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Journal of Chromatography A, 725 (1996) 177–187

JOURNAL OF CHROMATOGRAPHY A

Systematic analysis of glucoiridoids from *Penstemon serrulatus* Menz. by high-performance liquid chromatography with pre-column solid-phase extraction

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Abstract

Samples (122) of crude ethanol-extracts of callus tissues from *Penstemon serrulatus* Menz. were used to develope a solid-phase extraction (SPE) clean-up procedure using the octadecylsilica packed cartridge for removing a complex mixture of free phenolic acids and anthocyanine-like colour substances, for the isolation of a sufficiently clean glucoiridoid fraction (GF). An additional SPE sample pretreatment step of the crude extracts enabled the enhancement of selectivity and sensitivity on applied high-performance liquid chromatography (HPLC) for the identification and quantitation of the iridoid constituents of the GF fraction. In particular, the presence of five valeriana-type esterified glucoiridoids which consist of penstemide, serrulatoloside, 8-epi-valerosidate, 7-desoxy-8-epi-valerosidate and serrulatoside in prepared GF fractions were determined by isocratic HPLC measurements. During a single HPLC separation, the traces of four non-esterified glucoiridoids, i.e. harpagide, aucuboside, loganine and plantarenaloside, were satisfactorily resolved and detected on the registered HPLC chromatograms of investigated GF fractions. The HPLC analyses were carried out on an octadecylsilica column (25 × 0.4 cm I.D.) using methanol–water (30:70, v/v) as the mobile phase with a flow-rate of 2 ml/min and the absorbance was monitored at 220 nm using an UV detector. The described chromatographic assay for penstemide, which exhibits potential antitumor activity against the P-388 lymphocytic leukemia cells, was applied to the monitoring and standarization of growth conditions for the callus cultures of *P. serrulatus*. Penstemide contents were found to range from 0.05% to 2.7% of the fresh weight of the investigated callus samples. Multivariate statistical methods (principal components analysis) was applied to demonstrate the influence of a variety of compositions of growth media (especially the type and concentration of synthetic growth regulators, e.g. 3-indolylacetic acid, 2,4-dichlorophenoxyacetic acid or 1-naphthylacetic acid, on the formation of different profiles of glucoiridoids in the callus cultures of *P. serrulatus*. Calculated principal component values were found to be useful for the explanation of variations in the penstemide/serrulatoloside ratio in the investigated samples and for determining the most favourable growth conditions in plants leading to optimal glucoiridoid biosynthesis.

Keywords: *Penstemon serrulatus* Menz.; Solid-phase extraction; Sample preparation; Glucoiridoids; Monoterpenoids

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0021-9673/96/$15.00
SSDI 0021-9673(95)01057-2

1. Introduction

Glucoiridoids are a group of monoterpenoid compounds characterised by the presence of sugar moiety at the α-pyrone ring of the iridoid skeleton (see examples in Fig. 1) and constitute an important fraction of pharmacologically active substances isolated from such families of medicinal plants as the *Scrophulariaceae*, *Labiatae*, *Rubiaceae*, etc. [1]. The antibiotic, hypotensive, anti-inflammatory, fungistatic and antibacterial properties of some non-esterified glucoiridoids (IG) from plants, were well documented [1,2]. Antitumor activity against P-388 lymphocytic leukemia cells, or antiproliferative properties against the mouse spleen lymphocytes and the hamster hepatoma cells of the rare natural valeriana-type glucoiridoids (VIG), i.e. penstemide and serrulatoloside, containing an additional isovaleroyl substituent at the main α-pyrone ring (see Fig. 2), have been also reported [3,4].

The occurrence of both mentioned types of glucoiridoids, IG and VIG, in several species within the genus *Penstemon* Mitch. (*Scrophulariaceae*) were well documented by multi-step chromatographic analyses of plant extracts [4–6]. Wysokińska [4,7] has performed the most detailed studies on the conditions for cell growth and enhanced production of VIG in the callus cultures of *Penstemon serrulatus* Menz.

However, no reports were found on the application of an efficient separation procedure enabling the simultaneous and reliable single-run chromatographic determination of all types of glucoiridoid present in the callus cultures of genus *Penstemon* Mitch. The large amounts of different types of secondary metabolites (e.g. cardenolide and acetophenone glucosides [6,8], anthocyanines and flavonoids [4,9], free aglycons and phenolic acids [4]) present in the alcohol extracts of glucoiridoid fractions from intact plants, callus tissues and cell suspensions from *Penstemon* Mitch. was probably the main reason why it was impossible to obtain an optimized direct HPLC procedure for the sensitive determination of each pharmacologically valuable constituent of such an extract. Dzido et al. [10] developed and optimized an isocratic HPLC and a stepwise gradient TLC procedure for the selective separation, quantitation and micropreparative isolation of penstemide and serrulatoloside from glucoiridoid extracts of callus tissues from *Penstemon serrulatus* Menz. Iida et al. [11] used binary gradient elution for the separation of two closely related IG, verbenalin and 6-dihydroverbenalin, in a leaf-extract of *Symplocos glauca* by thermospray HPLC combined with mass spectrometry. Recently, Guillerault et al. [12] reported the use of solid-phase extraction (SPE) procedure for the selective clean-up of harpagide, a similar type of glucoridoid to the dry commercial

