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The extraction of yellow gentian root (*Gentiana lutea* L.)

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Abstract Several solvents have been investigated for the preparation of bitter compounds of gentian roots (*Gentiana lutea* L.) for food applications. The highest concentrations of the bitter compounds, amarogentin and gentiopicroside, were obtained with ethanol : water 55 : 45 (v/v), propylene glycol: water 30 : 70 (v/v) and ethanol: propylene glycol: water 20 : 20 : 60 (v/v/v). Enzyme treatment prior to solvent extraction gave a greater extract yield (3.5%) but the amarogentin and gentiopicroside concentrations remained the same. The volatile fraction was affected by the solvent used through the formation of esters of organic acids from the plant.

Key words Gentian root · Solvent extraction · Enzyme treatment · Bitter secoiridoids · Volatile fraction

Introduction

Commercial food grade extracts can contain ethanol or propylene glycol in different concentrations depending on the polarity of the compounds to be extracted. Commercial gentian root extracts are normally obtained by percolation with about 60–70% ethanol in water, yielding extracts with a characteristic bitter taste and aroma. When plant material contains high levels of insoluble constituents (pectin, cellulose, etc.) it is useful to pre-treat the material with an enzyme preparation to facilitate solute diffusion and increase the extraction yield [1].

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Gentiana lutea L. is a perennial plant that grows at altitudes of 900–2000 m. The adult plants have very thick roots and rhizomes which function as storage organs. These organs accumulate a variety of compounds: soluble carbohydrates (30–50% dry wt.), mainly gentianose and sucrose and a smaller percentage of glucose, fructose and gentiobiose [2, 3]; lipids (6–7% dry wt.) and insoluble carbohydrates, primarily pectin. The roots and rhizomes also contain small quantities of free amino acids [2], essential oil [4], polyphenolics, i. e. flavones, xanthones and their glycosides [5], and the bitter secoiridoids that give gentian root its characteristic bitter taste. The percentage of these compounds and their concentrations in dried roots are as follows: amarogentin (0.05–0.15%) with a bitterness index of 58×10^6, gentiopicroside (2.5–3.5%), swertiamarin (0.15–0.20%) and sweroside (<0.1%); the last three all have a bitterness index of 12×10^3 [6]. As these bitter compounds are more abundant in the outer layers of the root, their concentration is higher in the thinner roots relative to the wider ones [7].

The legal limitations on the use of ethanol and the high taxes involved have necessitated a search to find alternative solvents for extraction. In this paper we examine the effect of replacing ethanol, total or partially, by propylene glycol and water as an extraction solvent, on the yield of the bitter compounds amarogentin and gentiopicroside. We also examined the effect of plant enzyme treatment before solvent extraction on the yield of the bitter compounds extracted. Once the best extraction solvents had been selected, the volatile fraction of each extract was analysed to determine their qualitative and quantitative differences.

Materials and methods

Standards and reagents. Amarogentin and gentiopicroside standards were purchased from Boehringer-Mannheim (Mannheim, Germany) and Extrasynthese (Genay, France), respectively. All compounds used as standards for gas chromatographic analysis were obtained from Sigma-Aldrich Quimica, S. A. (Madrid, Spain). All other chemicals

