ELSEVIER

Journal of Chromatography A, 949 (2002) 249–262

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

# Simultaneous determination of *N*-oxides and free bases of pyrrolizidine alkaloids by cation-exchange solid-phase extraction and ion-pair high-performance liquid chromatography

Tomasz Mroczek, Kazimierz Glowniak*, Anna Wlaszczyk

*Department of Pharmacognosy, Medical University, 12 Peowiaków St., 20-007 Lublin, Poland*

**Abstract**

Cation-exchange solid-phase extraction using LiChrolut SCX (Merck, Darmstadt) cartridges filled with polymeric strong cation-exchanger enabled efficient isolation of both *N*-oxides and free bases of pyrrolizidine alkaloids (PAs). The recoveries were about 80% for retrorsine-*N*-oxide, 90% for retrorsine and 100% for senkirkine and were assessed both by TLC–densitometry and ion-pair high-performance liquid chromatography (HPIPC) on Hypersil BDS $C_8$ stationary phase and hexane-1-sulfonic acid as ion-pairing agent. The applied HPIPC gradient procedure was suitable for separation of PAs with various types of structures (*N*-oxides, free bases, otonecine-PAs). The method limits of detection and quantitation, respectively, ranged from 0.06 ng/μl (senecionine) and 0.2 ng/μl (senkirkine) to 0.1 and 0.35 ng/μl for retrorsine-*N*-oxide. For each component calibrated by linear regression method, correlation coefficients were higher than 0.9995 (six-point calibration from 4 to 100 μg/ml). The elaborated procedure was used in searching for PAs in plant derived samples from *Symphytum* sp. (comfrey), *Petasites hybridus* and *Petasites albus* (butterbur), *Tussilago farfara* (coltsfoot), *Emilia coccinea* (tassel flower) and *Doronicum columnae* (leopard's bane). For the last three samples macrocyclic PAs (senecionine, senecionine-*N*-oxide, senkirkine) have been detected for the first time. Details of precision of the analyses are also included.


*Keywords:* Ion-pair chromatography; Plant materials; Pyrrolizidines; Alkaloids; Hexanesulfonic acid

## 1. Introduction

Pyrrolizidine alkaloids (PAs) are natural compounds mainly distributed in Boraginaceae, Asteraceae and Fabaceae families [1–3]. They were among the first naturally occurring carcinogens found in products of plant origin [3], but some of them (i.e., platyphylline) are investigated for therapeutic use [1]. Their wide distribution, both geographical and botanical, makes their probable presence in herbal tea preparations a matter of public health significance [3]. Beside its hepatotoxic effect, treatment with PA-containing products proved to be hepatocarcinogenic and mutagenic [4–7] showing female rats to be more sensitive than males [4].

For PAs having an unsaturated basic moiety ($\Delta^3$-pyrroline ring) which are toxic, spectrophotometric color reactions were elaborated [8–11], whereby the base is oxidized to *N*-oxide which, when heated with acetic anhydride (or acetyl chloride and 4-dimethylaminopyridine [10] for PAs containing otonecine structure), is dehydrogenated to a pyrrole. The

*Corresponding author: Tel./fax: +48-81-53233-14.

*E-mail address:* glowniak@hipokrates.am.lublin.pl (K. Glowniak).

0021-9673/02/$ – see front matter 
PII: S0021-9673(01)01498-4

pyrrole is then coupled with Ehrlich reagent (4-dimethylaminobenzaldehyde) to give a colour adduct. The same conditions can be applied to detection of PAs on TLC plates [12–14].

For the analysis of PAs in natural samples GC–MS methods are often applied [15–24], but these methods are not suitable for *N*-oxides which are unstable at GC method conditions; usually *N*-oxide should be formerly reduced to free bases with Zn dust/acidic solution [15], but in such conditions some amounts of *N*-oxides are decomposed. In some cases, for GC determination of retronecine, bis-(heptafluorobutyrate) derivatives should be formed prior to analysis [18]. Gas chromatography matrix-isolation Fourier transform infrared spectroscopy (GC–MI-FT-IR) was also used to the identification of PAs from comfrey (*Symphytum officinale* L.) roots [25].

$C_{18}$ and amino-bonded RP-phases were applied in HPLC with UV detection determination of PAs in comfrey [26] but peak asymmetry factors obtained were unsatisfactory. HPLC coupled with mass spectrometry was also reported [27–29] usually with thermospray interface. Some papers already published have dealt with ion-pair HPLC analysis of PAs on $C_{18}$ [30] or cyano-bonded RP phases [31] but sample preparation still needs to be improved although solid-phase extraction procedures were applied [30,32]. Solid-phase extraction using $C_{18}$ cartridges can be easily performed [30] and gives good recoveries (>90%) both for *N*-oxides and free bases but, due to the amount of polar co-extractive compounds, accurate HPLC method is not feasible as high background baseline absorbance is always present.

The sample preparation method, proposed here, by using LiChrolut SCX (Merck, Darmstadt) polymeric strong cation-exchanger enabled very efficient isolation of both *N*-oxides and free bases of PAs which could be further analysed by newly elaborated gradient ion-pair HPLC on $C_8$ RP phase with baseline separation of the alkaloids quantified in various plant-derived samples.

## 2. Experimental

### 2.1. Chemicals and reagents

The standards of senkirkine, senecionine and seneciphylline were obtained from Carl Roth (Karlsruhe, Germany). Retrorsine and retrorsine-*N*-oxide were purchased from Sigma (St. Louis, MO, USA). Hexane-1-sulfonic acid sodium salt, LiChrolut SCX (500 mg, 3 ml) strong cation-exchange solid-phase extraction columns and TLC plates (20×10 cm, 0.25 mm thickness) coated with silica gel $F_{254}$ sorbent were from Merck. Methanol, acetonitrile and 85% phosphoric acid (each solvent was of HPLC gradient grade) were obtained from J.T.Baker (Gross-Gerau, Germany). Tartaric acid, 36% hydrochloric acid, 25% ammonia and chloroform (each solvent was of analytical grade) were from POCh (Gliwice, Poland). Double-distilled water was used in all experiments. In the recoveries studies the mixture of three standards (retrorsine, retrorsine-*N*-oxide and senkirkine) at the concentration of 0.2 mg/ml was prepared by dissolving 2 mg of each standard in 10 ml of methanol in a 10-ml volumetric flask. The investigated plant samples (excluding the leaves of coltsfoot purchased from Herbapol (Lublin, Poland) were correctly identified by the botanist (Ms Maria Byc) and collected from the Botanical Garden of the University of Maria Curie-Sklodowska in Lublin, Poland, in August 2000. Voucher specimens of these plants are deposited at the herbarium of the garden.

### 2.2. Extraction of plant materials

A 1-g (for the samples from comfrey) or 2-g sample (for the other samples but excluding the leaves from coltsfoot suited for the decoction) of dried at room temperature and powdered plant material was extracted in a 500-ml round-bottom flask for 2 h with boiling 1% solution of tartaric acid in methanol (100±5 °C) under reflux. After cooling at room temperature the extract was filtered and diluted to the mark with methanol in a 100-ml volumetric flask. Then the aliquots (10-ml) of each extract were transferred to a 50-ml round-bottom flask and evaporated to dryness under reduced pressure at 44 °C.

