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High-performance liquid chromatographic analysis and separation of *N*-feruloylserotonin isomers

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Abstract

The *N*-feruloylserotonin containing fraction was isolated from seeds of *Leuzea carthamoides* (Willd.) DC by solvent extraction followed by column chromatography on silica gel or on Sephadex LH-20. Nuclear magnetic resonance spectroscopic analysis of the isolated fraction showed the presence of four structurally related compounds. These compounds were identified as four isomers of *N*-feruloylserotonin: *N*-(*Z*)-feruloylserotonin, *N*-(*Z*)-isoferuloylserotonin, *N*-(*E*)-feruloylserotonin and *N*-(*E*)-isoferuloylserotonin. They were analyzed by HPLC on Separon SGX C_{18}, Separon SGX and Separon SGX phenyl, using various mobile phases. Separon SGX phenyl phase was found the most efficient for a rapid analysis and for the final separation of the *N*-feruloylserotonin isomers.

Keywords: Phenyl phase; *Leuzea carthamoides*; *N*-Feruloylserotonin; *N*-Isoferuloylserotonin

1. Introduction

Conjugated serotonins have been found so far only in *Carthamus tinctorius* L. of the family *Asteraceae* [1–10] and in *Amorphophallus konjac* K. Koch. of the family *Araceae* [11]. High content of serotonin was found in walnut (*Juglans regia* L.; *Juglandaceae*) and in banana (*Musa sapientum* L.; *Musaceae*) [12,13]. The various activities of these compounds were reported [1–11], e.g., cathartic effect, antioxidative activities, radical-scavenging activities (electron donating ability), anti-tyrosinase activity, inhibitory activity on melanine production, potent inhibitory activity to Epstein–Barr virus (an in vitro assay for anti-tumor-promoting activity), growth-promoting activity for human and mouse fibroblasts and inhibitory effect on proinflamatory cytokine production. *N*-(*p*-coumaroyl)serotonin *trans* and *cis* isomers were found in the plant *Amorphophallus konjac* in an almost equivalent proportion [11]. However, in the most cases only *trans* isomers were reported, the eventual low admixtures of *cis* isomers were not detected. Isolation of feruloylserotonin and coumaroylserotonin was in the most cases performed by column chromatography [1–10] and collected fractions were monitored by thin-layer chromatography (TLC) [2,3,6]. High-performance liquid chromatography (HPLC) was used in a few cases for separations, but mainly for analysis of these compounds [1,4,5,7–11]. However, only one paper reported information on HPLC analysis and isolation of *cis* and *trans* isomers [11].

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We report here HPLC analytical and preparative conditions for the separation of four *N*-feruloylserotonin isomers found in one of the column chromatography fractions obtained during the fractionation of the phytoecdysteroid containing part of the seed extract of *Leuzea carthamoides* (Willd.) DC [14].

2. Experimental

2.1. Extraction

Seeds (700 g) of *Leuzea carthamoides* (Willd.) DC. [syn. *Rhaponticum carthamoides* (Willd.) Iljin] were first extracted by CO_2 supercritical fluid extraction in order to obtain and utilize the seed oil, and then were extracted by MeOH (yielding 137 g of extract) for recovering the characteristic *Leuzea* phytoecdysteroids [15–17]. Two portions of this extract (see below) were separated by column chromatography on silica gel or by low-pressure liquid chromatography (LC) on Sephadex LH20.

2.2. Chromatographic process A

MeOH extract (1 g) was separated by LC on a column of 550×50 mm packed with Sephadex LH20 (UV detection at 244 nm, specific solvent gradient program: 600 min, from 10% MeOH to 18% MeOH, further 300 min with 80% MeOH, flow-rate of 4 ml min^{-1}). Sixteen fractions were collected. The 14th fraction, collected in the interval 600–730 min, was further separated by HPLC under the following chromatographic conditions: column packed with Separon SGX C_{18} (600×26 mm, 5 μm particle size), solvent gradient program: 300 min from 10 to 80% MeOH, flow-rate of 5 ml min^{-1}, UV detection at 244 nm. The fraction eluted at 235 min was again separated by HPLC on a Silasorb 600 column (500× 12.7 mm, 5 μm particle size, Lachema, Brno, Czech Republic) under the following conditions: mobile phase *n*-hexane–EtOH–water (834:160:6, v/v), flow-rate 2.3 ml min^{-1}. For final HPLC purification, a column packed with Separon SGX C_{18} (500×17 mm, 7 μm particle size, Tessek Prague, Czech Republic) was used with a mobile phase of MeOH–water (30:70, v/v), a flow-rate of 3 ml min^{-1}, and UV detection at 244 nm. Compound that eluted as a dominating peak at 230 min was identified by nuclear magnetic resonance (NMR) spectroscopy as *trans*-*N*-feruloylserotonin [14].