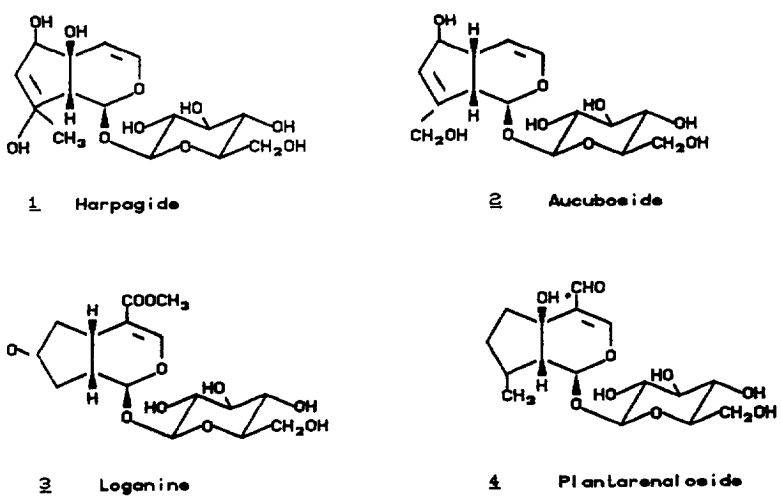

Fig. 1. Molecular structure of non-esterified glucoiridoids (IG): 1 = harpagide, 2 = aucuboside, 3 = loganine, 4 = plantarenaloside. Some hydrogen atoms have been omitted for clarity.

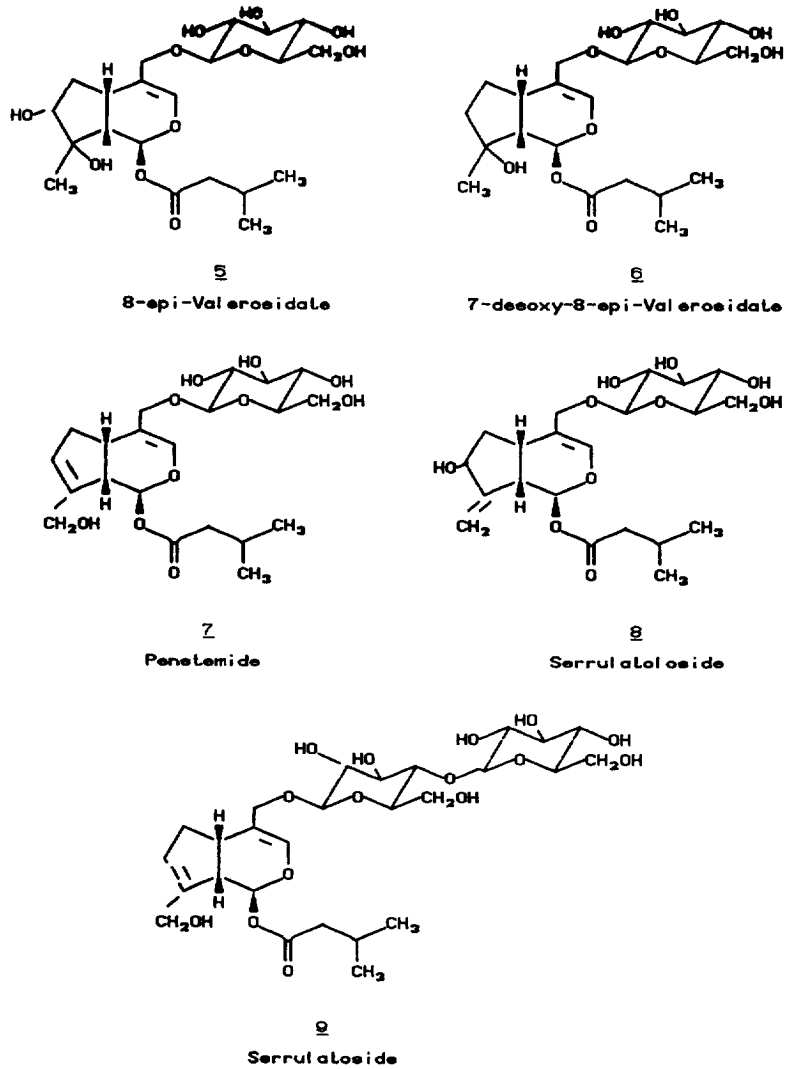

Fig. 2. Molecular structure of valeriana-type esterified glucoiridoids (VIG): 5 = 8-epi-valerosidate, 6 = 7-desoxy-8-epi-valerosidate, 7 = penstemide, 8 = serrulatoloside, 9 = serrulatoside. Some hydrogen atoms have been omitted for clarity.

extract of *Harpagophytum procumbens*, followed by their gradient HPLC separation and quantitative assay using a light scattering detector.