Decoction from leaves of coltsfoot (*Tussilago farfara*) was prepared from 5 g of plant material purchased from Herbapol by dipping the material in 200 ml of distilled water in a 350-ml beaker and heating the boiling extract for 15 min. After cooling at room temperature the decoction was filtered and diluted to the mark with methanol in a 200-ml

volumetric flask. Then the aliquots (50 ml) of each extract were transferred to a 200-ml round-bottom flask and evaporated to dryness under reduced pressure at 44 °C.

Aliquots (10 ml) of juice from the flowers and leaves of coltsfoot obtained from Phytopharm (Klęka, Poland) were used in the experiments and further elaborated in the same way as the aliquots from the leaves of coltsfoot.

### 2.3. Cation-exchange solid-phase extraction

#### 2.3.1. Method development

Cation-exchange solid-phase extraction (SPE) has been optimised at first by the application of different mixtures of ammonia in methanol for the elution of PAs from LiChrolut polymeric strong cation-exchanger columns. For this purpose 0.4-ml aliquots of the mixed standard solution (equivalent to the amount of 80 μg for senkirkine, retrorsine and retrorsine-*N*-oxide) were transferred to a 50-ml round-bottom flask and evaporated to dryness under reduced pressure at 44 °C. The residues were dissolved in 15 ml of 0.05 *M* hydrochloric acid for the cation-exchange SPE procedure. Two parallel samples were prepared and analysed in this way.

The cartridge was first conditioned with 10 ml of double-distilled water (0.04 MPa pressure was adjusted during the solvent application) and then very slowly (0.01 MPa pressure; the flow-rate was adjusted to 1 drop in 3 s), 15 ml of 0.05 *M* hydrochloric acid was applied. After the application of 0.05 *M* hydrochloric acid, a 3-mm layer of the solvent had to remain. Then the alkaloids dissolved in 15 ml of 0.05 *M* hydrochloric acid were also very slowly, at the same speed of dropping, transferred into the cartridge. After the transfer of the alkaloid standards, the cartridge was dried under reduced pressure for 1 min. Then the cartridge was eluted with 4 ml of methanol (the extract was removed). The alkaloids were eluted from the sorbent with various elution mixtures (0.02 MPa pressure was adjusted during elution which resulted in 1 drop in 2–3 s): (a) 15 ml of methanol–10% ammonia (3:1, v/v); (b) 15 ml of methanol–10% ammonia (3:2, v/v); (c) 15 ml of methanol–25% ammonia (8:1, v/v). The alkaloids fractions after cation-exchange SPE were evaporated to dryness under reduced pressure at 44 °C and dissolved in 2 ml of methanol for TLC–densitometry or ion-pair high-performance liquid chromatography (HPIPC).

#### 2.3.2. Clean-up of the samples analysed

The residues after evaporation of the solvent from the extracts analysed (three parallel samples) were dissolved in 15 ml of 0.05 *M* hydrochloric acid and applied into a LiChrolut SCX cartridge in the same way as it was described for the mixed standard solutions. The alkaloids were eluted from the cartridge with 15 ml of methanol–10% ammonia (3:1, v/v). Then, after cation-exchange SPE, the fractions were evaporated to dryness under reduced pressure at 44 °C and dissolved in 2 ml of methanol for HPIPC qualitative and quantitative analyses.

Fortified extracts from *Symphytum asperum* and *Tussilago farfara* roots as well as *Emilia coccinea* herbs (three parallel samples) were prepared by adding 0.4 ml (and in the case the extract from *Emilia coccinea* additionally also 0.6 ml) of the mixed standard solution of senkirkine, retrorsine and retrorsine-*N*-oxide (each compound was at the concentration of 0.2 mg/ml) to 10-ml aliquots of the methanolic extracts from these samples. Then the solutions were evaporated to dryness under reduced pressure at 44 °C, the residues were taken up in 15 ml of 0.05 *M* hydrochloric acid and cleaned as it was previously described.

### 2.4. Recoveries studies by TLC–densitometry

Six samples (10 μl were injected; three tracks for each sample after cation-exchange SPE) of the fractions after the development procedure were analysed in parallel with six standards (5 μl were injected) containing the mixture of senkirkine, retrorsine and retrorsine-*N*-oxide at concentrations of 4–100 μg/ml. The samples and the standards were applied to TLC silica gel 60 $F_{254}$ plates as 2-mm bands by means of a Camag (Muttenz, Switzerland) TLC III v. 2.12 autosampler controlled by ATS3 software. Plates were developed twice to a distance of 9 cm in a 20×20-cm horizontal DS. chamber (Chromdes, Lublin, Poland) with the mobile phase containing chloroform–methanol–25% ammonia (50:0.5:0.1, v/v/v). After development, plates were dried under the air and then scanned at $\lambda$=220 nm in absorption–reflection mode (slit dimensions 4×0.3

mm were adjusted) using a Camag TLC scanner v. 3 managed by a CATS 4.01 interface.

### 2.5. HPIPC analysis and quantitation

HPIPC was performed by the use of a HP 1100 chromatograph equipped with a gradient pump (G1311A), column oven (G1316A), membrane degasser (G1322A), photodiode array detector (G1328A) and 20-μl sample injector (Rheodyne, Cotati, CA). The analytical column was a 5-μm Hypersil BDS $C_8$ (Shandon, UK), 250×4.6 mm I.D. The column was held at 40 °C, and the mobile phase flow-rate was 0.8 ml/min. A two-pump gradient program was applied: reservoir A contained 5 m*M* hexane-1-sulfonic acid in 1% aqueous phosphoric acid (pH adjusted to 3.2), and reservoir B contained 100% acetonitrile. The injection volume was 10 μl (samples; in triplicate) and 5 μl (standards; in triplicate). The following gradient program was used: 0–7 min, isocratic at 20% B; 7–25 min, jump to 40% B; 25–28 min, jump to 60% B; 28–33 min, jump to 80%; 33–35 min, isocratic at 80% B; 35–40 min, jump to 20% B.

A 15-min delay was maintained for equilibration of the column and stabilisation of the baseline. The peaks were recorded at 220 nm. The scanning of UV spectrum was performed from 200 to 350 nm at 2-nm intervals. The total time of analysis was 40 min. Quantitation of the alkaloids found in plant samples was by a six-point linear regression curve in the range of 4–100 μg/ml of the standard examined.

