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Analysis of iridoids, secoiridoids and xanthones in *Centaurium erythraea*, *Frasera caroliniensis* and *Gentiana lutea* using LC–MS and RP-HPLC

Anita Aberham [a], Valerio Pieri [a], Edward M. Croom Jr. [b], Ernst Ellmerer [c], Hermann Stuppner [a,*]

[a] *Institute of Pharmacy/Pharmacognosy, Center of Molecular Biosciences Innsbruck, University of Innsbruck, Innrain 52c, 6020 Innsbruck, Austria*
[b] *School of Pharmacy, University of Mississippi, Oxford, MS, USA*
[c] *Institute of Organic Chemistry, University of Innsbruck, Innrain 52a, 6020 Innsbruck, Austria*

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ABSTRACT

This study presents a new and validated HPLC method for the simultaneous determination of bioactive compounds in *Centaurium erythraea*, *Frasera caroliniensis* and *Gentiana lutea*. The iridoid loganic acid, four secoiridoids and 29 xanthones were separated on a RP-18 column, using aqueous o-phosphoric acid (0.085%, v/v) and acetonitrile as mobile phase. Phytochemical investigation of *C. erythraea* herb and *F. caroliniensis* roots resulted into isolation of 25 xanthones and three secoiridoids the structure of which was elucidated by spectroscopic means (NMR, MS and UV). 1,3,8-Trihydroxy-5,6-dimethoxyxanthone, isolated from *C. erythraea*, turned out to be a novel xanthone. The stability of the analytes was tested by subjecting samples to light, moisture and different temperatures. After six months of storage, decomposition of gentiopicroside and sweroside was observed. The swertiamarin content was nearly unchanged when stored at room temperature or in the refrigerator, but high temperature conditions reduced the content to 85%. In contrast, xanthones were stable under long-term, refrigerated and accelerated conditions. The established chromatographic method has been successfully applied for the quantification of the bioactive compounds in the three plants. The presence and distribution of polyoxygenated xanthones within the three members of the Gentianaceae family and their significance as analytical markers are discussed.

1. Introduction

Centaurium erythraea Rafn. (small centaury), *Frasera caroliniensis* Walter (American columbo) and *Gentiana lutea* L. (gentian) are widely distributed herbaceous plants members of the Gentianaceae which are renowned for their bitter properties. These are popular ingredients of many gastric herbal preparations and dietary supplements. As natural sources of food flavouring they are utilized in alcoholic and nonalcoholic beverages [1–4]. The bitter taste is due to the secoiridoids (e.g. swertiamarin (**2**), gentiopicroside (**3**), sweroside (**4**) and amarogentin (**5**), see Fig. 1 for structures). Numerous pharmacological effects have been attributed to these plant species e.g. stomachic, digestive, antinflammatory and antipyretic effects [3,5,6,8–10] as well as cholagogue, hepatoprotective and wound-healing activities [4,5,7]. Single constituents have been identified as active principles: The iridoid loganic acid (**1**) showed anti-inflammatory activity [11], xanthones methylswertianin (1,8-dihydroxy-3,7-dimethoxyxanthone, **29**) and swerchirin (1,8-dihydroxy-3,5-dimethoxyxanthone, **30**) were found to be hepatoprotective [12,13] and isogentisin (1,3-dihydroxy-7-methoxyxanthone, **18**) exhibited protective effects against endothelial damage caused by cigarette smoking [14] and revealed potent MAO inhibition [15].

Analysis of secoiridoids in gentian by HPLC has been described several times previously, but only one report describes the simultaneous determination of secoiridoids, iridoids and xanthones in gentian roots, including the complete validation data [16–19]. Two HPLC methods are reported about *C. erythraea*, but these are limited to a single compound (gentiopicroside; partially validated) or a group of constituents (xanthones; not validated) [20,21]. Coscia et al. isolated loganic acid (**1**) and gentiopicroside (**3**) from the roots of *F. caroliniensis*, and Stout et al. described the isolation of several xanthones, but there is no report of analytical methods for the determination of the bitter substances and xanthones of this plant [22,23]. In the course of this project a new fully validated HPLC method was developed for the simultaneous determination of bioactive compounds in *C. erythraea*, *F. caroliniensis* and *G. lutea*. This new method can be used for the chemical characterization of the plants and for quality control of several commercially available products. Another issue addressed in this paper is stability of analytical markers which is crucial for quality control especially when

* Corresponding author. Tel.: +43 512 507 5300; fax: +43 512 507 2939.
E-mail address: Hermann.Stuppner@uibk.ac.at (H. Stuppner).

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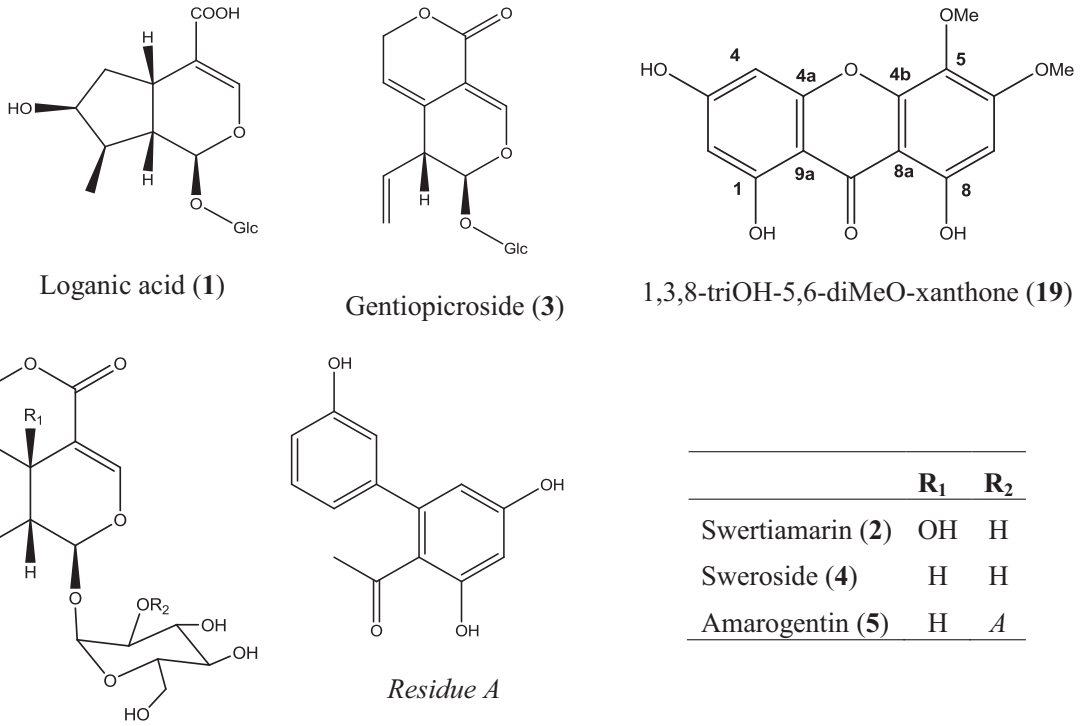

	R_1	R_2
Swertiamarin (**2**)	OH	H
Sweroside (**4**)	H	H
Amarogentin (**5**)	H	*A*

Fig. 1. Chemical structures of compounds **1**–**5** and the new xanthone (**19**).

dealt with iridoids and secoiridoid glycosides which decompose rather easily.