In view of the requirement for fast monitoring and characterization of the production conditions in an in vitro cultures of *Penstemon serrulatus* Menz. for the bioaccumulation of glucoiridoids with potential pharmacological interest, we performed a study on the application of an SPE procedure to preparations of the crude extract which enabled the direct, parallel, single-run and sensitive HPLC determination of all kinds of glucoiridoid in different species originated from this plant.

2. Experimental

2.1. Chemicals and standards

Spectrograde methanol (E. Merck, Darmstadt, Germany) was used in all experiments. Deionized and double redistilled water was used. All solvents

were filtered through Millipore filters (0.45 μm) and ultrasonically degassed before chromatographic use.

Glucoiridoid standards were purchased from Extrasynthese, Genay, France (harpagide) and Roth, Karlsruhe, Germany (aucuboside, loganine). Plantarenaloside, penstemide, serrulatoloside and serrulatoside standards were a generous gift from the Institute of Environmental Research and Bioanalysis, Medical University of Łódź, Łódź, Poland. The standards 8-epi-valerosidate and 7-desoxy-8-epi-valerosidate were obtained by preparative chromatography from *Penstemon serrulatus* plant extracts and the identity of their structures was confirmed by MS, ^{1}H NMR, ^{13}C NMR and IR spectra in accordance with previously reported results [4,7]. Anthocyanine standards (malvin and pelargonin) were obtained from Sigma (St. Louis, MO, USA). Phenolic acid standards (caffeic acid, ferulic acid and synapic acid) were obtained from Aldrich (Gillingham, UK).

Standard solutions of glucoiridoids. anthocyanines and phenolic acids were prepared in methanol within the concentration range of 0.1 mg/ml to 250 mg/ml and stored at −20°C until use.

2.2. Preparation of callus tissue samples

Samples of *Penstemon serrulatus* were gifts from Suchiro-cho, Kita-ku, Osaka, Japan. Callus cultures were initiated from the two-week-old flower buds of *P. serrulatus* which had anthers containing uninucleate microspores. The anther was placed on Murashige and Skoog (MS) agar medium supplemented with sucrose (50 mg/l) and one of the following growth regulators: 3-indolylacetic acid (IAA) (1 mg/l), 2,4-dichlorophenoxyacetic acid (DAA) (1 mg/l) or 1-naphthylacetic acid (NAA) (0.2 mg/l). After 5 weeks a light brown callus was transfered onto analogous medium as described above but containing a lower sucrose concentration (30 mg/l) and one of the specified growth regulators (0.2 mg/l). The callus tissues were subcultured every 5 weeks under continuous fluorescent light (about 10 μE/m^{2}s) at 25 ± 1°C. The callus samples numbered 1 to 31 were cultured on MS medium with IAA, those numbered 32 to 63 were cultured on MS medium containing DAA and those numbered 63 to 122 were cultured on MS medium fortified with NAA.

An experiment was perfomed to analyse the effect of decreasing concentration (in the range 1–0.02 mg/l, decreased in every sample by 0.03 mg/l) of DAA growth regulator in 29 callus cultures on the production efficiency and the ratio of penstemide to serrulatoloside.

2.3. Preparation of a crude extract of glucoiridoids

The dried and powdered callus tissues of *P. serrulatus* (2 g) were extracted twice with boiling ethanol for 3 h. Ethanol was then removed from the extracts using a rotary evaporator. The residue was washed with hot (80°C) water. The solid impurities were filtered off and the filtrate (aqueous solution) was evaporated under reduced pressure. Subsequent purification was performed using the alumina column (10 g Al_2O_3 with an activity of I, according to Brockmann [21]). Glucoiridoids were eluted from the column with 1 l of methanol (flow-rate of 3 ml/min). The fractions containing glucoiridoids were collected and evaporated to dryness. The residue was dissolved in 1 ml of methanol and analysed as the crude extract of glucoiridoids by HPLC, or was used for the clean-up SPE procedure followed by HPLC analysis.

2.4. Solid-phase extraction (SPE) clean-up of crude extracts

Solid-phase extraction of glucoiridoids from the crude extract of callus tissues of *P. serrulatus* was performed on the SPE cartridges packed with 200 mg of octadecylsilane C_{18}-bonded silica (J.T. Baker, Philipsburg, NJ, USA). The SPE cartridges were placed in the luer fittings of the Baker-10 extraction system manifold cover. A vacuum (1600 Pa) was applied and the cartridges were washed with two 3 ml portions of methanol and the vacuum was turned off so that the sorbent would not dry. The vacuum value remained the same during the subsequent cartridge elution. The 1 ml sample of crude extract of glucoiridoids was applied to the top of cartridge. The elution was started with 3 × 3 ml of water. The collected fractions were discarded. In the next step of

the SPE procedure the cartridges were eluted with 3 × 3 ml of methanol. The collected fractions were combined and evaporated under vacuum and dissolved in 300 μl of water. This final solution (containing glucoiridoids) was used for injection to the HPLC system.