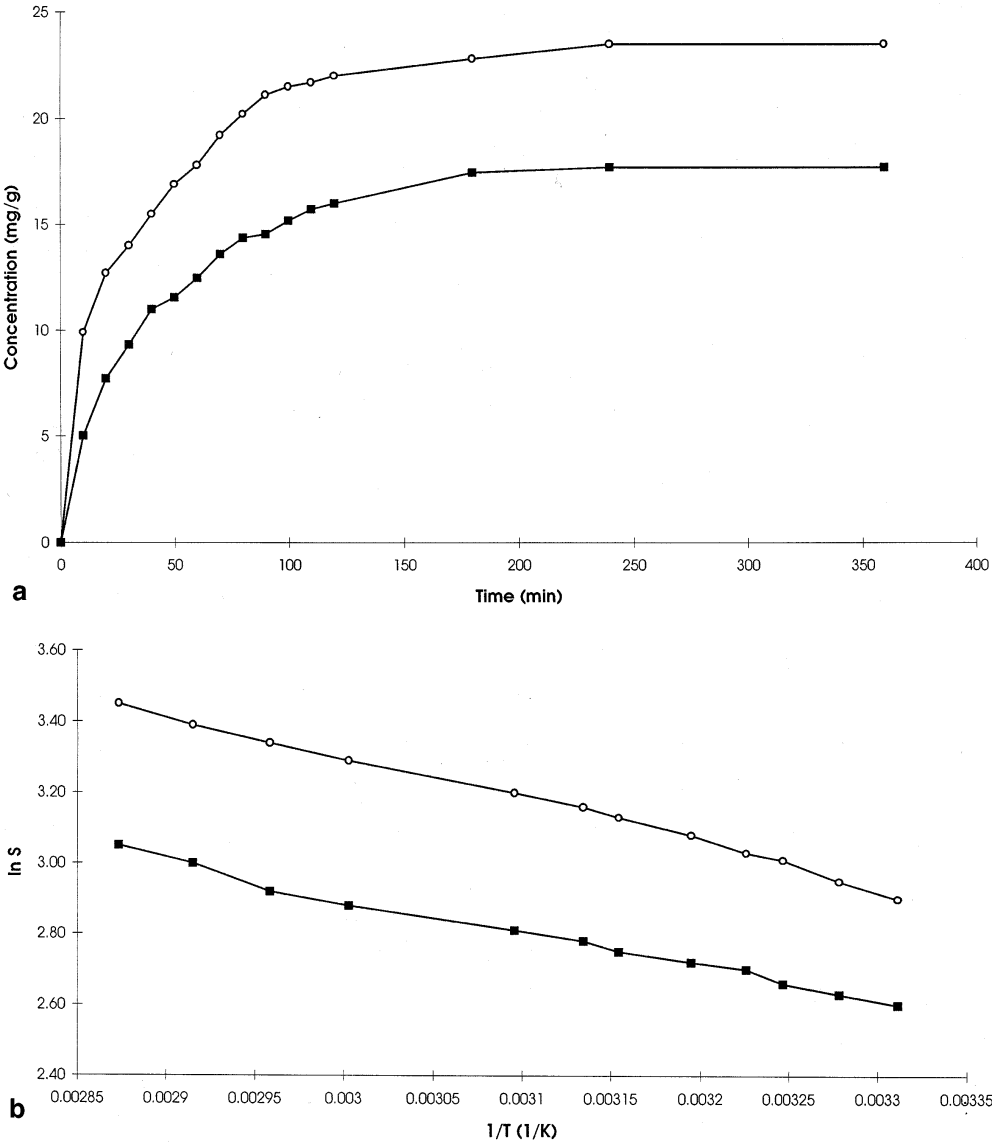

Fig. 1a, b Extraction of gentian root with ethanol: water 50 : 50 (v/v). **a** Concentration of amarogentin × 100 (○) and gentiopicroside (■) obtained at 35 °C at the times indicated. **b** Variation of solubility (lnS) of amarogentin +5 (○) and gentiopicroside (■) with temperature after a 2 h extraction

were analytical reagent grade, except for methanol which was chromatographic grade (Scharlau, S. A., Barcelona, Spain).

Plant material. All extracts analysed in this paper were obtained from commercial *G. lutea* L. roots collected in the north-west of Spain, which had been dried and ground. The dried material was passed through a sieve to obtain particles between 2.5 and 5.0 mm in diameter.

Plant extract preparation. Extractions were performed in triplicate: 100 ml of solvent was added to 10 g ground gentian root. Samples were shaken at 150 rpm in a temperature-controlled orbital incubator. After fixed times, samples were filtered and stored at room temperature until analysed. Firstly, the extraction time was optimized for ethanol: water 50 : 50 (v/v) at 35 °C. Then to optimize the extraction temperature, the same solvent: water ratio and extraction time were used. Once the parameters were optimized, extracts with various solvents were obtained. The solvents tested were ethanol, propylene glycol and a 50% (v/v) mixture of both, at different concentrations in water.