### 2.6. Linearity, limit of detection, and limit of quantitation studies

For each compound quantified (retrorsine, retrorsine-*N*-oxide, senkirkine and senecionine), standards solutions were made at six different concentrations between 4 and 100 μg/ml. Each standard solution was analysed in triplicate. The same standard concentrations were used in TLC–densitometry and HPIPC quantitative determinations but only for HPIPC were all curves linear, above the limit of detection, in the concentration range tested (*r* values were greater than 0.9995 in all cases and amounted: for retrorsine, $y = 6.297x + 5.2002$, $r = 0.99980$; for retrorsine-*N*-oxide, $y = 6.128x + 3.5468$, $r = 0.99980$; for senkirkine, $y = 7.261x + 3.8423$, $r = 0.99970$; for senecionine, $y = 7.452x + 3.756$, $r = 0.99970$).

In TLC–densitometric assay because the range of concentrations was higher than 1:5, and absorption–reflection mode was applied, all curves were polynomial in the concentration range tested, and *r* values were greater than 0.9955 in all cases and relative standard deviations of the slope amounted to 2–6%. In this method, when tests for linearity were performed, *r* values not exceeded 0.985 (relative standard deviations of the slope exceeded 10%). For these reasons in TLC–densitometric assays, polynomial regression curves were used.

The method limit of detection (MLOD) and limit of quantitation (MLOQ) were determined by analysis of the extracts purified by cation-exchange SPE and spiked with a mixture of the standards at an injected concentration of 40 ng/μl for senkirkine, senecionine, retrorsine and retrorsine-*N*-oxide, and 20 ng/μl for seneciphylline. The LOD and LOQ values were measured according to Ref. [33], where LOD is considered at a signal-to-noise ratio 3:1 and LOQ is defined as the concentration that gives rise to a signal, which is 10 times higher than the noise level.

## 3. Results and discussion

### 3.1. Optimisation of cation-exchange SPE

Solid-phase extraction of pyrrolizidine alkaloids is an important task due to the presence of various structural types of the alkaloids of different polarities (polar, water soluble *N*-oxides, i.e., retrorsine-*N*-oxide, versus free bases which are water insoluble, i.e., retrorsine) and otonecine-PAs (i.e., senkirkine) with moderate solubility in water (can be extracted by hot water). The structures of these representatives are shown in Fig. 1. Using a simple washing step of the $C_{18}$ sorbent with the mixtures of methanol or acetonitrile in water, the alkaloids can be readily eluted with good recoveries but also polar co-extractives are present, and for this reason accurate HPLC quantitation is not feasible due to high background baseline absorbance levels [30].

Another approach into SPE of PAs included

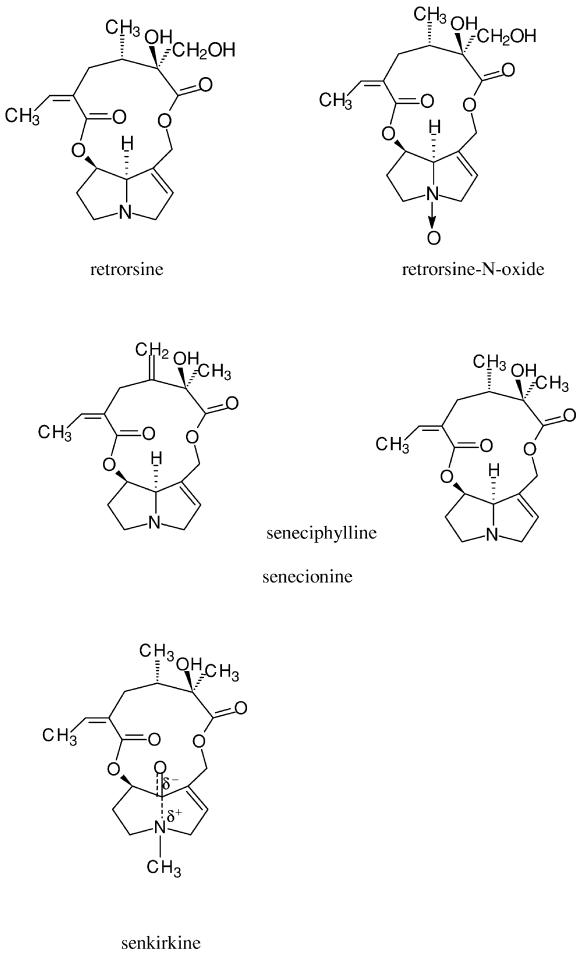


Fig. 1. The structures of pyrrolizidine alkaloids investigated.

cation-exchange SPE, but *N*-oxides could not be analysed together with free bases as they were formerly reduced to free bases by means of Serdoxit oxygen-absorbing resin [32].

When we applied LiChrolut polymeric strong cation-exchanger SPE columns, *N*-oxide sorption phenomena was found. This cation-exchange sorbent is a kind of tentacle exchanger, where the functional exchange groups do not lie in defined positions on the surface of the sorbent but along a flexible, covalent bonded chain with five to 20 monomer units [34]. The chemical stability of the sorbent is very high. The sorption of *N*-oxides can be explained rather by efficient mass transfer of polar analytes into the polar active sites of the sorbent than cation-exchange process, as these compounds possess a free pair of electrons on nitrogen atom coupled with oxygen atom by electron–donor bonding. On elution of various structural types of PAs from the sorbent, ammonia concentrations were influenced (Table 2). The most efficient elution mixture consisted of 75% of methanol and 25% of 10% ammonia. Elution volumes of 15 ml were sufficient for the desorption process. Increase in the concentration of 10% ammonia in methanol did not improve the amounts of PAs eluted from the sorbent, probably due to their worse solubility in the elution mixture.

Similar results were achieved when comparable concentration of 10% ammonia in methanol was replaced by 25% ammonia. This could result in worse availability of the active exchanger groups to smaller volumes of ammonia but also for the volumes of 25% ammonia equal to the volumes of 10% ammonia in methanol, no improvement in desorption of the alkaloids was observed.

### *3.2. Chromatograms, densitogram, $R_f$ values, retention times*

Separation of the five PA representatives by HPIPC gradient procedure is shown in Fig. 2. Retention times ($t_R$) and relative retention times (RRT) for the compounds separated are presented in Table 1. It is clearly demonstrated that separation of PAs varied only by the presence of additional substituents in dicarboxylic acid moiety is very efficient. By means of ion-pair formation with 1-hexane-sulfonic acid, and applied gradient procedure on $C_8$ stationary phase, peak asymmetry factors are excellent (between 0.7 and 1.0), even for later-eluting compounds. Retrorsine is eluted first due to the presence $-CH_2OH$ substituent. The retrorsine-*N*-oxide is eluted just after the free base, and although these two compounds create the ion pair with a very similar hydrophobicity and they are not baseline separated, the separation is quite sufficient ($\Delta t_R \approx$ 0.35 min) for proper quantification as was shown by peaks purity studies. Such ''not-baseline separation'' of the free base and the corresponding *N*-oxides can be suitable for the detection of the *N*-oxides of the free bases, when a standard is available only for free base. Seneciphylline, eluted after retrorsine-*N*-oxide, contains a $=CH_2$ substituent, and then senecionine