2. Experimental

2.1. General procedures

Column chromatography was performed using silica gel 60 (40–63 μm, 230–400 mesh; Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma–Aldrich, Vienna, Austria). Semi-preparative reversed phase HPLC was carried out on a Dionex system (Dionex, Vienna, Austria) with a P580 pump, ASI-100 autosampler, UVD 170U detector, Gilson 206 fraction collector (Gilson Inc., USA), a Phenomenex Synergi Max-RP column (250 mm × 10.0 mm; 10 μm particle size) and a mixture of water (A) and acetonitrile (B). The flow rate was adjusted to 3.5 ml/min, the detection wavelength set to 254 nm, and the temperature was 40 °C. Isolation of *F. caroliniensis* xanthones was done by means of a preparative liquid chromatography system with a Lobar® LiChroprep® RP-18 column (240 mm × 10 mm, 40–63 μm; Merck, Darmstadt, Germany) using a gradient of water (A) and methanol (B). The flow rate was adjusted to 1.0 ml/min, the detection wavelength set to 254 nm, and all separations were performed at room temperature. TLC was performed with silica gel 60 F_{254} plates (0.25 mm; Merck, Darmstadt, Germany). A compact inoLab pH Level 1 precision pH meter (WTW; Weilheim, Germany) was used for pH measurements. UV spectra were recorded on a Hitachi U-2000 spectrophotometer (Inula, Vienna, Austria). Melting point was determined on a Kofler melting point apparatus microscope (Reichert, Austria). The FTIR spectrum was recorded on a Bruker IFS 25 FTIR spectrometer in transmission mode within the range of 4000–600 cm^{-1}. The sample was applied to a ZnSe disk of 2 mm thickness. NMR spectra were recorded on a Bruker Avance 300 spectrometer at 300 MHz and a Bruker Avance AV 600 spectrometer at 600 MHz. High-resolution mass spectra were acquired on a micrOTOF-Q mass spectrometer (Bruker-Daltonics, Bremen, Germany). Ionization was performed in positive ESI mode. Nebulizer, dry-gas and probe temperature of the mass spectrometer were set to 10 psi (nitrogen), 4 l/min (nitrogen) and 180 °C, respectively; mass scan range was set in the range of *m/z* 100–1000.

2.2. Materials

C. erythraea plant material (CE-3) was purchased from Mag. Kottas & Sons (Vienna, Austria; batch: KLA70787). *F. caroliniensis* root material (samples FC-1 to FC-3) was collected in March 2007 (Blue Springs, Mississippi) and botanically identified by E.M. Croom, Jr. *G. lutea* plant material (GL-1) was purchased from Kräuter Mix (Abtswind, Germany; batch: B100778). Samples of *C. erythraea* herb (CE-1 to CE-11) and *G. lutea* roots (GL-2 and GL-3) were obtained from vendors in Germany and Austria. Samples CE-1 and CE-2 were kindly offered by Kräuter Mix (Abtswind, Germany; batch: P125977 and P116595). Samples CE-4 to CE-11 were a gift of Bionorica AG (Neumarkt, Germany). Gentian sample GL-2 was supplied by Alfred Galke (Gittelde, Germany; batch: 55801) and sample GL-3 by Drogerie Nierle (Freising, Germany). Reference specimens of all samples are deposited at the Institute of Pharmacy, University of Innsbruck, in Innsbruck, Austria.

2.3. Reagents

Acetonitrile (gradient grade), formic acid, o-phosphoric acid, sodium hydroxide, hydrochloric acid, sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate (all p.a. grade) were obtained from Merck (Darmstadt, Germany). Ethanol was obtained from Kögl Pharm (Innsbruck, Austria). Methanol, petroleum ether, diethyl ether, ethyl acetate, butanol, dichloromethane and acetone (all p.a. grade) were purchased from VWR (Vienna, Austria). All solvents for preparative separations were distilled prior to use, and water was purified by reverse osmosis followed by distillation. Reference compound **1** (loganic acid) was purchased from Extrasynthese (Genay, France), and reference

compound **5** was a gift of Bionorica AG (Neumarkt, Germany). Purity of all standard compounds was ≥97% (determined by HPLC).

2.4. Isolation procedures

2.4.1. Analyte isolation from C. erythraea

The dried and powdered plant material (500 g) was extracted with methanol by maceration. The resulting extract was dried *in vacuo* yielding 149.1 g of crude extract. This extract was redissolved in water and successively partitioned with petroleum ether, diethyl ether, ethyl acetate and butanol. The diethyl ether layer was brought to dryness *in vacuo* (5.8 g) and chromatographed on silica gel employing a gradient of dichloromethane and ethyl acetate (from 95:5 to 30:70, v/v), resulting into 19 fractions (A 1–19). Fraction A 4 (56.4 mg) was subjected to Sephadex LH-20 column chromatography (CC) using CH_2Cl_2 and acetone (85:15, v/v) as mobile phase, yielding six fractions (B 1–6). Fraction B 5 (11.2 mg) was dissolved in acetonitrile and separated by semi preparative HPLC (gradient: 0 min: 40A/60B; 15 min: 30A/70B) to give 7.2 mg of **14** (identified as 1,5-dihydroxy-3-methoxyxanthone by comparison of the NMR data with literature data [21]) and 3.7 mg of **19** (1,5,8-trihydroxy-3-methoxyxanthone [24]). Fraction A 5 (67.0 mg) was separated over Sephadex LH-20 CC using CH_2Cl_2 and acetone (85:15, v/v) as mobile phase, yielding 10 fractions (C 1–10). Compound **17** (19.7 mg; 1,6-dihydroxy-3,5-dimethoxyxanthone [25,26]) was obtained from fraction C 7 (22.1 mg) by crystallization.

Fraction A 8 (240.9 mg) enriched with four xanthones was subjected to Sephadex LH-20 CC using CH_2Cl_2 and acetone (85:15, v/v) as mobile phase, yielding 13 fractions (D 1–13). Fraction D 5 (97.5 mg) was dissolved in CH_3CN and separated by semi preparative HPLC (gradient: 0 min: 60A/40B; 15 min: 50A/50B) to give **9** (2.0 mg; 1,3,6-trihydroxy-2,5-dimethoxyxanthone [27]), **16** (7.5 mg; 1,6-dihydroxy-3,5,7,8-tetramethoxyxanthone [26,28]), **19** (4.9 mg; 1,3,8-trihydroxy-5,6-dimethoxyxanthone) and **21** (18.8 mg; 1,6,8-trihydroxy-3,5,7-trimethoxyxanthone [26]).