Enzyme treatment of plant material. Preliminary results indicated that some commercial pectolitic enzyme preparations degraded glucosides, most likely due to the presence of small amounts of β-glucosidase. Thus, the degradation of the secoiridoid glucosides, amarogentin and gentiopicroside, in aqueous solution was tested in the presence of β-glucosidase from almonds (Sigma-Aldrich Quimica, S. A., Spain). Eleven units of β-glucosidase were added to 8 ml of a 100 mg/l solution of amarogentin and gentiopicroside in water. The samples were shaken at 150 rpm for 2 h at 35 °C. Control samples contained no β-glucosidase. The concentration of glucosides was determined before and 2 h after extraction.

Sixteen commercial multi-enzyme preparations for plant tissue maceration were analysed to determine their β-glucosidase activity as described by Agrawal and Bahl [8]. These multi-enzyme complexes were Ultrazym 100 G, Pectinex Ultra SP-L and Extrazyme from Novo Nordisk (Bagsvaerd, Denmark); Rohapect D5L, C2L, TF, VR and PC from Rohm GmbH (Darmstadt, Germany); Pektolase LM, CA and 3PA from Grinsted Products (Brabrand, Denmark); Rapidase C80, BE and Press from Gistbrocades BV (Delft, The Netherlands); Pectinase 362 from Biocatalysts Ltd. (Pontipryd, Wales) and Sclase Liquid from Sankyo Co., Ltd. (Tokyo, Japan). The multi-enzyme preparation with the lowest β-glucosidase activity was selected and 10 g of gentian root in 20 ml of water was incubated with 1500 PSU (Pectinase S. units) of the enzyme at 35 °C for 30 min. At the end of the incubation, 80 ml of ethanol: water 69 : 31 (v/v) was added and the incubation was continued on a rotary shaker at 150 rpm, for another 90 min at 35 °C. The alcoholic extract was filtered and stored at room temperature until analysed.

High-performance liquid chromatography analysis of amarogentin and gentiopicroside. Amarogentin was present at very low concentrations in the extracts so a concentration step was introduced. Ten-mililitres aliquots of each sample were diluted with water to decrease the alcohol concentration to 2–3% (v/v). Aliquots were then passed through a C18 Sep-Pak cartridge (Millipore, Milford, Mass., USA) previously activated with 2 ml methanol and washed with 10 ml distilled water. Amarogentin was eluted with 1.5 ml methanol. Gentiopicroside was analysed directly in the original extract, after filtering through a 0.45-µm filter.

Identification of amarogentin and gentiopicroside was carried out in a Beckman HPLC apparatus (Beckman Instruments, San Ramon, Calif., USA) equipped with a diode array detector (model 168), a programmable solvent module with two pumps (model 126) and an autosampler (model 502). The column used was a LiChrosorb RP18 (250 mm × 4 mm i. d.), with a 10-µm pore size and a guard-column (30 mm × 4 mm) of the same packing material. The mobile phase for the determination of amarogentin was methanol: water 43 : 57 (v/v) and for gentiopicroside methanol: water 30 : 70 (v/v), with a flow rate of 1 ml/min. The injection volume was 20 µl. The detection wavelength selected for amarogentin was 233 nm and 270 nm for gentiopicroside [9]. All analyses were carried out at room temperature. Both compounds were identified by comparing their retention times and ultraviolet (UV) spectra with those of authentic standards and, finally, by chromatography of extract samples spiked with authentic standards.

Quantification was achieved by the external standard method using a Waters HPLC apparatus (Milford, Mass., USA) equipped with two pumps (model 510), a UV detector (model 486) and an automatic sampler (model 717). Amarogentin and gentiopicroside were dissolved in their mobile phases at concentrations between 0.1–0.25 g/l and 1–5 g/l, respectively. Calibration curves were obtained by linear regression between concentration and area units for each peak.

Gas chromatographic analysis of the volatile fraction. A simultaneous distillation-extraction apparatus was used to obtain the volatile fraction from 200 ml of extract, previously diluted with distilled water to decrease its alcohol concentration to 5% (v/v). Distillation was carried out for 40 min; the volatile compounds were collected in about 20 ml pentane, which was evaporated by gentle heating.