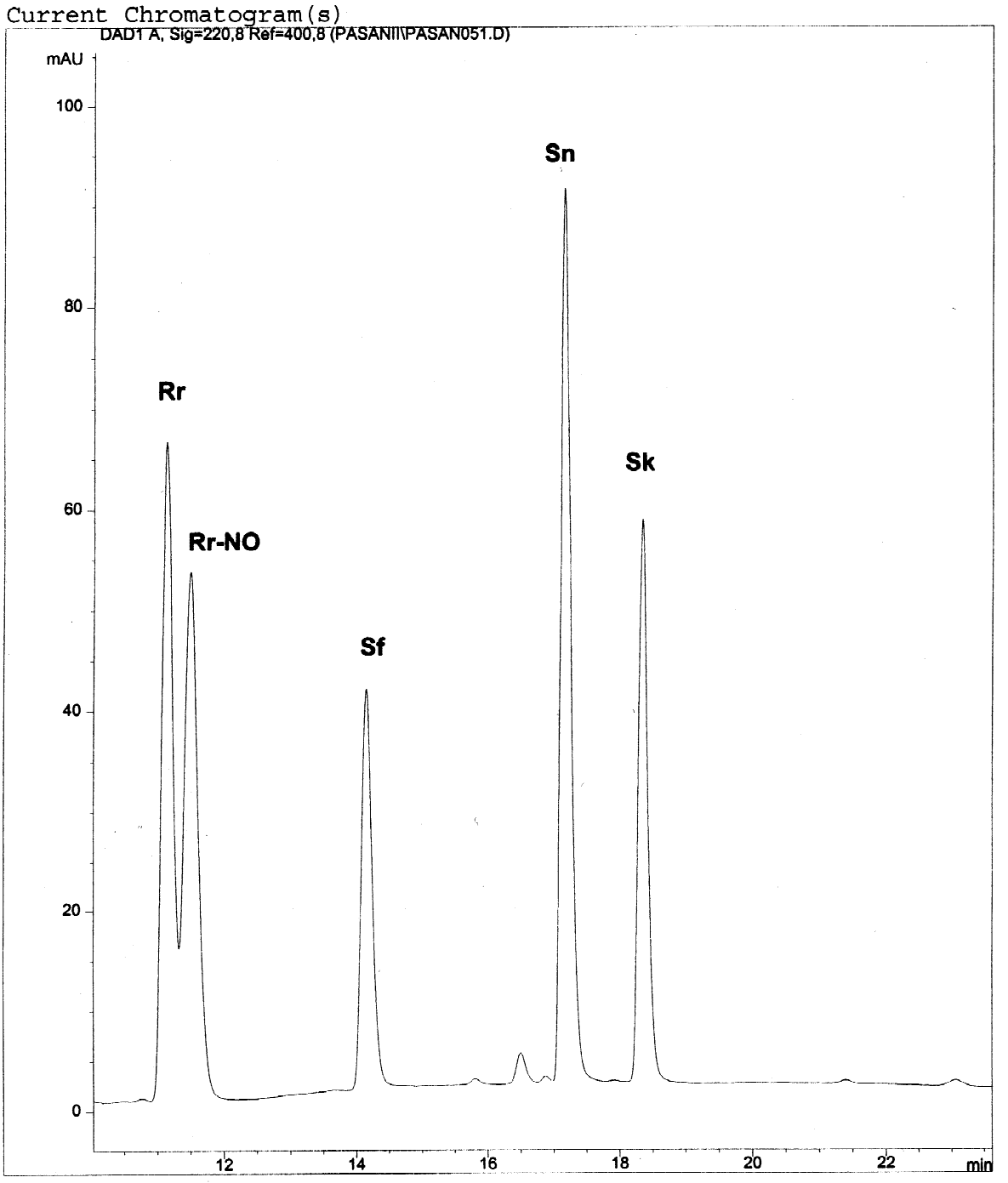


Fig. 2. HPIPC separation of the alkaloid standards. Stationary phase: Hypersil BDS $C_8$ (Shandon, UK), 250×4.6 mm I.D; $d_p$=5 μm. Mobile phase: gradient of acetonitrile in 1% aqueous phosphoric acid (pH adjusted to 3.2) containing 5 m*M* hexane-1-sulfonic acid (see text). Flow-rate, 0.8 ml/min; column temperature, 40 °C; injection volume, 5 μl. UV trace recorded at 220 nm. The concentrations injected for retrorsine, retrorsine-*N*-oxide, senkirkine and senecionine were about 80 μg/ml, and for seneciphylline 40 μg/ml. Abbreviations: Rr, retrorsine; Rr-NO, retrorsine-*N*-oxide; Sf, seneciphylline; Sn, senecionine; Sk, senkirkine.

Table 1
Retention times ($t_R$) and their statistical significance including standard deviation (SD), relative standard deviation (RSD) both for inter- and intra-day variations of RT and relative retention (RRT)

| Compound | $t_R$ (min) | RRT[a] | SD | RSD (%) | No. of replicates | LOD (ng/μl) | LOQ (ng/μl) |
|---|---|---|---|---|---|---|---|
| Retrorsine | 11.126 | 1.0 | 0.162 | 1.43 | 9 | 0.08 | 0.29 |
| Retrorsine-*N*-oxide | 11.483 | 1.032 | 0.147 | 1.26 | 9 | 0.1 | 0.35 |
| Seneciphylline | 14.143 | 1.271 | 0.11 | 0.77 | 6 | 0.07 | 0.26 |
| Senecionine | 17.145 | 1.541 | 0.067 | 0.39 | 9 | 0.06 | 0.22 |
| Senkirkine | 18.329 | 1.647 | 0.046 | 0.25 | 23 | 0.07 | 0.20 |

The table includes also limits of detection (LOD) and limits of quantitation (LOQ) assessed according to Ref. [33].
[a] (Retention time of component)/(Retention time of retrorsine).

which at the same place of dicarboxylic acid contains a $-CH_3$ substituent. The most hydrophobic ion-pair for senkirkine is created, and this compound is eluted at the end. The *N*-methyl structure is probably responsible for an increase of hydrophobic properties. The standard deviations of $t_R$ for overall analyses were relatively low and amounted to between 0.046 (senkirkine) and 0.162 (retrorsine).

For all peaks analysed in the extracts the proper identification was based both by the comparison of retention times of the peak and the standard as well as UV spectra taken from a diode array detector. Additionally, spiking of the analytical standards into the plant samples have been done to test the identity of the sample peak observed.

In Fig. 3 the detection of senkirkine and senecionine in rhizome of *Tussilago farfara* is presented. In Fig. 4 the presence of senecionine-*N*-oxide in the herb of *Emilia coccinea* was concluded by the analysis of UV spectrum taken using a diode array detector (typical 2–3 nm right shift for *N*-oxide in comparison with free base) and elution order, just after senecionine (the difference between $t_R$ of senecionine and its *N*-oxide concluded was about 0.3 min). Traces of senkirkine were also found in this material and confirmed both in overlaid chromatograms (Fig. 4) of the extract and the extract fortified with the standards, as well as by comparison of UV spectra of the peak in the extract and the peak of senkirkine standard (Fig. 5).