Fraction A 11 (1.0 g) was further chromatographed on silica gel CC using CH_2Cl_2 and MeOH (from 98:2 to 70:30, v/v), yielding five fractions (E 1–5). Compound **10** (13.8 mg; 1,3,5-trihydroxy-2-methoxyxanthone [29]) was obtained from fraction E 3 (14.2 mg) by crystallization. Fraction A 15 (332.3 mg) was subjected to Sephadex LH-20 CC using CH_2Cl_2 and acetone (85:15, v/v) as mobile phase, yielding 11 fractions (F 1–11). Fraction F 7 (67.5 mg) was dissolved in CH_3CN and separated by semi preparative HPLC (gradient: 0 min: 60A/40B; 15 min: 50A/50B) to give **8** (1.6 mg; 3-hydroxy-1,5,6-trimethoxyxanthone [29]) and **11** (2.5 mg; 1,5,6-trihydroxy-3-methoxyxanthone [30]).

The petroleum ether layer was brought to dryness *in vacuo* (13.9 g) and chromatographed on silica gel CC by means of a gradient of CH_2Cl_2 and MeOH (from 95:5 to 50:50, v/v), yielding five fractions (G 1–5). Fraction G 5 (957.2 mg) was dissolved in CH_3CN and separated by semi preparative HPLC (gradient: 0 min: 35A/65B; 20 min: 5A/95B) to give **25** (1.5 mg; 1-hydroxy-3,5-dimethoxyxanthone [26]), **29** (9.1 mg; 1,8-dihydroxy-3,7-dimethoxyxanthone [31]), **30** (3.5 mg; 1,8-dihydroxy-3,5-dimethoxyxanthone [31]), **31** (1.4 mg; 1,8-dihydroxy-2,3,6-trimethoxyxanthone [32]) and **32** (1.0 mg; 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone [33]). The isolation processes were monitored by TLC, LC–DAD and LC–DAD–MS. TLC was performed on silica gel plates, with CH_2Cl_2 and ethyl acetate (9:1 or 4:6, v/v) as mobile phase and vanillin/sulfuric acid as detection reagent.

The butanol layer enriched with the bitter substances **2**–**4** was brought to dryness *in vacuo* (39.9 g) and subjected to Sephadex LH-20 CC with MeOH, yielding seven fractions (H 1–7). Fraction H 3 (123.5 mg) was subjected to semi preparative HPLC (gradient: 0 min: 90A/10B; 15 min: 50A/50B) to give **2** (20.0 mg; swertiamarin [34]), **3** (5.0 mg; gentiopicroside [35]) and **4** (20.0 mg; sweroside [35]). Secoiridoids were further purified by repeated Sephadex LH-20 CC (eluent: MeOH). The isolation process was monitored by TLC, HPLC–DAD and HPLC–DAD–MS. TLC was performed on silica gel plates, with ethyl acetate, MeOH and H_2O (77:15:8, v/v) as mobile phase and vanillin/sulfuric acid as detection reagent.

2.4.2. Analyte isolation from F. caroliniensis

Dried and grounded plant material (150 g) was extracted with CH_2Cl_2 by maceration. After evaporation of the combined extracts *in vacuo* 7.6 g of crude dichloromethane extract was obtained. A portion (151.3 mg) of the extract was dissolved in 3 ml of 60% (v/v) MeOH and separated on a Lobar column with water (A) and MeOH (B) as mobile phase (gradient: 0 min 40A/60B; 120 min: 30A/70B) to give five fractions (A 1–5). Lobar CC (gradient: 0 min: 40A/60B; 120 min: 0A/100B) of fraction A 1 (7.8 mg) resulted into the isolation of **12** (1.0 mg; 1,3-dihydroxy-2,4,7-trimethoxyxanthone [36]), **13** (4.1 mg; 1,3-dihydroxy-4,5-dimethoxyxanthone [23]) and **15** (1.0 mg; 1,3-dihydroxy-2,5-dimethoxyxanthone [37]). Compound **20** (10.2 mg; 1-hydroxy-2,3,5-trimethoxyxanthone [23]) was obtained from fraction A 2 (19.6 mg) by crystallization. Fraction A 3 (25.6 mg) was further purified by repeated Lobar CC (from 40A/60B to 0A/100B) to give **22** (1.1 mg; 1,8-dihydroxy-2,5,6-trimethoxyxanthone [38]), **23** (2.2 mg; 1-hydroxy-2,3,7-trimethoxyxanthone [23]) and **24** (7.3 mg; 1-hydroxy-2,3,4,5-tetramethoxyxanthone [39]). Fraction A 4 (19.4 mg) was subjected to repeated Lobar CC (from 40A/60B to 0A/100B) to give **26** (1.2 mg; 1-hydroxy-3,5,6,7-tetramethoxyxanthone [21]), **27** (9.3 mg; 1-hydroxy-2,3,4,7-tetramethoxyxanthone [39]) and **28** (1.0 mg; 1-hydroxy-2,3,4,6-tetramethoxyxanthone [40]). Compound **30** (15.4 mg; 1,8-dihydroxy-3,5-dimethoxyxanthone [31]) was obtained from fraction A 5 (29.1 mg) by crystallization. The isolation process was monitored by HPLC–DAD and HLC–DAD–MS.

2.4.3. Analyte isolation from G. lutea

Isolation of compounds **6**, **7** and **18** was performed by chromatographic methods as described earlier [14]. Identity and purity of the compounds were confirmed by chromatographic (TLC, HPLC) and spectroscopic (1D- and 2D-NMR, LC–MS) methods in reference to literature values [41].

2.5. Spectroscopic data of the new xanthone

1,3,8-Trihydroxy-5,6-dimethoxyxanthone (**19**): yellow needles; mp 267–268 °C ($CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 210 (3.65), 234 (3.73), 254 (4.05), 278 (4.05), 334 (4.05); FTIR ν_{max}: 3413, 3083, 2992, 2935, 2850, 2582, 2359, 2326, 1987, 1660, 1622, 1463, 1437, 1328, 1252, 1228, 1208, 1184, 1147, 1071, 1024, 1005, 969, 923, 868, 845, 806, 734, 687, 656, 627 cm^{-1}; MS (ESI) *m/z* 304 $[M]^+$, 327 $[M+Na]^+$, 631 $[2M+Na]^+$, 213, 157, 139; HR-MS (ESI) *m/z* 631.0946 $[2M+Na]^+$ (calc. for $C_{15}H_{12}O_7$ + Na, 631.4920). ^1H NMR (CD_3COCD_3): δ 3.98 (3H, s, OCH_3-6), 3.81 (3H, s, OCH_3-5), 11.97 (1H, s, OH-1), 11.90 (1H, s, OH-8), 6.26 (1H, d, J = 1.9 Hz, H-2), 6.47 (1H, d, J = 1.9 Hz, H-4), 6.48 (1H, s, H-7). ^{13}C NMR (CD_3COCD_3): δ 61.2 (OCH_3-5), 56.6 (OCH_3-6), 163.5 (C-1), 99.0 (C-2), 166.6 (C-3), 95.0 (C-4), 158.3 (C-4a), 150.0 (C-4b), 129.4 (C-5), 160.7 (C-6), 95.6 (C-7), 158.7 (C-8), 101.6 (C-8a), 183.9 (C-9), 101.2 (C-9a).

