa *et al.*, 1984; De Groot *et al.*, 1988; Okamura *et al.*, 2001), the proposed method is simpler and requires less expensive instrumentation. The sensitivity compared with the method of De Groot *et al.* (1988) is significantly higher and allows the quantivation of traces of 2 in liquorice roots. Moreover, the extraction procedure developed for roots and confectionery products allows for the quantitative extraction of 1 and 2 in a single step. The described HPLC method may also be applicable for the simultaneous determination of 1 and 2 levels in plasma for toxicological and pharmacokinetic studies: further studies are in progress in order to adapt the method to this type of biological matrix.

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