On silica gel plates, the separation especially of free bases and their *N*-oxides is quite different (Fig. 6). When the mobile phase containing chloroform–methanol–25% ammonia (50:0.5:0.1, v/v/v) was applied twice over a distance of 9 cm, the retardation factors for free different PAs representatives amounted to 0.07 for retrorsine-*N*-oxide, 0.32 for retrorsine and 0.36 for senkirkine. This clearly shows that on a polar stationary phase *N*-oxides are the most strongly retained, and the separation of free bases and *N*-oxides is much more efficient than on hydrophobic stationary phases, due to interactions of N–O groups and HO–Si groups of silica. For this reason *N*-oxides are strongly retained. But the separation of PA analogues (i.e., retrorsine and senkirkine) on silica is less efficient.

### *3.3. Simultaneous cation-exchange SPE recoveries studies by TLC–densitometry and HPIPC*

For the most efficient elution mixture consisting of methanol–10% ammonia (3:1, v/v), the recoveries after cation-exchange SPE for three PAs (retrorsine-*N*-oxide, retrorsine and senkirkine) were estimated both by TLC–densitometry and HPIPC (Table 2). Similar results were obtained by means of these various chromatographic methods, although different calibration procedures were applied (polynomial regression for TLC–densitometry and linear regression for HPIPC). Additionally, in fortified extracts from *Symphytum asperum* and *Tussilago farfara* roots as well as *Emilia coccinea* herbs (for this plant extract, two concentrations of the standards were added: 80 and 120 μg) the recoveries were also measured by HPIPC. In general, in such a multidimensional approach, the recoveries for retrorsine-*N*-oxide were between 73 and 89%, for retrorsine between 80 and 90%, and for senkirkine between 86 and 110%. For these reasons, LiChrolut SCX cartridges and applied elution mixture are very rec-

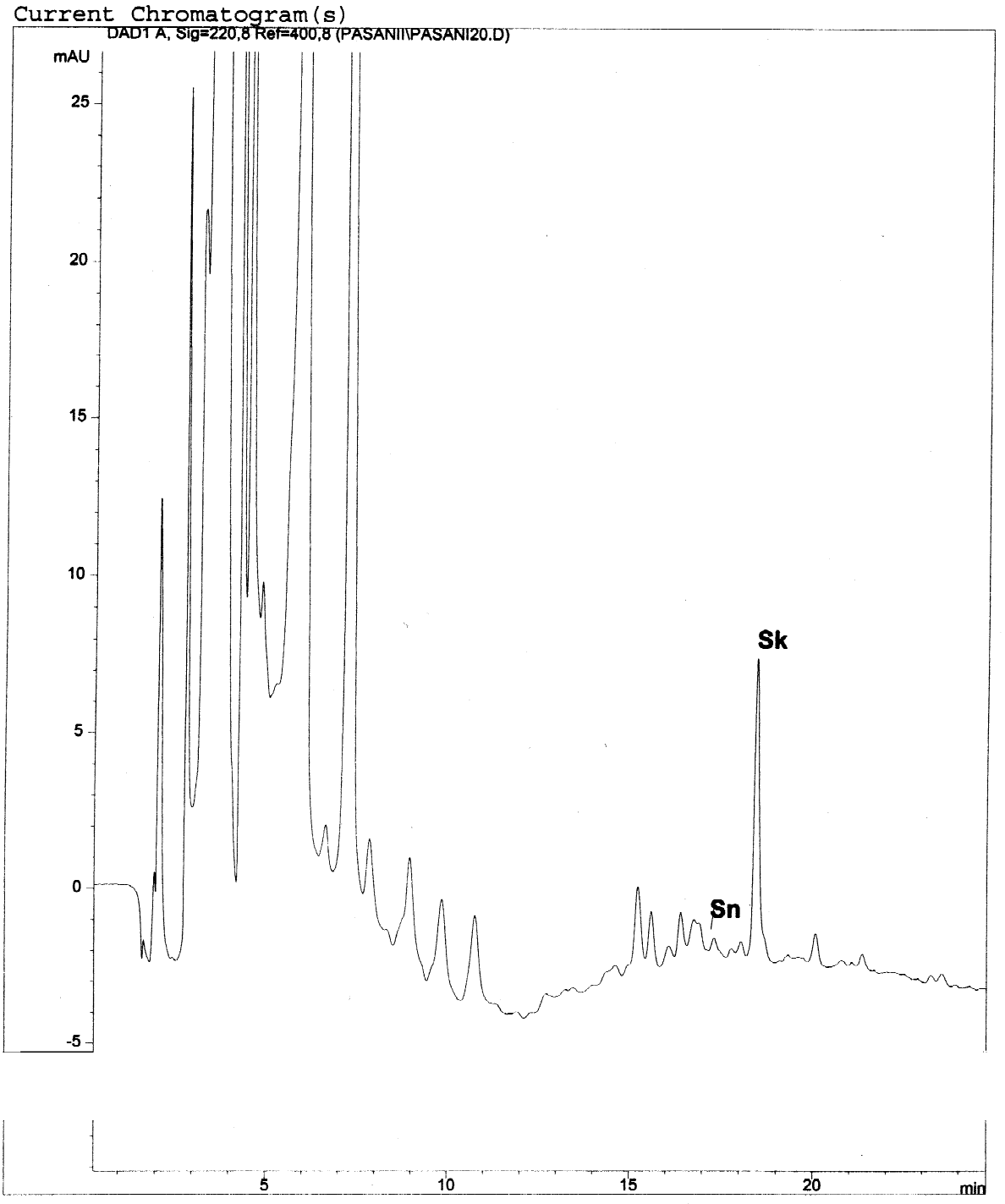


Fig. 3. HPIPC separation of the alkaloid fractions from rhizomes of *Tussilago farfara* purified by cation-exchange SPE (see conditions in the text). Stationary phase: Hypersil BDS $C_8$ (Shandon, UK), 250×4.6 mm I.D; $d_p$=5 μm. Mobile phase: gradient of acetonitrile in 1% aqueous phosphoric acid (pH adjusted to 3.2) containing 5 m*M* hexane-1-sulfonic acid (see text). Flow rate, 0.8 ml/min; column temperature, 40 °C; injection volume, 10 μl. UV trace recorded at 220 nm. Abbreviations: Sn, senecionine; Sk, senkirkine.