2.6. Stability study

The ethanol content of liquid formulations of bitter drugs currently available on the market ranges from 19 to 40% (v/v). Therefore, stability tests were carried out with an aqueous ethanolic extract of small centaury (20%, v/v; unbuffered sample). The aqueous ethanolic solution was prepared as follows: Powdered aerial parts of *C. erythraea* (1450.0 mg) were macerated with

30.0 ml of 60% (v/v) ethanol, adding water gradually in order to obtain 100.0 ml of an aqueous solution with a final alcohol content of 20% (v/v). This aqueous ethanolic solution (pH 5.36) was subjected to stability tests.

To investigate the influence of pH on the stability of analytes, two times 100 ml of the aqueous ethanolic (20%, v/v) extract were prepared, each extract evaporated *in vacuo* to dryness and the residues redissolved in 80.0 ml of 0.1 M phosphate buffer solutions with pH values of 5 and 7. To each buffer solution 20.0 ml ethanol was added in order to obtain a final alcohol content of 20% (v/v).

Buffered and non buffered samples were stored in climatic chambers under the following conditions: Long-term stability studies were conducted by storage at 25 °C and 60% relative humidity (RH). Accelerated stability testing was conducted by storage at 40 °C and 75% RH. Furthermore, samples were stored in a cool, dark place under refrigerated conditions (refrigerator at +4 °C). Samples were withdrawn at 0 day, 1, 3 and 6 months, and the content of secoiridoids and xanthones was analyzed by HPLC. The samples were also checked for appearance.

2.7. Sample preparation for quantification purposes

The finely powdered plant material (600.0 mg) was extracted with methanol using accelerated solvent extraction (ASE 110; Dionex, Idstein, Germany) equipped with 10-ml stainless-steel cells. The cell was filled with methanol and pressurized (1500 psi). Three static cycles of 10 min and ambient temperature were used. After each extraction step, the pressure was released and the extract was collected in a 60 ml glass vial. The cell was rinsed with fresh solvent and purged using pressurized nitrogen gas at 1500 psi. The extracts were combined; each sample solution was assayed in triplicate.

2.8. HPLC and HPLC–MS conditions

2.8.1. Stability studies

Evaluation of stability was performed by means of an Agilent 1200 series HPLC instrument, equipped with binary pump, autosampler, column oven and photodiode array detector (Agilent, Waldbronn, Germany). A Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm particle size) from Agilent was utilized as stationary phase, the mobile phase comprised 0.085% (v/v) of o-phosphoric acid in water (A) and acetonitrile (B). Separations were performed by gradient elution (0 min: 99A/1B, 40 min: 5A/95B, 45 min: 5A/95B, 45.5 min: 99A/1B, 55 min: 99A/1B). Flow rate, temperature and injected sample volume were adjusted to 1.0 ml/min, 30 °C and 10 μl, respectively. Detection wavelength was set to 254 nm.

2.8.2. Quantitative HPLC and HPLC–MS

Quantification studies were performed on a LaChrom Elite HPLC system (Merck-Hitachi, Tokyo, Japan), equipped with L-2200 autosampler, L-2100 quaternary pump, L-2300 column oven and L-2400 UV-detector. Optimum separation of the compounds of interest was achieved on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm) from Agilent and a mobile phase comprising 0.085% (v/v) of o-phosphoric acid in water (A) and acetonitrile (B). Separations were performed by gradient elution (0 min: 95A/5B, 12 min: 80A/20B, 20 min: 70A/30B, 40 min: 20A/80B, 45 min: 2A/98B, 53 min: 2A/98B, 53.5 min: 95A/5B, 65 min: 95A/5B). Flow rate, temperature and injected sample volume were adjusted to 1.0 ml/min, 40 °C and 10 μl, respectively. Detection was performed at 254 nm.

HPLC–MS experiments were performed on an Esquire 3000 iontrap mass spectrometer (Bruker-Daltonics, Bremen, Germany), which was coupled to the HPLC (split ratio 1:3). HPLC separation conditions were identical with those described above, but eluent A had to be replaced by a mixture of water and formic acid in the ratio of 99.9:0.1 (v/v). For optimum MS results ionization was performed in positive ESI mode. Nebulizer, dry-gas and probe temperature of the mass spectrometer were set to 30 psi (nitrogen), 10 l/min (nitrogen) and 350 °C, respectively; mass scan range was set in the range of *m/z* 100–1000.

2.9. Validation

The HPLC method was validated for linearity, limit of detection and quantification, peak purity, accuracy, precision, repeatability and ruggedness. To produce the calibration line, a standard stock solution was prepared by dissolving six standard compounds in 5.00 ml methanol (1.00 mg of **1** and **5**, 2.00 mg of **21**, 10.0 mg of **2**, **3** and **4**). Six additional calibration levels were prepared by diluting this solution 1:2 with methanol (for HPLC chromatogram see Supplementary Fig. S-1). The standard solutions were stable for at least 1 month if stored at +4 °C (confirmed by re-assaying). All the xanthones were identified by comparing their retention times, UV and MS spectra with those of the previously isolated compounds and quantified based on the calibration data of 1,6,8-trihydroxy-3,5,7-trimethoxyxanthone (**21**). Limit of detection and limit of quantification for each analyte were determined by serial dilution of standard solutions containing the relevant compounds and calculated as three and ten times of the signal-to-noise ratios (based on a 10 μl injection). Peak purity and identity was confirmed by UV, HPLC–MS and NMR spectroscopy. Accuracy was determined by spiking sample CE-1 with three concentrations of standard compounds (low-, medium-, high spike). For this purpose known amounts of **1**–**5** and **21** were added to the dry, powdered plant material, which was then extracted and assayed as described before. The actually found amounts in relation to the theoretically present ones were expressed as percent of recovery.

Precision (intra- and inter-day) of the method was verified by analyzing sample CE-1 five-fold on three consecutive days. By comparing variations within the same days intra-day precision was determined, by observing differences within the three days inter-day precision was deduced. Repeatability was confirmed by evaluating consistency of retention times and standard deviations.

3. Results and discussion

3.1. Isolation and structure elucidation of the compounds

Chromatographic separation of the methanol extract of small centaury and the dichloromethane extract of American columbo resulted in the isolation of 25 xanthones and three secoiridoid glycosides. Identity and purity of the isolated compounds were confirmed by chromatographic (TLC, HPLC) and spectroscopic (1D- and 2D-NMR, HPLC–MS) methods in reference to literature values. Purity of all isolated compounds was ≥90% (determined by HPLC).