In next step of the SPE procedure, the free phenolic acids (FA) retained on the SPE column were eluted according to the modified method of Schultz and Albroscheit [19], by rinsing the SPE cartridge slowly (under a vacuum of 10 kPa) with 4 ml of a methanol–25% ammonia (80:1, v/v) solution. The collected eluate was evaporated to dryness and dissolved in 1 ml of methanol.

2.5. HPLC measurements

A Liquochrom 2010 chromatograph (Labor-MIM, Budapest, Hungary) equipped with a Rheodyne 7010 sample loop injector (20 μl) (Supelco, Gland, Switzerland), a multiwavelength UV detector and a computing integrator CI 100A (Laboratorni Pristroje, Prague, Czech Republic) were used. Separations were carried out at 21°C on a thermostated LiChrosorb RP-18 (E. Merck) stainless-steel column (25 × 0.4 cm I.D.) packed with octadecylsilica gel with a particle size of 10 μm. The isocratic mobile-phase consisted of methanol–water (30:70, v/v) which was pumped at a flow-rate of 2 ml/min. The detector was set at a wavelenght of 220 nm and 0.5 AUFS. The hold-up time was determined by injection of sodium nitrate solution (0.1 mol/l) as a non-adsorbed solute. Retention times for the identification of glucoiridoids were established as the mean of four parallel measurements.

2.6. Quantitation

Calibration standards were situated at the beginning and at the end of the HPLC runs that were carried out each day. Linear regression analyses were performed for the mean peak areas and concentrations of both sets of glucoiridoid standards. This was used to calculate each analyte concentration in the crude extracts before and after the application of the SPE procedure.

2.7. Gas chromatographic assay of free phenolic acids (FA)

The content of free phenolic acids in the methanol–ammonia eluted fraction of crude callus extracts on SPE cartridges (see Section 2.4) was verified by GC after derivatization of FA with N,O-bis-(trimethylsilyl)acetamide (BSA) (Hicol B.V., Oud-Beijerland, Holland) according to the procedure described by Morita [20]. Anthranilic acid was used as the internal standard in these measurements. In GC experiments a modified CHROM 5 chromatograph (Laboratorni Pristroje, Prague, Czech Republic), equipped with a FID detector and a CI 100A computing integrator, was used. The mid/high-polarity polysiloxane type (25% cyanopropyl–25% phenyl–50% methyl) stationary phase DB-225 (J & W Scientific, Folsom, CA, USA) was applied to a prepacked fused-silica megabore column (15 m × 0.53 mm I.D.) as 1 μm film thickness. The temperatures of the injection port and the detector were 120 and 260°C, respectively. The following programme for the column temperature was employed: initial temperature 120°C (mantained for 1 min), increasing at a rate 5°C/min to 250°C. Argon was used as the carrier gas with a flow-rate of 5 ml/min. Samples (1 μl) were injected onto the column and nitrogen (30 ml/min) was used as the make-up gas.

2.8. Mulivariate statistics

The matrix of nine glucoiridoid concentrations (as presented in Fig. 1 and Fig. 2), was determined using the developed SPE and HPLC analysis in 122 samples of callus tissues from *P. serrrulatus* and was subjected to principal components analysis [13–15] using a specially written program in Pascal 6.0 and performed on an IBM AT 386/SX personal computer.

3. Results and discussion

In Table 1 the retention times of standard phenolic acids, anthocyanines and glucoiridoids obtained using the applied HPLC system have been presented. The retention of these compounds increased with increasing hydrophobic character of the free phenyl

Table 1
Retention times of standard compounds in an applied HPLC system[a]

No[b]	Compounds	Retention time[c] (t_R) min	
		Single	With SPE[a]
	Phenolic acids (FA)		
1.	Caffeic acid	1.3	–[d]
2.	Ferulic acid	1.8	–
3.	Synapic acid	4.3	–
	Anthocyanines (AC)		
4.	Pelargonin	7.8	–
5.	Malvin	14.3	–
	Glucoiridoids (Non-esterified) (IG)		
6.	Harpagide	8.1	8.2
7.	Aucuboside	14.3	14.5
8.	Loganine	13.9	14.0
9.	Plantarenaloside	16.9	17.1
	Valeriana-type (VIG)		
10.	8-epi-Valerosidate	26.1	26.0
11.	7-desoxy-8-epi-Valerosidate	29.2	29.3
12.	Penstemide	33.2	33.5
13.	Serrulatoloside	35.7	35.9
14.	Serrulatoside	48.1	48.3

[a]See Experimental (Section 2.5).
[b]Not related to the numbers used in Figs. 1–4.
[c]Mean of four determinations.
[d]Retained on the SPE cartridge.

ring, and of the flavandiol or iridoid skeleton in the respective class of considered solutes. It is clearly seen from this data that retention of anthocyanines strongly interferes with IG making the identification and quantitation of individual IG components impossible.