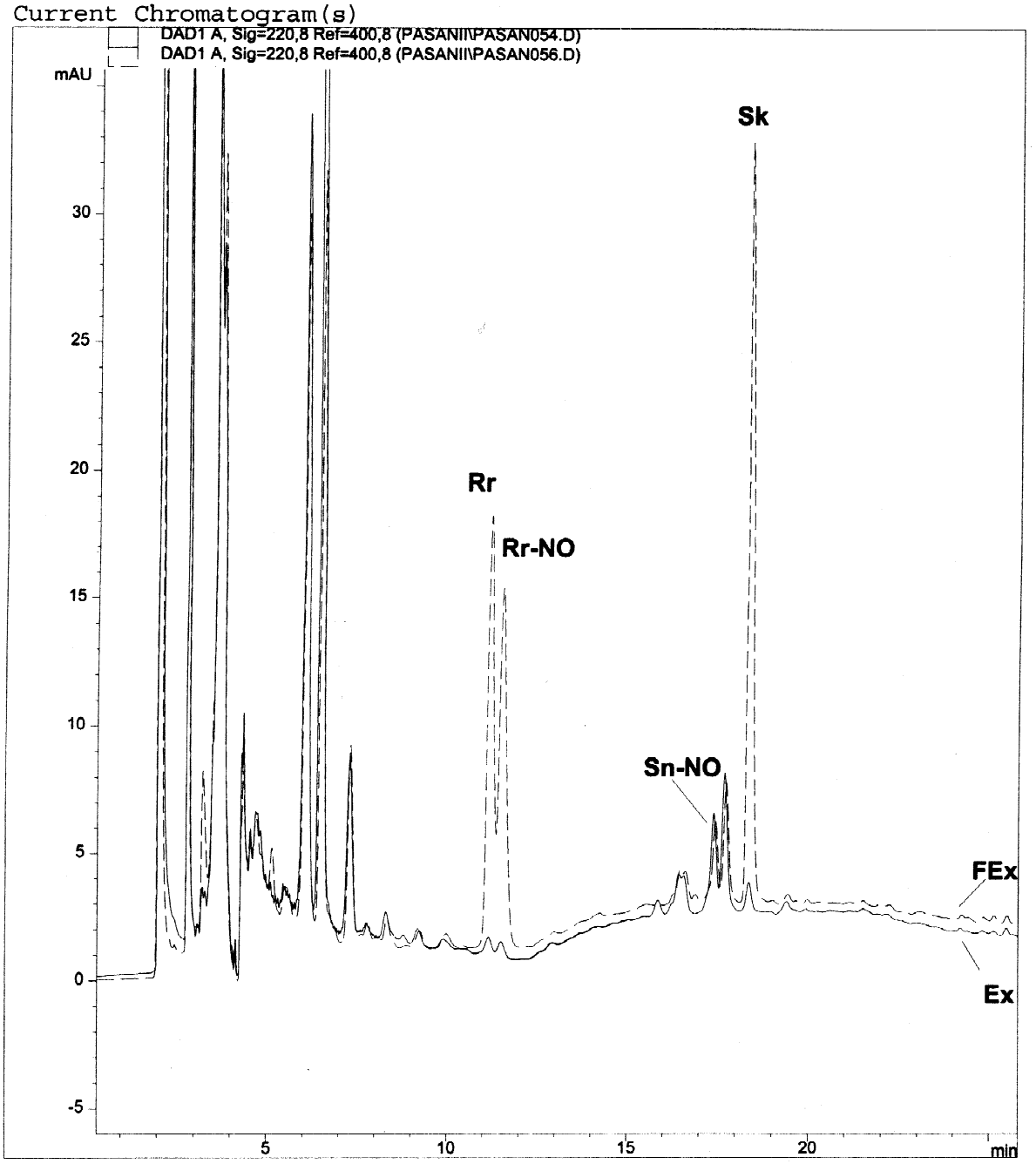


Fig. 4. HPIPC overlaid chromatograms of the alkaloid fractions from herbs of *Emilia coccinea* purified by cation-exchange SPE (Ex, see conditions in the text) and the same extract fortified with the mixture of retrorsine, retrorsine-*N*-oxide and senkirkine and then purified by cation-exchange SPE (FEx). Stationary phase: Hypersil BDS $C_8$ (Shandon, UK), 250×4.6 mm I.D; $d_p$=5 μm. Mobile phase: gradient of acetonitrile in 1% aqueous phosphoric acid (pH adjusted to 3.2) containing 5 m*M* hexane-1-sulfonic acid (see text). Flow rate, 0.8 ml/min; column temperature, 40 °C; injection volume, 10 μl. UV trace recorded at 220 nm. Abbreviations: Rr, retrorsine; Rr-NO, retrorsine-*N*-oxide; Sn-NO, senecionine-*N*-oxide; Sk, senkirkine.

Target Library Spectrum

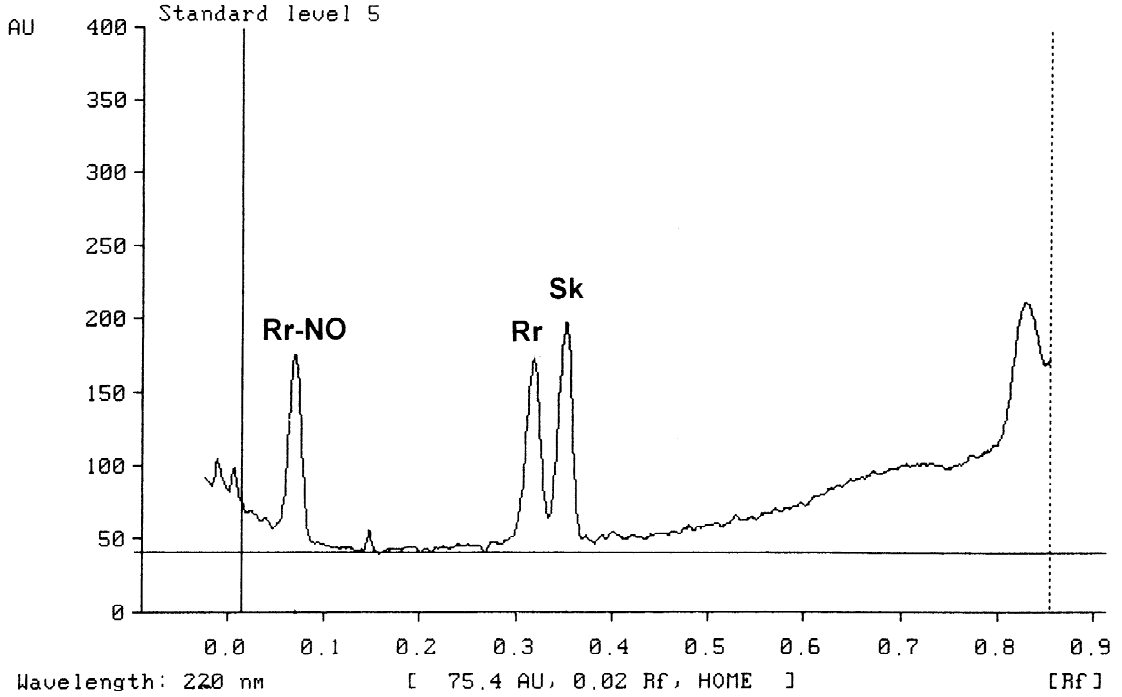


Fig. 6. TLC–densitometric assay of the mixture of retrorsine-*N*-oxide (Rr-NO), retrorsine (Rr) and senkirkine (Sk). Stationary phase: silica gel 60 $F_{254}$ plates, 10×20 cm; 0.25 mm thickness; mobile phase: chloroform–methanol–25% ammonia (50:0.5:0.1, v/v/v). A distance of development, 2×9 cm. Scan was recorded at 220 nm. Standards concentration was about 80 μg/ml. Injection volume was 5 μl.

ommended for efficient isolation of PAs with various types of polarity.