1,3,8-Trihydroxy-5,6-dimethoxyxanthone (**19**) is a new naturally occurring xanthone and has not been described in the literature before. The UV spectrum exhibited five characteristic absorption bands of xanthones. The molecular formula of **19** was established as $C_{15}H_{12}O_7$ by HRESIMS. Further ESI-MS data indicated the quasi-molecular ion peak at *m/z* 304 $[M]^+$ and fragment ions at 327 $[M+Na]^+$. The FTIR spectrum indicated the presence of hydroxyl groups (broad valence vibration at 3413 cm^{-1}). A strong absorption band at 1660 cm^{-1} due to the stretching of the C=O group in xanthones was detectable. NMR data revealed the presence of 13 aromatic carbon signals, corresponding to 10 quaternary and three methine carbons. Additionally, two methoxy groups appeared at δ 3.98 and 3.81. The ^1H NMR spectrum displayed the signals of

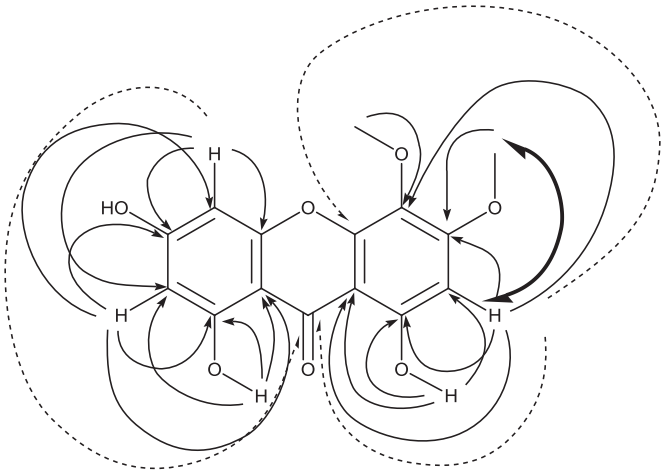

Fig. 2. Observed 1D-NOESY- and HMBC correlations of 1,3,8-trihydroxy-5,6-dimethoxyxanthone (1D-NOESY, bold; HMBC 2J/3J, black; 4J, dashed).

two chelated protons at δ 11.97 and 11.90, suggesting the presence of two hydroxyl groups at C-1 and C-8. A pair of *meta* coupled doublets at δ 6.26 and 6.47 (J = 1.9 Hz, ring A) were also observed in the ^{1}H NMR spectrum, integrating for one proton, each. HMBC correlations supported that ring A is 1,3-disubstituted bearing two hydroxyl groups (Fig. 2). The proton at δ 6.26 (H-2) was coupled to the carbons at δ 95.0 (C-4), 101.2 (C-9a), 163.5 (C-1) and 166.6 (C-3), whereas the proton at δ 6.47 (H-4) was coupled to the carbons at δ 99.0 (C-2), 101.2 (C-9a), 158.3 (C-4a), 166.6 (C-3) and 183.9 (C-9). The HMBC correlation between OH-8 (δ 11.90) and the carbon at δ 95.6 enabled the unambiguous assignment of the remaining methine group (δ_H 6.48, δ_C 95.6) to position 7. In the NOESY experiment (Fig. 2) mutual enhancement of the signals at δ 6.48 and 3.98 confirmed the presence of one methoxyl group (δ 56.6) at C-6. Therefore, the second methoxyl group (δ 61.2) must be placed at C-5. Further HMBC correlations between δ 6.48 (H-7) and carbons at δ 101.6 (C-8a), 129.4 (C-5), 158.7 (C-8), 160.7 (C-6) and 183.9 (C-9) were consistent with the 8-hydroxy-5,6-dimethoxy substitution pattern. Thus, the new xanthone was determined as 1,3,8-trihydroxy-5,6-dimethoxyxanthone.

3.2. Stability studies

Stability of analytes in samples is a prerequisite for the reliable quantification. According to the ICH-guidelines [42], samples were stored under different stress conditions for six months. Results of long-term, accelerated and refrigerated stability studies conducted with extracts of small centaury are shown in the supplementary information (see Supplementary Table S-1). Phosphate buffer solutions showed a similar behavior to citrate/borate buffer solutions (data not shown). At the end of six months, sample solutions kept at 40 °C were opalescent and did not show any precipitation. Solutions kept under refrigerated conditions showed some precipitation while the samples stored at room temperature exhibited a slight opalescence, but no precipitation.

In aqueous ethanolic and in buffer solution swertiamarin was stable for up to six months when stored at room temperature or at +4 °C (recovery rates ≥ 95%). Accelerated storage conditions led to decomposition of the compound. To further pursue the degradation behavior of swertiamarin, the solutions were analyzed after 12 months of storage. The content of the substance decreased about 50%. Accordingly, swertiamarin is able to withstand several storage conditions after an exposure time of at least six months, but after one year the secoiridoid underwent decomposition.

Table 1
Iridoids, secoiridoids and xanthones separated by HPLC.

No.	Compound	Rt (min)	*m/z*
1	Loganic acid	7.4	376
2	Swertiamarin	9.2	374
3	Gentiopicroside	10.4	356
4	Sweroside	10.8	358
5	Amarogentin	19.8	586
6	Gentioside	20.3	552
7	Gentioside isomer	20.8	552
8	3-OH-1,5,6-triMeO-xanthone	23.4	302
9	1,3,6-triOH-2,5-diMeO-xanthone	24.9	304
10	1,3,5-triOH-2-MeO-xanthone (tovopyrifolin C)	25.3	274
11	1,5,6-triOH-3-MeO-xanthone	26.3	274
12	1,3-diOH-2,4,7-triMeO-xanthone	29.1	318
13	1,3-diOH-4,5-diMeO-xanthone	29.8	288
14	1,5-diOH-3-MeO-xanthone (mesuaxanthone A)	30.0	258
15	1,3-diOH-2,5-diMeO-xanthone	30.1	288
16	1,6-diOH-3,5,7,8-tetraMeO-xanthone	30.2	348
17	1,6-diOH-3,5-diMeO-xanthone	30.4	288
18	1,7-diOH-3-MeO-xanthone (gentisin)	30.5	258
	1,3-diOH-7-MeO-xanthone (isogentisin)		258
19	1,5,8-triOH-3-MeO-xanthone (bellidifolin)	31.1	274
	1,3,8-triOH-5,6-diMeO-xanthone		304
20	1-OH-2,3,5-triMeO-xanthone	32.5	302
21	1,6,8-triOH-3,5,7-triMeO-xanthone	32.8	334
22	1,8-diOH-2,5,6-triMeO-xanthone (davaditin A)	33.9	318
23	1-OH-2,3,7-triMeO-xanthone	34.3	302
24	1-OH-2,3,4,5-tetraMeO-xanthone	34.5	332
25	1-OH-3,5-diMeOH-xanthone	34.8	272
26	1-OH-3,5,6,7-tetraMeO-xanthone	34.9	332
27	1-OH-2,3,4,7-tetraMeO-xanthone	35.3	332
28	1-OH-2,3,4,6-tetraMeO-xanthone (dulcisxanthone C)	35.5	332
29	1,8-diOH-3,7-diMeO-xanthone (methylswertianin)	36.0	288
30	1,8-diOH-3,5-diMeO-xanthone (swerchirin)	36.3	288
31	1,8-diOH-2,3,6-triMeO-xanthone	36.9	318
32	1,8-diOH-3,5,6,7-tetraMeO-xanthone (demethyleustomin)	37.4	348

In contrast, gentiopicroside turned out to be rather unstable. Storage of unbuffered and buffered solutions at 25 and 40 °C resulted into recovery rates for gentiopicroside in the range from 36.9 to 60.4%. Low temperature conditions did not stop degradation processes, but a slowdown of the decomposition took place. When stored at +4 °C the gentiopicroside content remained nearly unchanged (recovery rates ≥ 94%). Buffered solutions did not improve the results. Thus, because of their instability, the secoiridoids are not useful as analytical identifiers. In contrast, xanthones were stable under long-term, accelerated and refrigerated conditions (data not shown). For example, at the end of six months the content of compound **29** was more than 99% of the original one.