Samples were analysed in a Hewlett-Packard 5890 series II gas chromatograph directly coupled with a 5971A mass selective detector on an HP-1 capillary column (50 m × 0.2 mm i. d., 0.11 µm film thickness), temperature programmed from 65 °C (4 min) to 240 °C (15 min), at increments of 4 °C/min. The injector temperature was 250 °C and the carrier gas (He) flow rate was 0.4 ml/min. Mass spectra were obtained by electron ionization at 70 eV; the ion source temperature was 260 °C. Identification of compounds was achieved by comparing their mass spectra with those of the commercial Wiley Mass Spectral Database (John Wiley and Sons, 1986) and with a library of standards compiled in our laboratories. Percent composition of samples was determined in a Hewlett-Packard 5880A gas chromatograph equipped with a flame ionization detector on an HP-101 capillary column (50 m × 0.32 mm, 0.30 µm film thickness). The operation conditions were those described above. The carrier gas (He) flow rate was 2 ml/min.

Results and discussion

Solvent extraction

The concentration of amarogentin and gentiopicroside in ethanol : water 50 : 50 (v/v) increased with time following a normal saturation kinetics curve for 2–3 h, at which point they reach their highest level (Fig. 1 a). At this point the concentration of each compound corresponded to its solubility in that solvent at 35 °C. On increasing the temperature to 75 °C the concentration of these compounds increased steadily, the rate for amarogentin being higher than that for gentiopicroside. The concentration of each compound obtained at each temperature corresponded to its solubility (S) at that temperature. If we present ln S versus $1/T$, where T is absolute temperature (Fig. 1 b), it can be concluded that these compounds have an ideal behaviour in ethanol : water and are not degraded. From linear regression, we can calculate a and b from the formula: $S = a \times e^{-b/T}$ where $a = 5.79$, $b = 962$ for gentiopicroside; and $a = 2.24$, $b = 1187$ for amarogentin.

The solubility of amarogentin and gentiopicroside obtained in a series of ethanol: water, propylene glycol: water and propylene glycol: ethanol: water mixtures is shown in Fig. 2. While gentiopicroside exhibited the expected solubility behaviour in ethanol-water mixtures, amarogentin showed very low values up to ethanol: water ratios of 40 : 60 (v/v). The highest concentration of amarogentin

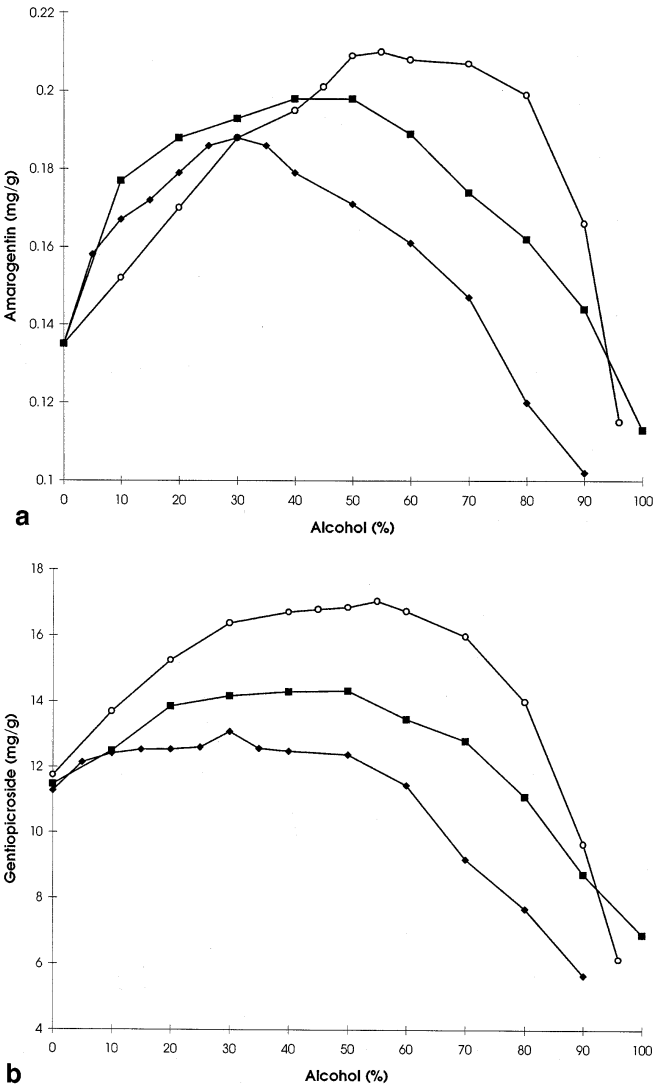

Fig. 2a Amarogentin and **b** gentiopicroside concentration in gentian root extracts obtained at 35 °C after 2 h extraction with different ethanol (○), propylene glycol (◆) and ethanol: propylene glycol 50 : 50 (v/v) (■) mixtures in water