2.3. Chromatographic process B

2.3.1. Separation by column chromatography (CC)

MeOH extract (10 g) was separated on a silica gel column (450 g, ICN SiliTech, particle size 32–63 nm, activated 15 h at 120 °C) eluted with increasing percentage of MeOH in $CHCl_3$. The solvent system was $CHCl_3$–MeOH at 20:1 (1400 ml), 18:1 (900 ml), 16:1 (900 ml), 14:1 (1000 ml), 12:1 (1850 ml), 10:1 (1400 ml), 8:1 (700 ml), 6:1 (900 ml), 4:1 (450 ml), and 2:1 (600 ml), followed by mixing the distilled eluent from previous fractions with MeOH in ratios of 4:1 (600 ml), 3:1 (350 ml), 2:1 (600 ml), 1:1 (400 ml) and MeOH 400 ml. Fractions (20 ml) were collected and distributed after a TLC monitoring into 24 collective fractions. Fractions 6–10 (1.4 g) contained one major compound with similar chromatographic properties as the standard compound *N*-feruloylserotonin from Section 2.2. These fractions were further separated on a silica gel column (60 g) with a mobile phase containing 2% MeOH in $CHCl_3$ (1000 ml), and 5% (900 ml) giving after TLC monitoring one large and unified *N*-feruloylserotonin containing fraction (420 mg).

2.3.2. Analysis and separation by HPLC

'Knauer—Modular HPLC System' equipment was used for all analyses and separations. Selected fractions from Section 2.3 were analyzed using system I: column packed with Separon SGX C_{18} (250×4 mm, 7 μm particle size), mobile phase A: water–MeOH (50:50, v/v), mobile phase B: MeOH, gradient program: initial conditions hold at 10% B for 1 min, with linear increase to 40% B over 21 min, and hold for 4 min, with linear increase to 100% B over 6 min, and hold for 3 min, return to initial conditions of 10% B over 4 min, and hold for 5 min, flow-rate of 1 ml min^{-1}, UV detection at 220 nm. The *N*-feruloylserotonin containing fractions (from Section 2.3) were also purified by preparative HPLC using system II: column packed with Separon SGX C_{18} (250×8 mm, 7 μm particle size), with mobile phase A: water–MeOH (50:50, v/v), mobile phase B:

MeOH, gradient program: initial conditions hold at 10% B for 1 min, with linear increase to 25% B over 19 min, and with linear increase to 100% B over 5 min, and hold for 4 min, return to initial conditions of 10% B over 5 min, and hold for 4 min, flow-rate of 3 ml min^{-1}, UV detection at 220 nm. This purification resulted in obtaining one single-peak fraction containing *trans-N*-feruloylserotonin.

2.3.3. Analysis and separation of the feruloylserotonin isomers

The *N*-feruloylserotonin containing fraction (from Section 2.3.2) was further analyzed and consequently also separated using various columns and solvent systems summarized in Table 1. For columns packed with Separon SGX C_{18} (7 μm particle size) systems 1 and 2 (see Table 1) were applied. For columns packed with normal-phase Separon SGX (250×4 mm, 7 μm particle size, Tessek) system 3 (Table 1) was applied and for columns packed with Separon SGX Phenyl (7 μm particle size, Tessek) systems 4–6 (Table 1) were applied. These three systems yielded the best results.

Table 1
HPLC retention times of the *N*-feruloylserotonin isomers **1**–**4** (see Fig. 2) in various systems

System	Retention time of isomers (min)			
	1	**2**	**3**	**4**
1	33.1	36.0	77.3	90.7
2	22.4	26.0	72.0	87.4
3	–	–	101.8	97.7
4	52.0	160.8	274.4	340.4
5	44.2	47.5	62.8	73.9
6	17.2	18.1	20.4	23.1

For columns packed with Separon SGX C_{18} (7 μm particle size) the following conditions were applied: system 1: column (250× mm), mobile phase MeOH–water (30:70, v/v) at 0.5 ml min^{-1}; system 2: column (250×8 mm), mobile phase MeOH–water (30:70, v/v) at 5 ml min^{-1}.