The proposed SPE of glucoiridoids was tested using the model mixture of all standard substances used in this study. In the developed SPE procedure (see Section 2) the standard phenolic acids and anthocyanines were completely, and selectively, retained on the octadecylsilica cartridge. Reproducibility of retention times for both groups of glucoiridoids (IG and VIG) was satisfactory after the SPE procedure. Linearity of calibration curves for each glucoiridoid was calculated by correlating the peak area against the corresponding concentrations in the range of 0.2–250 mg/ml (Table 2). The limits of detection for individual glucoiridoids were observed with a minimum signal-to-noise ratio of 2:1 and are presented in Table 2.

The effect of the use of the C_{18} cartridge on the amount of glucoiridoids detected has been studied using the model standard mixture and samples of crude extract. For the model standard mixtures, the use of SPE treatment tended to decrease the level of some VIG compounds (see Table 3). For serrulatoloside and serrulatoside the decrease was statistically significant ($P < 0.05$). In the case of crude extract samples, significantly lower levels ($P < 0.05$) of 8-epi-valerosidate were found when the SPE clean-up step was applied. The difference in the amounts of each VIG obtained between using both methods of glucoiridoid extraction (without or with SPE) was less than 5%, which compares well with the accuracy found in other studies using the SPE procedure for highly polar compounds [16,17].

Table 2
Coefficients of regression equations for linear calibration curves in the range 0.2 to 250 mg/ml ($y=ax+b$) and detection limits (d.l.) for glucoiridoids in HPLC measurements[a]

No.[b]	Glucoiridoid	Slope	Intercept	R^2	d.l. (ng/ml)
	Non-esterified (IG)				
1.	Harpagide	24047	8.93	0.9997	5
2.	Aucuboside	20776	7.26	0.9867	5
3.	Loganine	16623	6.77	0.9989	5
4.	Plantarenaloside	18434	5.58	0.9996	5
	Valeriana-type (VIG)				
5.	8-epi-Valerosidate	5738	−1.55	0.9998	2
6.	7-desoxy-8-epi-Valerosidate	5308	−1.63	0.9944	2
7.	Penstemide	4810	−1.71	0.9999	2
8.	Serrulatoloside	4529	−1.81	0.9988	2
9.	Serrulatoside	4675	−1.75	0.9943	5

[a] See Experimental (Section 2.5).
[b]Numbers as in Figs. 1–4.

Table 3
Mean concentration of glucoiridoids in a model standard mixture and crude extract sample (No. 23) prepared with, or without, SPE pretreatment[a] and followed by HPLC determination[a]

No[b]	Glucoiridoid	Concentration (mg/ml)			
		Model mixture (n=5)		Crude extract No. 23 (n=5)	
		Without SPE	With SPE	Without SPE	With SPE
	Non-esterified (IG)				
1.	Harpagide	3.2	3.3	N.D.[d]	1.7
2.	Aucuboside	10.1	10.0	2.4	15.2
3.	Loganine	2.5	2.6	N.D.	N.D.
4.	Plantarenaloside	15.2	15.2	3.1	16.3
	Valeriana-type (VIG)				
5.	8-epi-Valerosidate	8.0	8.2	3.1[5,c]	1.7[5]
6.	7-desoxy-8-epi-Valerosidate	5.0	5.1	2.3	2.4
7.	Penstemide	120.5	120.5	180.2	180.7
8.	Serrulatoloside	80.0[5]	75.6[5]	90.7	91.2
9.	Serrulatoside	10.0[5]	8.1[5]	9.5	9.7

[a]For details, see Experimental (Section 2.5).
[b]Numbers as in Figs. 1–4.
[c]Values with superscript numbers differ significantly (p<0.05).
[d]N.D.=not detected.

In Fig. 3, samples 19 and 56 are shown as representative HPLC chromatograms of crude extracts of glucoiridoids (without SPE pretreatment) from different cultures of callus tissues from *P. serrulatus*. The presence of a complex mixture of