Table 1 Main compounds identified in the different extracts of *Gentiana lutea* (*Rt, retention time, A.U.*, area units, *PG 30%*, propylene glycol: water 30 : 70 (v/v), *ETOH 55%*, ethanol: water 55 : 45 (v/v), *ETOH-PG 40%, ethanol: propylene glycol: water 20 : 20 : 60 (v/v/v), trace, n. d., not detected)*

		Rt (min)	PG 30% A. U. (%)	ETOH 55% A. U. (%)	ETOH-PG 40% A. U. (%)
1	Hexanal	7.3	n. d.	0.5	0.5
2	n-Hexanol	9.0	0.2	0.3	0.2
3	Unknown	10.7	tr	n. d.	0.3
4	Benzaldehyde	11.3	0.3	0.4	0.4
5	Heptanol	12.1	tr	0.1	0.1
6	1-Octen-3-ol	12.4	n. d.	0.1	n. d.
7	Hexanoic acid	12.6	3.2	0.7	0.8
8	Hexanoic acid ethyl ester	13.1	n. d.	8.6	14.3
9	Phenylacetaldehyde	14.2	0.9	0.3	0.5
10	2-Ethyl-1-hexanol	14.4	0.4	0.9	1.1
11	*cis*-Linalyl furanic oxide	16.0	1.3	0.8	0.7
12	Hexanal propylene glycol acetal	16.2	1.2	n. d.	0.5
13	*trans*-Linalyl furanic oxide	16.6	1.0	0.5	0.6
14	Unknown	16.8	1.2	n. d.	n. d.
15	Heptanoic acid ethyl ester	17.0	n. d.	0.2	0.3
16	Linalool	17.2	15.5	9.2	7.4
17	Alpha-cyclocitral	17.6	1.0	0.5	0.4
18	Unknown	18.8	1.7	0.8	0.7
19	Terpinen-4-ol	20.0	n. d.	0.2	n. d.
20	Octanoic acid	20.3	0.6	0.2	0.2
21	Alpha-terpineol	20.5	2.1	1.8	0.9
22	Octanoic acid ethyl ester	20.8	n. d.	1.1	1.2
23	Decanal	20.9	n. d.	0.2	n. d.
24	*trans*-2-*trans*-4-Nonadienal	21.1	tr	0.1	n. d.
25	Beta-cyclocitral	21.6	0.6	0.3	0.3
26	Nerol	22.0	n. d.	0.2	n. d.
27	Hexanoic acid propylene glycol ester	22.6	2.1	n. d.	0.5
28	Geraniol	22.9	1.1	0.5	0.4
29	Nonanoic acid	24.0	0.7	0.9	0.4
30	Nonanoic acid ethyl ester	24.4	n. d.	0.8	0.6
31	*trans*-2-*trans*-4-Decadienal	25.2	0.2	0.3	0.1
32	Eugenol	26.6	0.4	0.2	0.1
33	Beta-damascenone	27.5	1.1	0.5	0.6
34	Decanoic acid ethyl ester	28.0	n. d.	0.2	0.2
35	Unknown	29.4	12.0	10.3	6.3
36	Elemicine	32.5	47.8	48.6	49.1
37	Unknown	36.3	2.4	6.5	2.5
38	Nonanedioic acid diethyl ester	36.6	n. d.	2.2	6.8
39	Tetradecanoic acid ethyl ester	40.2	n. d.	0.4	n. d.

was obtained with ethanol: water 55 : 45 (v/v). With ethanol: propylene glycol: water 20 : 20 : 60 (v/v/v), the concentration of this bitter compound was only 5% lower than that obtained with ethanol: water 55 : 45 (v/v). When propylene glycol: water 30 : 70 (v/v) was used as can be seen in Fig. 2 a, the maximum concentration of amarogentin obtained was 12% lower than the concentration obtained with ethanol: water 55 : 45 (v/v) (Fig. 2 a).