For column with Separon SGX (250×4 mm, 7 μm particle size) the following conditions were applied: system 3: mobile phase hexane–EtOH (90:10, v/v) at 0.9 ml min^{-1}.

For columns with Separon SGX Phenyl (7 μm particle size) the following conditions were applied: system 4: column (250×4 mm), mobile phase MeOH–water (30:70, v/v) at 0.5 ml min^{-1}; system 5: column (250×4 mm), mobile phase MeOH–water (40:60, v/v) at 0.5 ml min^{-1}; system 6: column (250×4 mm), mobile phase MeOH–water (50:50, v/v) at 0.5 ml min^{-1}.

UV detection at 220 nm was used in all cases.

3. Results and discussion

The roots of *Leuzea carthamoides* contain various ecdysteroids [15–17]. Our interest was to compare the content and composition of ecdysteroids in the different organs of this plant. Seeds were selected for comparison, because of their high content and structural variability of ecdysteroids [18]. Seeds were in our case extracted and separated in a similar procedure (chromatographic process A) as was described earlier for roots [16]. However, in the obtained fractions were detected besides ecdysteroids [15,16] also phenylpropanoids of lignan and *N*-feruloylserotonin type [14]. In order to investigate biological activities of these compounds we scaled up separations by a modified procedure (chromatographic process B), using *trans N*-feruloylserotonin (obtained from process A) as internal standard. The *N*-feruloylserotonin containing fraction obtained from the column chromatography was analysed and purified by HPLC (in systems I and II) and resulted in a one single-peak product. NMR spectra showed, however, that the obtained sample was a mixture of several compounds [14]. The previously described HPLC conditions as reported in papers [1,4,5,7–10] and also those used in our first experiments (systems I and II) were found insufficient for separation of this family of compounds.

It was observed that even the modified conditions for reversed-phase (RP) HPLC (e.g., system 1 in Table 1) did not significantly improve the separation efficiency. The calculated values characterizing chromatographic separation (resolution, capacity, separation and retention factors summarized Tables 2 and 3), as well as our repeated analyses have shown that the separation efficiency of this system was for the separation of *N*-feruloylserotonin isomers insufficient. Similar results were observed after re-examination of fractions separated by system 2. The separation conditions for *trans*-feruloylserotonin and *trans*-cumaroylserotonin, as applied for the column Nova-Pak C_{18} by Kang et al. [7] containing only 7% carbon load, were found to be very little promising. Such a low content of carbon load (compared with our Separon SGX C_{18} column) would very probably result in a decreased effectiveness of separation.

HPLC performed on Separon SGX (normal phase) was found unsuitable for a good separation of the

Table 2
Capacity factor k_i'[a] and separation factor α_{ji}[b] of the *N*-feruloylserotonin isomers **1**–**4** (Fig. 2) in various systems

System	k_1'	k_2'	k_3'	k_4'	α_{21}	α_{32}	α_{43}
1	12.2	13.4	29.9	35.3	1.10	2.23	1.18
2	23.6	25.5	53.6	63.0	1.08	2.10	1.17
3	–	–	35.8	37.4	–	–	1.05
4	26.5	84.7	145.4	181.1	3.20	1.72	1.25
5	22.6	24.4	34.4	38.5	1.08	1.41	1.12
6	8.5	9.0	10.2	11.6	1.06	1.13	1.14

[a] $k'=(t_{Ri}-t_{R0})/t_{R0}$=capacity factor for the first-, the second-, the third- and the fourth-eluted isomers, respectively.
[b] $\alpha_{ji}=k_j'/k_i'$=separation factor for the eluted *N*-feruloylserotonin isomers in various systems.

isomers, too. As demonstrated in Tables 1–3 (for system 3), the *trans* isomers did not separated each other sufficiently, and the *cis* isomers were hidden in the tailing part of the second *trans* isomer peak. After decreasing the EtOH content in the eluent (*n*-hexane–EtOH) from 90:10 to 92:8, the retention times and the separation effectiveness of *trans* isomers significantly increased, but the *cis* isomers appeared only as inflexes on the second *trans* isomer peak.