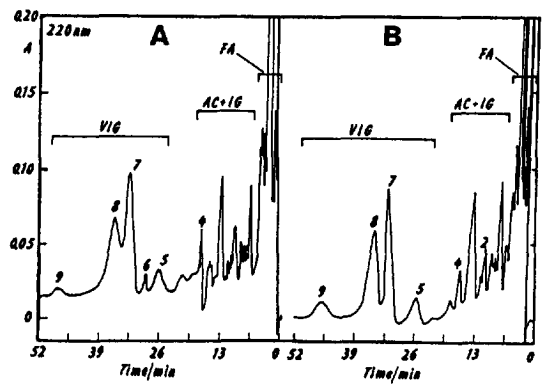

Fig. 3. Representative chromatograms from a crude methanolic extracts of glucoiridoids from the callus tissues of *P. serrulatus* before using a SPE clean-up pretreatment. The extracts contain the mixture of free phenolic acids (FA), anthocyanines (AC), non-esterified glucoiridoids (IG) and valeriana-type esterified glucoiridoids (VIG).(A) sample No. 19; (B) sample No. 56. Peaks: 2 = aucuboside, 4 = plantarenaloside, 5 = 8-epi-valerosidate, 6 = 7-desoxy-8-epi-valerosidate, 7 = penstemide, 8 = serrulatoloside, 9 = serrulatoside. For measurement details, see Section 2.5.

unidentified anthocyanine-like (AC) substances and large amounts of FA caused the higher level of recorded base-line, especially during the first minutes of chromatographic analysis, which reduced the possibility of identifying and quantitating the traces of the IG.

The high level of FA in callus tissues from *P. serrulatus* have been reported earlier [4,7] (with a total concentration of 0.185 mg per gram of dry weight of callus cultures) and was confirmed by our gas chromatographic analyses of N,O-bis-(trimethylsilyl)acetamide (BSA) derivatives of FA present in the collected methanol–ammonia fractions eluted in the SPE procedure (see Section 2.4 and Section 2.7). The total content of FA was determined as 0.215 mg/g and 0.240 mg/g in samples 19 and 56, respectively.

As can be seen, the HPLC analysis of the crude extract of glucoiridoids only allows the reliable separation and quantitation of the VIG group of compounds. The differences in the production abilities of 7-desoxy-8-epi-valerosidate (peak number 6 on Fig. 3A) by considering callus tissue No. 19 can be unequivocally stated. However, knowledge of the full glucoiridoid profile that could be potentially biosythesised in callus tisues of *P. serrulatus* is necessary for the evaluation of the synergistic effects

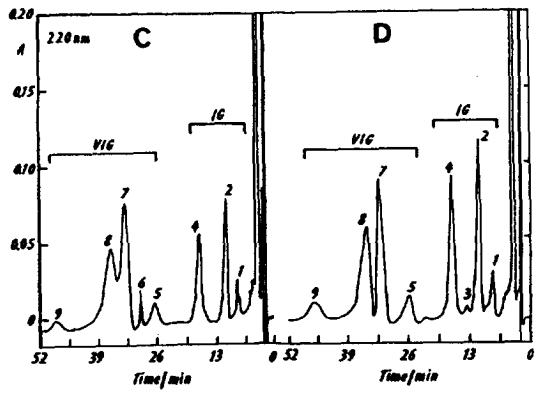

Fig. 4. Representative chromatograms from a crude methanolic extract of glucoiridoids from the callus tissues of *P. serrulatus* after using the SPE clean-up pretreatment. (C) sample No. 19; (D) sample No. 56. Peaks: 1 = harpagide, 2 = aucuboside, 3 = loganine, 4 = plantarenaloside, 5 = 8-epi-valerosidate, 6 = 7-desoxy-8-epi-valerosidate, 7 = penstemide, 8 = serrulatoloside, 9 = serrulatoside. For details of chromatographic analysis, see Section 2.5.

in such potentially pharmacologically active compouds.

Fig. 4 shows the HPLC chromatograms recorded after the proposed SPE pretreatment of the crude extracts of glucoiridoids (number 19 and 56) as previously described. Both specific groups of glucoiridoids, IG and VIG, were successfully separated here. The individual components of each glucoiridoid fraction could be reliably identified and quantitated using a monochrome UV detector operating at a wavelength of 220 nm, which is optimal in view of the maximal molar absorbance of such analytes. In particular, the differences in the composition of the IG fraction from callus samples 19 (Fig. 4C) and 56 (Fig. 4D) that have different nutritional factors in the growth media can be seen. The lack of a loganine peak (peak 3) and reduced amounts of aucuboside (peak 2) and plantarenaloside (peak 4) on the HPLC chromatogram of callus sample No. 19 (Fig. 4C) compared to the respective chromatogram of callus sample No. 56 leads to the conclusion that the type of growth regulator present in the MS agar medium controls the type and level of glucoiridoids biosynthesised by tissues of *P. serrulatus*. The significance of the type of growth regulator or of the source of nitrogen in the growth medium, on the diversified biosynthesis and bioaccumulation of VIG, anthocyanines and phenolic acids by callus tissues from *P. serrulatus*, have been reported previously [4,7]. However, the absence or presence of non-esterified glucoiridoids in callus tissues from *P. serrulatus* (stressed in the previously published reports [2,4,7]) were based on the results of non-selective thin-layer chromatographic procedures on extracts contaminated by anthocyanine-like compounds and FA.