3.3. Method development and validation

Optimum separation of the constituents of small centaury, American columbo and gentian including secoiridoids, iridoids and xanthones was achieved by means of a rather complicated solvent gradient consisting of aqueous o-phosphoric acid (0.085%, v/v) and acetonitrile as mobile phase (Table 1). Concerning the stationary phase, out of a number of different columns tested (C-8, C-12, C-18, phenyl-hexyl and cyano column), best results were obtained with a Zorbax Eclipse XDB-C18. Only 1,5,8-trihydroxy-3-methoxyxanthone and 1,3,8-trihydroxy-5,6-dimethoxyxanthone eluted as one peak (**19**); this was also the case with gentisin and isogentisin (**18**). Separation of gentisin and isogentisin was achieved by replacing acetonitrile with a 1:1 (v/v) mixture of CH_3CN and n-propanol as described earlier [19], but this mobile phase was not

Table 2
Calibration data of compounds **1**–**5** and **21**, including regression equation, correlation coefficient (R^2), extinction coefficient (EC), linear range (in μg/ml), limit of detection (LOD; in μg/ml) and limit of quantification (LOQ; in μg/ml).

	Regression equation	R^2	EC[a]	Linear range	LOD	LOQ
1	$y = 6.3881E-5X$	0.9991	1.249	330–0.5	0.017	0.051
2	$y = 5.1127E-5X$	0.9999	1.000	2100–2.9	0.016	0.049
3	$y = 3.3374E-5X$	0.9998	0.650	1900–2.6	0.012	0.035
4	$y = 2.9639E-5X$	0.9998	0.578	1600–2.2	0.012	0.037
5	$y = 1.9494E-5X$	0.9999	0.381	200–0.3	0.020	0.061
21	$y = 1.0104E-5X$	0.9997	0.200	310–0.4	0.005	0.016

[a] Determined at 254 nm, in relation to swertiamarin (**2**).

suitable for separation of the other compounds. Thus, both isomers eluted as one peak when applying the described conditions.

The established analytical method was validated according to ICH-guidelines [42]. Data presented in Table 2 indicate linearity of the method within the tested range (0.3 – 2100 μg/ml), with a correlation coefficient of 0.9991 and higher. UV and MS spectra showed that the method is specific and selective, and there is no interference or overlapping of co-eluting impurities with the compounds response at 254 nm detection wavelength. Accuracy was assessed at three different concentration levels of spiked analytes in triplicate and ranged from 98.0 to 101.3% for all compounds (see Supplementary Table S-2). Relative standard deviations below 5.2% and very stable retention times over the whole study period (approx. 300 injections) indicated the methods repeatability. The overall intra- and inter-day variations are less than 4.9%, indicating satisfactory precision of the instrumentation and the stability of the samples (see Supplementary Table S-3). Finally, ruggedness of the developed method could be concluded by the fact that the same results were obtained on two different HPLC-instruments (LaChrom Elite and HP 1200).

3.4. Analysis of samples

Prior to sample analysis the optimum extraction conditions were determined. Different solvents (methanol, water and mixtures thereof) and extraction procedures (maceration, sonication, accelerated solvent extraction or refluxing) were evaluated. Since secoiridoids are rather unstable substances, a relative soft extraction method at room temperature was employed instead of refluxing on boiling water bath or time-consuming maceration. Accelerated solvent extraction with methanol showed to be most efficient and rapid (three cycles of extraction for 10 min each).

Table 3
Quantitative results for samples CE-1 to CE-11; values in μg/g; relative standard deviations are given in parenthesis ($n = 3$).

Samples											
No.	CE-1[a]	CE-2[a]	CE-3	CE-4	CE-5	CE-6	CE-7	CE-8	CE-9	CE-10	CE-11
2	30,698	79,195	102,580	63,231	64,823	45,065	34,493	44,170	37,197	89,373	111,191
	(0.8)	(0.6)	(0.7)	(1.0)	(0.3)	(0.9)	(1.8)	(0.3)	(0.1)	(1.3)	(0.7)
3	5409	2915	4264	1401	4202	974	6231	5182	4721	3301	4541
	(0.7)	(0.8)	(0.3)	(0.3)	(0.2)	(1.1)	(2.4)	(0.4)	(0.5)	(1.4)	(0.6)
4	29,156	13,003	16,305	3873	26,523	2858	35,238	26,672	24,426	2944	3303
	(0.7)	(0.7)	(0.7)	(0.8)	(0.2)	(1.1)	(1.5)	(0.3)	(0.03)	(1.0)	(0.3)
8	27.0	6.4	6.9	8.5	14.3	4.8	13.0	14.9	15.0	7.6	5.3
	(1.7)	(0.3)	(0.8)	(5.2)	(1.8)	(3.1)	(1.7)	(2.7)	(1.1)	(2.1)	(0.8)
9	1.7	4.1	8.0	10.0	2.4	11.1	22.3	6.6	10.4	11.5	12.9
	(3.0)	(1.0)	(3.3)	(3.3)	(0.3)	(1.9)	(4.2)	(2.2)	(1.9)	(4.5)	(2.1)
10	104.4	57.5	249.1	111.7	121.2	89.4	80.6	126.7	111.3	86.0	82.1
	(4.4)	(1.6)	(1.0)	(3.2)	(1.8)	(2.1)	(0.4)	(0.8)	(0.9)	(0.6)	(0.2)
11	12.5	36.1	46.6	84.5	44.2	61.2	25.2	38.9	33.5	55.2	59.7
	(4.3)	(2.3)	(0.3)	(1.0)	(0.3)	(2.7)	(2.0)	(0.7)	(0.1)	(1.3)	(2.4)
14	67.0	38.5	90.0	66.3	75.5	62.9	75.4	84.5	79.1	43.5	35.6
	(0.8)	(2.0)	(0.2)	(1.1)	(0.7)	(3.6)	(1.9)	(1.9)	(1.1)	(0.9)	(0.8)
16	16.1	20.6	26.1	21.6	25.7	25.9	17.0	15.7	12.8	28.5	35.0
	(0.2)	(3.9)	(1.7)	(0.5)	(1.1)	(2.8)	(3.1)	(4.5)	(0.6)	(2.6)	(2.0)
17	74.4	71.7	127.4	127.4	97.6	113.9	111.5	116.4	113.7	79.7	83.4
	(2.6)	(1.0)	(4.5)	(1.4)	(0.2)	(1.9)	(1.3)	(0.7)	(2.6)	(3.8)	(1.3)
19	76.0	86.0	45.5	58.8	66.5	71.1	55.9	56.2	57.5	80.8	91.6
	(1.1)	(0.9)	(0.6)	(0.4)	(0.6)	(1.1)	(2.7)	(1.4)	(1.8)	(1.9)	(0.8)
21	224.8	94.0	136.5	50.2	183.2	48.2	163.7	156.2	150.6	58.8	70.0
	(1.0)	(2.6)	(0.5)	(1.6)	(0.5)	(1.4)	(1.3)	(0.3)	(0.9)	(0.8)	(0.9)
25	12.2	6.4	13.8	8.7	18.6	9.1	12.2	10.6	10.7	4.1	2.2
	(1.2)	(1.0)	(1.6)	(0.4)	(0.4)	(0.9)	(1.9)	(1.5)	(2.7)	(2.3)	(4.1)
29	123.4	68.3	54.1	110.0	107.7	88.9	82.1	84.3	81.9	105.4	102.5
	(1.0)	(1.3)	(0.5)	(1.6)	(0.3)	(1.4)	(1.8)	(0.5)	(1.5)	(1.3)	(0.6)
30	97.3	49.9	30.4	33.1	86.2	28.4	113.8	108.8	105.0	27.6	27.3
	(5.2)	(1.0)	(0.7)	(0.7)	(0.3)	(1.6)	(1.7)	(0.6)	(1.4)	(1.5)	(1.5)
31	92.4	73.8	62.5	106.5	74.4	98.9	61.9	69.5	66.9	110.4	114.2
	(1.9)	(0.8)	(0.5)	(1.0)	(0.4)	(1.6)	(1.6)	(0.6)	(1.4)	(1.1)	(1.0)
32	1026.0	406.6	464.4	240.4	718.1	212.2	606.3	644.5	632.3	206.0	199.2
	(2.0)	(0.9)	(0.2)	(1.2)	(0.1)	(1.1)	(1.6)	(0.2)	(1.3)	(1.4)	(0.7)
X[b]	0.20	0.10	0.14	0.10	0.16	0.09	0.14	0.15	0.15	0.09	0.09