The maximum concentrations of gentiopicroside and amarogentin were obtained with approximately the same mixtures of solvents. The decrease in gentiopicroside concentration obtained with ethanol: propylene glycol: water 20 : 20 : 60 (v/v/v) and with propylene glycol: water 30 : 70 (v/v) is about 16% and 25% respectively, in relation to the highest level obtained with ethanol: water 55 : 45 (v/v) (Fig. 2 b).

Although the differences in gentiopicroside concentration obtained with the three different solvent series were quite high in absolute terms, they had little or no impact on the bitter taste of the extracts due to the considerably low bitterness index of this compound as compared to that of amarogentin. From these results, the use of gentian root extracts with no or low ethanol content for different applications could be interesting, considering the 5% or 12% concentration reduction of the bitter compound amarogentin mentioned above.

Enzyme extraction

While amarogentin was not affected by enzyme extraction gentiopicroside was immediately degraded by β-glucosidases in aqueous solution. Most of the different commercial enzyme preparations tested showed β-glucosidase activity. Extrazyme was selected because it had the lowest β-glucosidase specific activity (0.12 μmol · min^{-1} · mg $prot^{-1}$). Extrazyme is a commercial multi-enzyme complex containing a wide range of carbohydrases, including araba-

nase, cellulase, β-glucanase, hemicellulase, pectinase and xylanase, and has an activity of 7500 PSU/g.

The analysis of bitter compounds in the extracts obtained after enzyme treatment of the plant material showed no difference to the compounds obtained without enzyme pretreatment. However, the enzyme treatment improved maceration of the plant material, increasing the final yield by 3.5% and reducing waste.

Extract aroma

Due to their sensorial importance, the volatile fractions of the different gentian root extracts were analysed. Table 1 summarizes the main components identified in the three selected extracts, together with their retention times and percent composition. It can be seen from Table 1, that the gentian root used in this study has a very high content of elemicine, which is characteristic of plants collected in the north-west region of Spain. Most of the compounds identified in all the extracts were also found in dried gentian roots and are responsible for their flavour, i. e. linalool and their furanic oxides, alpha-terpineol, alpha- and beta-cyclocitral and some other aldehydes, alcohols and acids [4]. However, there was a difference between the ester profile of solvent extracts and those of dried plant material. When ethanol or propylene glycol were present, most of the acids were converted to their corresponding ethyl or propylene glycol ester. Thus when alcohols were present, esters were formed in the gentian root extracts. On ageing of the extracts, the ester content increased until the acids disappeared completely.

The ethanol: water 55 : 45 (v/v) extract also had a higher lipid content, due to some less volatile aldehydes, acids and ethyl esters which were also found in the gentian root. However, although the other two extracts had a higher dry matter content, their lipid content was lower but they were richer in more polar components, due to the higher ratio of water in the solvent.

Sensorial analysis of these extracts showed some differences due mainly to the presence of ethanol and its esters. When ethanol was absent, the earthy dried leaf notes were stronger, but when the ethanol content increased, there was an augmentation in the fruity notes.

In conclusion, the highest level of amarogentin was obtained with an ethanol: water 55 : 45 (v/v) solvent mixture, but it was possible to reduce the ethanol content to 20% ethanol: propylene glycol: water 20 : 20 : 60 (v/v/v) and have only a 5% reduction in amarogentin concentration. When ethanol was completely substituted by propylene glycol, the highest concentration of amarogentin, obtained with propylene glycol: water 30 : 70 (v/v), was about 12% lower than that obtained with ethanol: water 55 : 45 (v/v).

Treatment of the plant tissue with Extrazyme increased the total yield by 3.5%, but the concentration of bitter compounds did not increase. Although small, the increase in yield could justify the use of an enzyme treatment step prior to extraction.

Volatile fraction analysis of the three different extracts showed differences, mainly in ester composition, which affected their sensory characteristics. However, all the extracts were acceptable and could be used in different food and beverage formulations.

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