Table 4 summarizes the selected results obtained on isocratic HPLC determination of the whole glucoiridoid profile in the callus tissue extracts of *P. serrulatus* using the proposed SPE clean-up pretreatment of crude extracts. The whole spectrum of VIG (with the highest concentration) have been observed only in the series of callus cultures initiated on MS medium containing 3-indolylacetic acid (IAA). In contrast, all of the IG (with the highest concentration) were determined only in the series of callus tissues grown on MS medium containing DAA. However, none of the analysed callus cultures contained full representatives of both classes of glucoiridoids. The lowest concentrations of penstemide and serrulatoloside were recorded in the callus cultures grown on medium supplemented with NAA. In earlier reports [4,7] the presence of 8-epivalerosidate and 7-desoxy-8-epivalerosidate in callus tissues from *P. serrulatus* were excluded and were detected only in different parts of the whole plant. As seen from the results presented here, the initiation of biosynthesis of both types of glucoiridoid could be strictly related to the composition of growth medium used.

The last conclusion is valid also in the case of harpagide and loganine, which have been not previously identified in callus tissues from *P. serrulatus* [2,4,7]. The concentrations of aucuboside and plantarenaloside were almost constant in all three series of callus cultures differing in the type of stimulating factor present in the agar medium.

The role of advanced finger-printing and statistical procedures, e.g. principal components analysis (PCA) or polar qualification system (PQS), was emphasized [18] for the standarization of the starting material for the production of pharmaceuticals of consistent quality. The data in Table 4 demonstrate that mean values of glucoiridoid content in specified series of callus cultures of *P. serrulatus* do not

Table 4
Mean level (% w/w of fresh weight of biomass) of glucoiridoids in callus tissues of *P. serrulatus* obtained on the Murashige–Skoog (MS) medium with different types of growth regulator

No[a]	Glucoiridoid	Type of growth regulator[b]					
		IAA[c]		DAA[c]		NAA[c]	
		Sample[d] No 19[f]	Mean[e]	Sample[d] No 56[g]	Mean[e]	Sample[d] No 96	Mean[e]
	Non-esterified (IG)						
1.	Harpagide	0.2	0.3	0.4	0.4	N.D.	N.D.
2.	Aucuboside	2.5	2.5	2.9	3.0	3.2	3.3
3.	Loganine	N.D.[h]	N.D.	0.05	0.06	N.D.	N.D.
4.	Plantarenaloside	2.3	2.3	2.6	2.7	3.5	3.6
	Valeriana-type (VIG)						
5.	8-epi-Valerosidate	0.4	0.5	0.8	0.8	N.D.	N.D.
6.	7-desoxy-8-epi-Valerosidate	0.3	0.3	N.D.	N.D.	N.D.	N.D.
7.	Penstemide	2.7	2.7	2.4	2.5	1.0	1.1
8.	Serrulatoloside	2.1	2.2	2.0	2.0	1.3	1.3
9.	Serrulatoside	0.6	0.6	0.7	0.8	0.1	0.1

[a]Numbers as in Figs. 1–4.
[b]Concentration in MS medium: IAA=1 mg/l, DAA=1 mg/l, NAA=0.2 mg/l.
[c]Abbreviations: 3-indolylacetic acid (IAA); 2,4-dichlorophenoxyacetic acid (DAA); 1-naphthylacetic acid (NAA).
[d]The most representative sample of callus.
[e]Mean determined for 31 samples of callus (n=4).
[f]Compare Fig. 3A and Fig. 4C.
[g]Compare Fig. 3B and Fig. 4D.
[h]N.D.=not detected.

distinguish the whole variation in the profiles of these bioactive compounds between the series members and whole batches. The three compact principal component (PC) values (Fig. 5), calculated in our studies, enable a better understanding of the diversity of callus cultures grown under different medium conditions. PCA was performed using the nine variables that were used to determine the concentrations of IG and VIG in all of the callus tissue samples investigated. The three principal components (PC1, PC2 and PC3) accounted for 86.15% of the original data variability. The first principal component, PC1, accounted for 61.10% of the total variance and correlated well with the concentration of VIG. The second principal component, PC2, accounted for 19.35% of the variance in the original data and was strongly (but negatively) correlated with the concentration of IG. The third principal component, PC3, accounted for 5.7% of the total variance in the original data and was caused by the concentration of penstemide and serrulatoloside. Fig. 5 shows a plot of the scores for these three main principal components and shows that the samples of