### *3.4. Linearity, limits of detection, limits of quantitation*

Response (peak area) was linear in the range of standards analysed by HPIPC (between 4 and 100 μg/ml) for each component but in TLC–densitometry polynomial regression was the most suitable.

In Table 1 limits of detection and quantitations are summarised. Similar LODs (between 0.06 and 0.1 ng/μl) and LOQs (between 0.20 and 0.35 ng/μl) are caused only by small differences in the responses from UV detector (for PAs $\lambda_{max}$ was between 216 and 221 nm, and similar values of molar adsorptivity $\varepsilon$ are present). Due to relatively low LOQ values for PAs quantified, it was possible to quantify 1–2 ng of the component in the injection volume (5 μl).

### *3.5. Precision*

Precision of the analyses was described by standard deviation (SD) and relative standard deviation (RSD). RSD values for each point (injected in triplicate) calibrated by linear regression method were between 1 and 3%. In the cation-exchange SPE recoveries studies (Table 2) RSD values did not exceed 14% ($n=5$), and usually amounted to about 10% or less. For the natural samples analysed in triplicate (Table 3) SD values were between 0.02 and 15.5.

### *3.6. Screening studies on PAs in natural sources by HPIPC*

Three parallel samples of different plant derived sources were analysed by HPIPC for the presence of the most common macrocyclic PAs (senecionine, or its *N*-oxide, senkirkine, seneciphylline; Figs. 3 and 4

Table 2
The recoveries of pyrrolizidine alkaloids after cation-exchange SPE on LiChrolut SCX (Merck) cartridges eluted with various elution mixtures: the mixtures of the compounds were applied together to the cartridges at the amount of 80 μg per compound[c,d] (corresponding to 0.12% in the extract) and 120 μg (corresponding to 0.18% in the extract)[e]

| Compound | Elution mixture[a] | Analysed sample (μg of the standard) | No. of replicates | Average recovery (%) | Standard deviation (SD) | Relative standard deviation (RSD), as percent of mean |
|---|---|---|---|---|---|---|
| Retrorsine | Methanol– | Standard (80) | 5 | 88.3 | 4.0 | 4.5 |
| Retrorsine–NO[b] | 10% $NH_3$ | Standard (80) | 6 | 72.9 | 11.2 | 15.3 |
| Senkirkine | (3:1, v/v) | Standard (80) | 5 | 110.1 | 10.3 | 9.4 |
| Retrorsine[c] | Methanol– | FSAR (80) | 5 | 90.0 | 13.1 | 14.5 |
| Retrorsine–NO[c] | 10% $NH_3$ | FSAR (80) | 5 | 79.9 | 13.6 | 17.0 |
| Senkirkine[c] | (3:1, v/v) | FSAR (80) | 5 | 91.4 | 10.9 | 11.9 |
| Retrorsine[c] | Methanol– | FTFR[f] (80) | 4 | 87.9 | 2.7 | 3.1 |
| Retrorsine–NO[c] | 10% $NH_3$ | FTFR (80) | 4 | 88.6 | 4.1 | 4.7 |
| Senkirkine[c] | (3:1, v/v) | FTFR (80) | 4 | 99.5 | 3.4 | 3.4 |
| Retrorsine[c] | Methanol– | FECH[g] (80) | 4 | 84.7 | 6.9 | 8.1 |
| Retrorsine–NO[c] | 10% $NH_3$ | FECH (80) | 4 | 82.9 | 5.9 | 7.1 |
| Senkirkine[c] | (3:1, v/v) | FECH (80) | 4 | 89.9 | 7.4 | 8.2 |
| Retrorsine[e] | Methanol– | FECH (120) | 4 | 79.3 | 6.7 | 8.4 |
| Retrorsine–NO[e] | 10% $NH_3$ | FECH (120) | 4 | 77.7 | 5.8 | 7.5 |
| Senkirkine[e] | (3:1, v/v) | FECH (120) | 4 | 85.7 | 5.4 | 6.3 |
| Retrorsine[d] | Methanol– | Standard (80) | 5 | 89.4 | 9.0 | 10.1 |
| Retrorsine–NO[d] | 10% $NH_3$ | Standard (80) | 5 | 81.2 | 7.6 | 9.3 |
| Senkirkine[d] | (3:1, v/v) | Standard (80) | 5 | 100.5 | 8.8 | 8.8 |
| Retrorsine[d] | Methanol– | Standard (80) | 5 | 63.5 | 3.4 | 5.3 |
| Retrorsine–NO[d] | 10% $NH_3$ | Standard (80) | 5 | 63.0 | 1.1 | 1.8 |
| Senkirkine[d] | (3:2, v/v) | Standard (80) | 5 | 73.5 | 2.9 | 3.9 |
| Retrorsine[d] | Methanol– | Standard (80) | 5 | 60.1 | 2.1 | 3.5 |
| Retrorsine–NO[d] | 25% $NH_3$ | Standard (80) | 5 | 53.3 | 5.1 | 9.6 |
| Senkirkine[d] | (8:1, v/v) | Standard (80) | 5 | 66.9 | 7.8 | 11.6 |

[a]For each elution mixture 15 ml volume were applied.
[b]Retrorsine–*N*-oxide.
[c]The recoveries of the alkaloids from the fortified extracts of *Symphytum asperum* roots (FSAR).
[d]The recoveries of PAs analysed by TLC–densitometric assay.
[f]FTFR ([g]FECH), the fortified extracts of *Tussilago farfara* roots[f] (*Emilia coccinea* herb[g]).

and Table 3). The amounts of senkirkine found in these samples varied from 0.45 ppm (μg/g of dry mass) for the decoction from leaves of *Tussilago farfara* and the methanolic extract from flowers of the same species to 92.8 ppm for the extracts from rhizomes of this plant. Senecionine was usually detected in smaller amounts not exceeded 12 ppm (in the extracts from rhizomes of *Petasites hybridus*). In the closely related *Petasites albus*, the presence of only senkirkine (11.6 ppm) was confirmed [35]. Relatively high amounts of senecionine-*N*-oxide were measured in the extracts from herb of *Emilia coccinea* together with small levels of senkirkine (9.4 ppm). In the present study, there is first report on PAs in the following plant materials: *Tussilago farfara* rhizome, *Emilia coccinea* herb and *Doronicum columnae* leaf (senkirkine, 20.3 ppm). Although these plants have not been investigated yet for the presence of PAs, now PAs were found in such amounts that the further consumption of these materials cannot be recommended, as toxic effects may be considered for people and animals.