The majority of the above mentioned negative effects of separation was removed by using a column packed with Separon SGX phenyl phase. In this case the separation effectiveness of isomers significantly improved (see systems 4–6 in Tables 1–3). The optimum fast separation was found using system 6, as demonstrated by the chromatogram (Fig. 1) and by data in Tables 1–3. Four compounds were detected in this chromatogram, which were after their separation identified by NMR spectroscopy [14] as *N*-(*Z*)-feruloylserotonin (**1**), *N*-(*Z*)-isoferuloylserotonin (**2**) *N*-(*E*)-feruloylserotonin (**3**), and *N*-(*E*)-isoferuloylserotonin (**4**) (Fig. 2).

The isomers were eluted from the phenyl phase (similarly as from the RP) in two groups. In the first group are present *Z*, i.e., *cis* isomers (feruloyl followed by the isoferuloyl isomer), in the second group are *E*, i.e., *trans* isomers (again feruloyl followed by isoferuloyl isomer).

The content of *cis* isomers (of cumaroyl-, feruloyl- or isoferuloylserotonin) in plants is usually much lower than the content of *trans* isomers. Therefore, it is possible that *cis* isomers as minor components were in some of the earlier analyses not detected and thus in the respective papers not reported. The identification of *cis* and *trans* isomers of *N*-(*p*-cumaroyl)serotonins, present in *Amorphophallus*

Table 3
Resolution R_{ij}[a] of the *N*-feruloylserotonin isomers **1**–**4** (Fig. 2) in various systems

System	R_{12}	R_{23}	R_{34}
1	1.1	11.3	2.5
2	0.5	4.4	1.0
3	–	–	0.4
4	11.6	12.6	4.7
5	1.7	7.5	2.1
6	1.5	2.2	1.6

[a] $R_{ij}=2\cdot(t_{Rj}-t_{Ri})/(Y_j+Y_i)$=resolution for the eluted *N*-feruloylserotonin isomers in various systems.

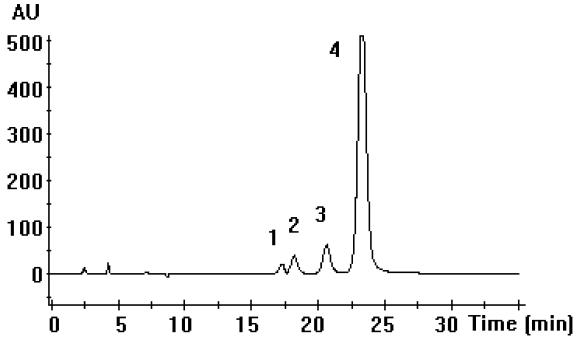
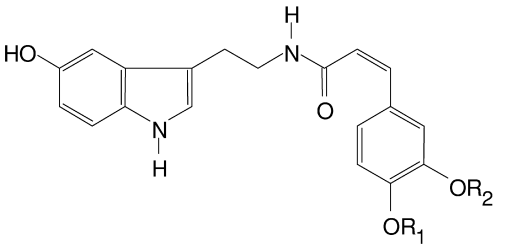
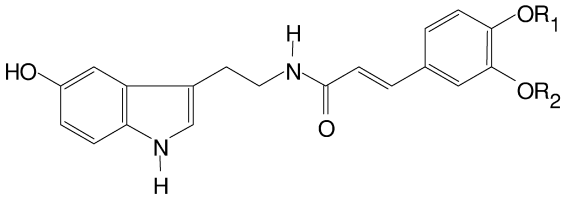


Fig. 1. HPLC separation of *N*-feruloylserotonin isomers **1**–**4** in the *N*-feruloylserotonin fraction on a Separon SGX Phenyl column (250×4 mm) eluted with methanol–water (50:50, v/v) at 0.5 ml min^{-1} (system 6). The peaks 1–4 represent the corresponding isomers **1**–**4** (see Fig. 2).



1. $R_1 = H$, $R_2 = CH_3$ N-(Z)-feruloylserotonin

2. $R_1 = CH_3$, $R_2 = H$ N-(Z)-isoferuloylserotonin



3. $R_1 = H$, $R_2 = CH_3$ N-(E)-feruloylserotonin

4. $R_1 = CH_3$, $R_2 = H$ N-(E)-isoferuloylserotonin

Fig. 2. Chemical structures of *N*-feruloylserotonin isomers **1**–**4**.

konjac in an almost equivalent ratio [11], as well as our results, support indirectly such an assumption.

4. Conclusion

Chromatographic analysis of *N*-feruloylserotonin isomers **1**–**4** has shown that columns packed either with a usual reversed phase or with a normal phase allow only isolation of the *N*-feruloylserotonin fraction from the other compounds of the extract. Final separation of individual isomers can be completed only on a column filled with a phenyl phase.

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