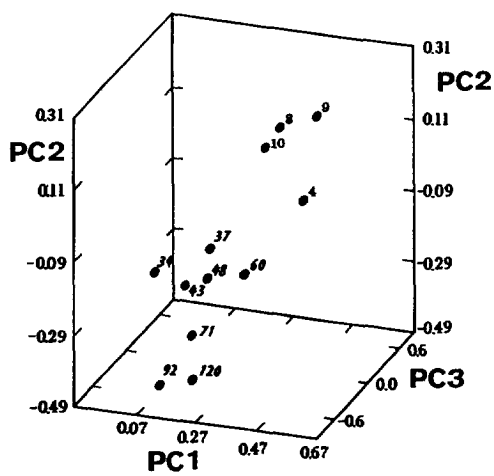

Fig. 5. Results of PCA on differences in bioaccumulation profile of nine glucoiridoids from the callus tissues of *P. serrulatus*. Numbers denote the callus cultures differing in the type of sythetic growth regulator used in MS growthing medium (see Section 2.2): Nos. 4–10 used 3-indolylacetic acid (IAA) as regulator, Nos. 34–60 used 2,4-dichlorophenoxyacetic acid (DAA) as regulator and Nos. 71–120 used 1-naphthylacetic acid (NAA) as regulator. Some numbers from each group of callus cultures were omitted for clarity.

callus tissues from *P. serrulatus* were fairly distinctly aggregated according to species. The series grown on IAA could be differentiated from the series of the other two species, based on the variables correlated with the second and third principal components, whereas variables correlated with the first and third principal component differentiated the DAA series from the NAA-grown series of callus tissues. Fig. 5 also implies that the profile of the glucoiridoids in callus cultures of *P. serrulatus* grown in medium supplemented with IAA were most homogeneous (points numbered 4–10) compared to the DAA (points 34–60) and NAA (points 71–120) series.

The influence of growth conditions on the standarization of the glucoiridoid profile in callus tissues from *P. serrulatus* was based on the results of PCA of 29 samples grown with decreasing concentrations of DAA in MS medium (see Section 2.2). Principal component scores were calculated for 29 previously mentioned callus tissue samples using a covariance matrix (29 × 6) in which the concentration of DAA growth factor, the penstemide/serrulatoloside ratio and the individual concentrations of the four IG were taken as variables. The position of each sample, as determined by the three main principal component axes (SPC1, SPC2 and SPC3), are shown in Fig. 6.

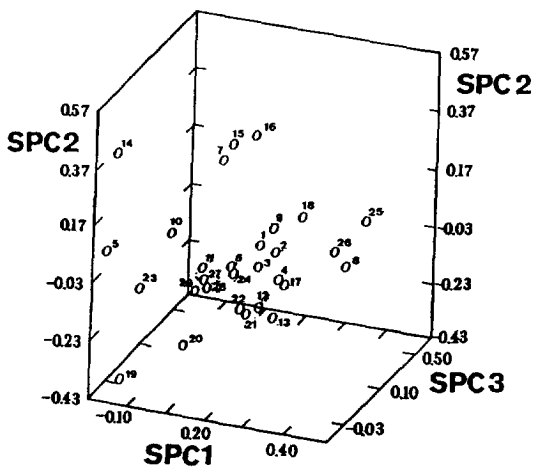

Fig. 6. Results of PCA on differences in penstemide/serrulatoloside ratio as determined in the callus tissues of *P. serrulatus* using decreasing concentrations of 2,4-dichlorophenoxyacetic acid (DAA) growth regulator (in the range 1 to 0.02 mg/l) added to MS growth medium (see Section 2.2). The increasing number on the plane denotes decreasing level of DAA used in MS medium.

The first abstract factor, SPC1, accounted for 71.0% of the observed variance of the original data matrix. The value of the DAA concentration in MS medium had a highly positive effect on the calculated SPC1 component. The second factor, SPC2, accounted for 16.9% of the observed varability of data and was highly negatively correlated to the concentration of IG. The third factor, SPC3, accounted for 7.2% of the variance and was related to the penstemide/serrulatoloside ratio. It can be seen from Fig. 6 that very similar glucoiridoid profiles (with a high penstemide/serrulatoloside ratio and a high concentration of IG) could be obtained using the lowest concentration of DAA (approximately 0.1 mg/l) in the MS medium (samples numbered from 27 to 29). Therefore, PCA facilitated the classification of callus tissue samples from *P. serrulatus* grown on DAA-containing medium, satisfying the closely similar pentemide/serrulatoloside ratio or IG fraction profile.

4. Conclusions

The use of a developed SPE procedure has given rise to a highly selective and robust analytical method for the simultaneous determination of different types of glucoiridoids bioaccumulated in the *P. serrulatus* species. PCA performed on the collected chromatographic data revealed additional valuable information on the glucoiridoid profile modifications caused in the callus tissues from *P. serrulatus* by different types and concentrations of synthetic growth substances in the agar medium.

Acknowledgements

The authors wish to thank the Crafoord Foundation (Lund, Sweden) for the kind support enabling one of them (G.B.) to participate and present this paper at the poster session of the 7th Symposium on Handling of Environmental and Biological Samples in Chromatography, May 7–10, 1995, Lund, Sweden. Dr J.A. Jönsson and Yin Shen from the Department of Analytical Chemistry, Chemical Center, Lund University (Lund, Sweden) are gratefully acknowledged for their helpful comments and suggestions on the material presented in this manuscript.

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