## 4. Conclusions

For fast and efficient sample preparation of extracts containing PAs with various types of structures and polarities (free bases, *N*-oxides, otonecine-PAs), cation-exchange solid-phase extraction using LiCh-

Table 3
The levels of pyrrolizidine alkaloids found in the investigated plant samples estimated quantitatively by HPIPC based on six-point linear calibration method in the range of 4–100 μg/ml of the standards examined

| Investigated plant sample | The compound analysed | Average conc. (ppm, $\times 10^{-4}$%) | No. of replicates | Standard deviation (SD) | Relative standard deviation (RSD) (%) |
|---|---|---|---|---|---|
| *Petasites* | Senkirkine | 25.8 | 3 | 3.9 | 15.3 |
| *hybridus* (rh)[a] | Senecionine | 11.3 | 3 | 1.6 | 13.8 |
| *Petasites* | Senkirkine | 11.6 | 3 | 1.5 | 13.0 |
| *albus* (rh)[a] | Senecionine | n.d.t.[b] | 3 | | |
| *Tussilago* | Senkirkine | 0.45 | 3 | 0.03 | 6.1 |
| *farfara* (leaf)[c] | Senecionine | n.d.t. | 3 | | |
| *Tussilago* | Senkirkine | n.d.t. | 3 | | |
| *farfara*[d] | Senecionine | n.d.t. | 3 | | |
| *Tussilago* | Senkirkine | 92.8 | 3 | 1.2 | 1.3 |
| *farfara* (rh)[a] | Senecionine | 1.4 | 3 | 0.02 | 2.0 |
| *Tussilago* | Senkirkine | 0.45 | 3 | 0.08 | 18.6 |
| *farfara* (fl)[e] | Senecionine | n.d.t. | 3 | | |
| *Emilia* | Senkirkine | 9.4 | 3 | 0.2 | 2.4 |
| *coccinea* (hb)[f] | Senecionine–NO[g] | 93.5 | 3 | 15.5 | 16.6 |
| *Doronicum* | Senkirkine | 20.3 | 3 | 2.0 | 9.7 |
| *columnae* (leaf) | Senecionine | n.d.t. | 3 | | |

[a]Rhizome.
[b]Under detection limit.
[c]Water extract (decoction) from leaves.
[d]Juice from flowers and leaves produced by Phytopharm.
[e]Flowers.
[f]Herb.
[g]Senecionine-*N*-oxide estimated according to retrorsine-*N*-oxide calibration curve.

rolut SCX polymeric strong cation-exchanger is highly recommended.

In searching for PAs in different plant materials, ion-pair high-performance liquid chromatography on $C_8$ stationary phase and hexane-1-sulfonic acid as pairing agent applied in the gradient mode, can be an important tool, due to efficient separation of PAs investigated and relatively low values of detection and quantitation limits.

## References


[1] E. Roeder, Pharmazie 50 (1995) 83.
[2] Environmental Health Criteria 80, Pyrrolizidine alkaloids, World Health Organization, Geneva, 1988.
[3] R. Manteiga, D.L. Park, S.S. Ali, Rev. Environ. Contam. Toxicol. 150 (1997) 1.
[4] H. Habs, M. Habs, H. Marquardt, E. Roeder, D. Schmähl, H. Wiedenfeld, Arzneim.-Forsch./Drug Res. 32 (1982) 144.
[5] B.N. Tandon, R.K. Tandon, H.D. Tandon, M. Narndranathan, Y.K. Joshi, Lancet 7 August (1976) 271.
[6] A.R. Mattocks, Nature 217 (1968) 723.
[7] P.S. Chu, M.W. Lame, H.J. Segall, Arch. Toxicol. 67 (1993) 39.
[8] A.R. Mattocks, Anal. Chem. 39 (1967) 443.
[9] E. Roeder, K. Liu, R. Mütterlein, Fresenius J. Anal. Chem. 343 (1992) 621.
[10] J.P. Barko Bartkowski, H. Wiedenfeld, E. Roeder, Phytochem. Anal. 8 (1997) 1.
[11] N.M. van Dam, R. Verpoorte, E. van der Meijden, Phytochemistry 37 (1994) 1013.
[12] R.K. Sharma, G.S. Khajuria, C.K. Atal, J. Chromatogr. 19 (1965) 433.
[13] A.T. Dann, Nature 4730 (1960) 1051.
[14] A.R. Mattocks, J. Chromatogr. 27 (1967) 505.
[15] M.E. Stelljes, R.B. Kelley, R.J. Molyneux, J.N. Seiber, J. Nat. Prod. 54 (1991) 759.
[16] E. Roeder, V. Neuberger, Dtsch. Apoth. Ztg. 39 (1988) 1991.
[17] C.M. Paßreiter, Phytochemistry 31 (1992) 4135.
[18] J.T. Hovermale, A.M. Craig, Fresenius J. Anal. Chem. 361 (1998) 201.
[19] P. Stengl, H. Wiedenfeld, E. Roeder, Dtsch. Apoth. Ztg. 16 (1982) 851.
[20] E. Roeder, H. Wiedenfeld, R. Kersten, R. Kröger, Planta Med. 56 (1990) 522.
[21] C.K. Winter, H.J. Segall, A.D. Jones, Biomed. Environ. Mass Spectrom. 15 (1988) 265.
[22] J.M. Betz, R.M. Eppley, W.C. Taylor, D. Andrzejewski, J. Pharm. Sci. 83 (1994) 649.

[23] C. Bicchi, R. Caniato, R. Tobacchi, G. Tsoupras, J. Nat. Prod. 52 (1989) 32.
[24] C. Bicchi, P. Rubiolo, C. Frattini, P. Sandra, F. David, J. Nat. Prod. 54 (1991) 941.
[25] M.M. Mossoba, H.S. Lin, D. Andrzejewski, J.A. Sphon, J.M. Betz, L.J. Miller, R.M. Eppley, M.W. Trucksess, S.W. Page, J. AOAC Int. 77 (1994) 1167.
[26] G. Tittel, H. Hinz, H. Wagner, Planta Med. 37 (1979) 1.
[27] R. Verpoorte, W.M.A. Niessen, Phytochem. Anal. 5 (1994) 217.
[28] C. Crews, J.R. Startin, P.A. Clarke, Food Addit. Contam. 14 (1997) 419.
[29] C.E. Parker, S. Verma, K.B. Tomer, R.L. Reed, D.R. Buhler, Biomed. Environ. Mass Spectrom. 19 (1990) 1.
[30] G. Hosch, H. Wiedenfeld, T. Dingermann, E. Roeder, Phytochem. Anal. 7 (1996) 284.
[31] M.S. Brown, R.J. Molyneux, J.N. Roitman, Phytochem. Anal. 5 (1994) 251.
[32] R. Chizzola, J. Chromatogr. A 668 (1994) 427.
[33] C.M. Riley, T.W. Rosanske, in: Development and Validation of Analytical Methods, Pergamon–Elsevier, Oxford, 1996.
[34] ChromBook 2, Merck, Darmstadt, 1998.
[35] J. Luethy, U. Zweifel, P. Schmid, C. Schlatter, Pharm. Acta Helv. 58 (1983) 98.