[a] Same supplier, but different batches.
[b] Total amount of xanthones in percent (m/m); xanthones quantified as compound **21**.

HPLC chromatograms of selected samples of centaury, American columbo and gentian are shown in Fig. 3. All of the quantified compounds were separated well, except peak **18** and **19**. The MS signals of each compound were assignable as $[M+H]^+$ or as sodium adduct ions, and deduced molecular masses are in good agreement to reported literature values (see Supplementary Tables S-4–S-6). As only small amounts of most of the xanthones were available they were quantified based on the calibration data of 1,6,8-trihydroxy-3,5,7-trimethoxyanthone (**21**). The obtained results are only approximate, because variations in substitution patterns will definitely have an influence on UV absorbance. Yet, considering their general structural similarity (see Supplementary Table S-7),

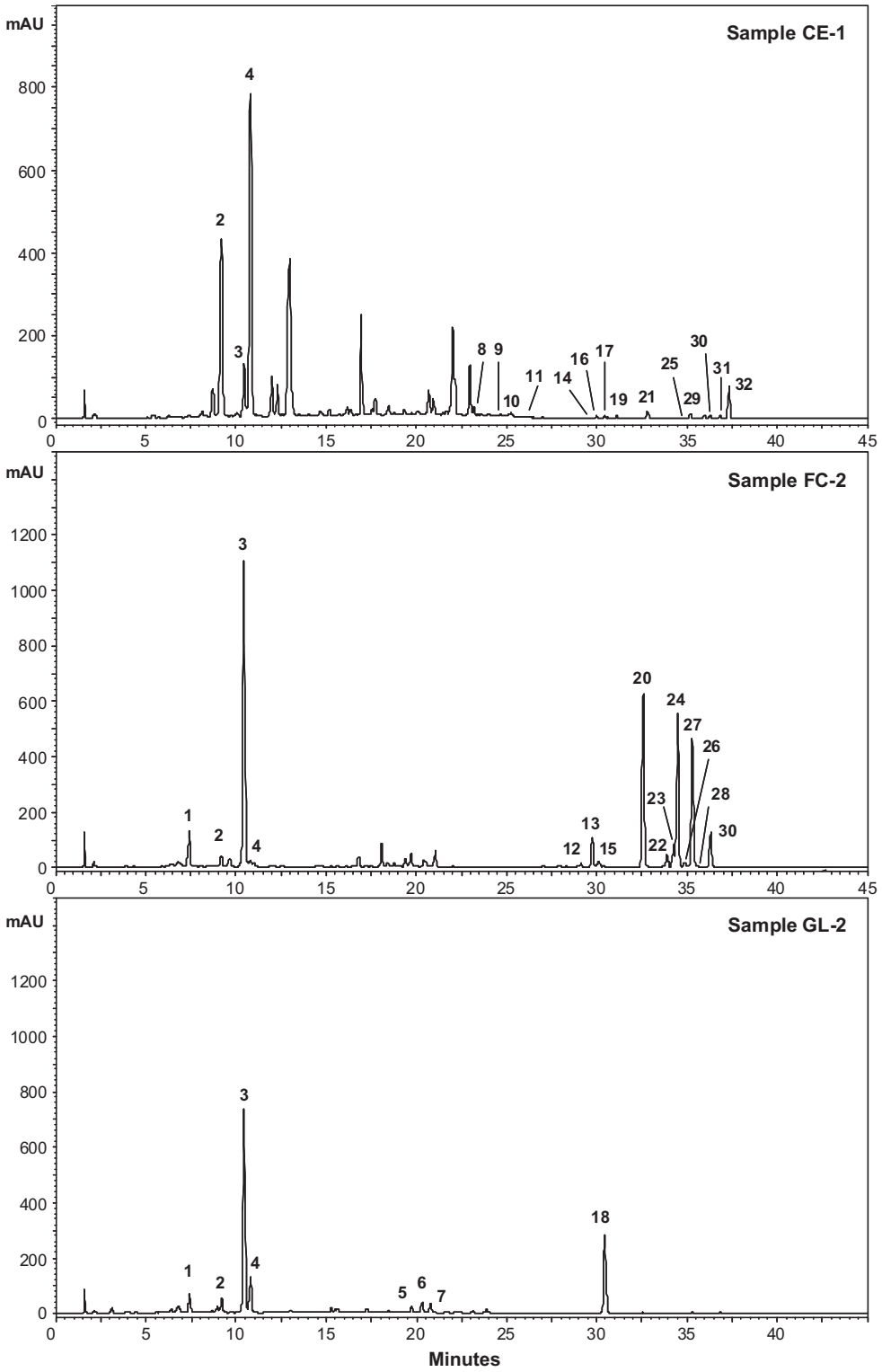

Fig. 3. Separation of samples CE-1, FC-2 and GL-2 under optimized conditions (column: Zorbax Eclipse XDB-C18 column, 150 × 4.6 mm, 5 μm; mobile phase: 0.085% o-phosphoric acid in water (A) and acetonitrile (B); gradient: 0 min: 95A/5B, 12 min: 80A/20B, 20 min: 70A/30B, 40 min: 20A/80B, 45 min: 2A/98B, 53 min: 2A/98B; flow rate: 1.0 ml/min; sample volume: 10 μl; temperature: 40 °C; detection: 254 nm).

Table 4
Quantitative results for samples FC-1 to FC-3; values in μg/g; relative standard deviations are given in parenthesis ($n = 3$).

Samples			
No.	FC-1	FC-2	FC-3
1	11,855 (1.0)	1064 (1.9)	11,230 (0.3)
2	2274 (0.3)	2604 (2.5)	2014 (0.3)
3	41,700 (0.3)	53,175 (1.7)	33,895 (0.4)
4	1025 (2.2)	1130 (4.3)	821 (0.5)
12	161.1 (0.01)	173.9 (1.0)	166.8 (0.3)
13	1197 (0.3)	1334 (1.3)	1315 (0.5)
15	369.9 (0.3)	406.9 (0.7)	374.9 (0.1)
20	8768 (0.4)	8664 (1.4)	8216 (0.4)
22	704 (0.7)	644.8 (1.4)	654.0 (0.3)
23	1228 (0.8)	1139 (0.7)	1185 (0.7)
24	7483 (0.5)	7644 (1.0)	7110 (0.2)
26	257.1 (0.4)	282.5 (1.6)	283.4 (0.2)
27	6399 (0.6)	6534 (1.1)	6137 (0.4)
28	90.3 (1.5)	89.7 (2.7)	86.4 (0.9)
30	1745 (0.4)	1801 (1.0)	1840 (0.1)
X[a]	2.84	2.87	2.74

[a] Total amount of xanthones in percent (m/m); xanthones quantified as compound **21**.

Table 5
Quantitative results for samples GL-1 to GL-3; values in μg/g; relative standard deviations are given in parenthesis ($n = 3$).

Samples			
No.	GL-1	GL-2	GL-3
1	10,922 (2.1)	4696 (0.3)	8628 (0.5)
2	6255 (1.5)	3308 (1.1)	3919 (0.7)
3	72,339 (0.7)	31,488 (0.6)	53,375 (1.3)
4	19,923 (0.7)	4911 (1.1)	1347 (4.2)
5	764 (0.8)	589 (0.8)	106.0 (0.2)
6	4256 (1.1)	438.6 (0.8)	4393 (1.0)
7	878 (0.7)	413.7 (0.7)	3251 (1.1)
18	1452 (0.9)	3990 (1.1)	2289 (0.7)
X[a]	0.66	0.48	0.99

[a] Total amount of xanthones (aglycons and xanthone glycosides) in percent (m/m); xanthones quantified as compound **21**.

an estimation of the xanthone content still is possible. All other compounds were quantified based on calibration data presented in Table 2. Additionally provided extinction coefficients will allow analysis of all compounds in case only swertiamarin (**2**) is available as standard.

Eleven samples of *C. erythraea* herb, three samples of *F. caroliniensis* roots and three samples of *G. lutea* roots were analyzed by the developed HPLC method. Quantitative results are given in Tables 3–5.

All analyzed samples of *C. erythraea* exhibited the presence of three secoiridoids (**2**–**4**) and swertiamarin (**2**), which was the major constituent. Xanthones showed the broadest deviation of all compounds, but all of the samples showed a common pattern with compound **32** as major representative, followed by **10**, **21** and **29**. Among the 15 xanthones found in small centaury herb 1,3,8-trihydroxy-5,6-dimethoxyxanthone (**19**) was not encountered before in nature. Xanthones isolated from *C. erythraea* are characterized by a high number of OH and/or methoxyl groups oxygenation pattern including tri-, tetra-, penta- and hexaoxygenation. Most of the xanthones were 1,3-dihydroxy or 1-hydroxy-3-methoxy derivatives with methoxyl substituents located in ring B.

Root samples of *F. caroliniensis* were collected from a very small area on the same day. Accordingly, no significant differences in the content of all 15 constituents could be detected. All analyzed samples exhibited the secoiridoids **2**–**4** and the iridoid **1.** In comparison to small centaury smaller amounts of **2** were observed and the main dominant bitter substance was **3.** Compounds **20**, **24** and **27** were the predominant xanthones. All the xanthones isolated from *F. caroliniensis* were tetra- or pentaoxygenated xanthones. The most dominant pentaoxygenated xanthones were based on a 1,2,3,4-oxygenation system with an additional methoxy group at C-5, C-6 or C-7 (e.g. compounds **24**, **27** and **28**).

Several xanthone glycosides were detected in American columbo roots but neither isolated nor unambiguously identified. HPLC analysis of samples FC-1 and FC-2 showed peaks eluting at a retention time of 15–22 min (see sample FC-2 in Fig. 3). UV spectra were characteristic of xanthones, and ESI-MS spectra displayed $[M+H]^+$ ion peaks at *m/z* 619 or 649, and signals at *m/z* 303 or 333, corresponding to the aglycones. The difference of 316 amu between two peaks in the MS spectrum is characteristic of the loss of a primverosyl moiety. Likewise, the presence of a gentiobiosyl moiety can be deduced from the characteristic loss of 346 amu. Sample FC-3 was nearly glycoside-free; in the chromatogram of the sample there were no peaks in the region of interest.

Quantitative data obtained for gentian roots were in good agreement with data published previously [19]. The main dominant bitter substance was **3**. Amarogentin (**5**), the bitterest known natural substance, is specific for gentian roots and was found in small amounts. Further, gentian has been shown to contain four xanthones (two aglycons and two xanthone glycosides). Gentisin (1,7-dihydroxy-3-methoxyxanthone) and its isomer isogentisin (1,3-dihydroxy-7-methoxyxanthone) are trioxygenated xanthones with a simple 1,3,7-oxygenation pattern and one methoxyl substituent.

The substitution pattern of the xanthones can be used for determination of the evolutionary position of a taxon. The higher the oxidation states of the compounds, the more evolutionary advanced taxa are [43]. Trisubstituted xanthones occurring in *G. lutea* are simple compounds and represent a primitive state. *Frasera* contains tetra- and pentasubstituted xanthones which demand many oxidation steps, and has therefore an indermediate position. *C. erythraea* produces different polysubstituted (tri- to hexaoxygenated) xanthones, and the hexaoxygenated ones are the most advanced. Hexaoxygenation as well as co-occurrence of tri-, tetra-, penta- and hexasubstituted compounds are an advanced character state in Gentianaceae.

4. Conclusions

The here presented HPLC method enables for the first time the simultaneous determination of several bioactive constituents of *C. erythraea*, *F. caroliniensis* and *G. lutea*. The method is fully validated and applicable to commercially available specimens. Because of their instability, the secoiridoids cannot be considered ideal analytical markers for the plant material. In contrast, polymethoxylated xanthones are stable and therefore may be suitable analytical markers, depending on the concerned preparation (e.g. sufficiently high absolute content for reliable quantification). Qualitative and quantitative analysis of polyoxygenated xanthones by HPLC is useful for chemical characterization and botanical classification of the plants. A new natural xanthone aglycone was isolated from *C. erythraea* herb and identified as 1,3,8-trihydroxy-5,6,-dimethoxyxanthone.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.09.030.

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