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# CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY OF PYRROLIZIDINE ALKALOIDS

G. HOLZER*
*School of Applied Biology, Georgia Institute of Technology, Atlanta, GA 30332 (U.S.A.)*
and
L. H. ZALKOW and C. F. ASIBAL
*School of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332 (U.S.A.)*

## SUMMARY

The application of capillary supercritical fluid chromatography (SFC) for the separation of pyrrolizidine alkaloids (PAs) is examined. Most of these compounds are powerful toxins. Because they are present in at least 3% of all flowering plants, they are responsible for serious losses of livestock and human disease. Capillary SFC, with its high separation efficiency, is shown to be an excellent method for the chromatographic analysis of complex PA mixtures. Complete separation of the PAs of the retronecine and otonecine family has been achieved by pressure-programmed capillary SFC. The operating conditions are mild enough to prevent thermal decomposition of the alkaloids during analysis.

## INTRODUCTION

It is estimated that at least 3% of all flowering plants contain pyrrolizidine alkaloids (PAs), most of which are toxic. Since these plants are found throughout the world, this is a problem of global concern[1]. Plants containing PAs have long been known to be responsible for serious losses of livestock, but only recently has there been increased recognition of their importance as a cause of human disease. The chronic and progressive character of their effects, including carcinogenicity, means that in the type of hazard they present, they resemble the mycotoxins rather than the main body of alkaloids. Metabolic activation of the alkaloids to the pyrrolic dehydroalkaloids is the main basis of alkaloid toxicity in animals[2]. The toxic PAs are esters of unsaturated amino alcohols (the necines), usually with unique types of branched-chain hydroxylated acids (the necic acids). They may be mono- or diesters with monocarboxylic acids or macrocyclic diesters, formed from dicarboxylic acids. The latter are considered to be the most toxic[3].

Because of the toxicity of PAs and as an aid in chemotaxonomy of the large number of plants that contain them, it is important to have rapid and sensitive methods for their analysis. A number of reports have appeared in the literature describing applications of modern analytical techniques to this problem, including thin-

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layer chromatography (TLC)[4], high-performance liquid chromatography (HPLC)[5–7], gas chromatography combined with mass spectrometry (GC–MS)[8,9] and $^1$H Fourier transform nuclear magnetic resonance spectroscopy ($^1$H FT-NMR)[10,11].

Complex mixtures of pyrrolizidine alkaloids are generally not resolved by TLC; however, a comparison of $R_F$ values of sample peaks and known standards can provide supportive evidence for compounds already identified by other methods.

A recent report describes the utilization of capillary GC–MS for the separation and identification of four twelve-membered macrocyclic ring diesters of retronecine from *Senicio inaeguidens*[9]. Because of its high resolution and fast analysis times, GC should be an ideal method for the determination of PAs, but a number of problems restrict a more general use of this technique. Packed columns cannot be used because of severe tailing and even loss of compound by adsorption. Fused-silica bonded-phase capillary columns have reduced or eliminated some of these adverse effects; however, the high temperatures required for the analysis often result in sample decomposition. On-column injection systems can eliminate decomposition during injection, and thermal degradation during chromatography can be controlled to some extent by careful deactivation of the capillary column. Because of these difficulties, gas chromatographic analysis of PAs has not become widely popular among the investigators in this field.

The most frequently used technique for the determination of PAs remains HPLC. A number of studies have been performed on the separation of the retronecine as well as the otonecine type PAs[5,6,12]. The most recent one describes a comparison of $^1$H NMR and HPLC in the quantitative analysis of similar macrocyclic diesters from *S. vulgaris*[13].

Reversed-phase HPLC systems, which are generally employed, have certain drawbacks. The column lifetime is limited by the relatively high pH of the buffer required for the separation. Tailing, a common phenomenon, can lower the resolution, and the sensitivity of the UV detection is decreased if methanol- or tetrahydrofuran-containing solvent systems are used. We have observed transesterifications in certain instances when methanolic solvents are used in HPLC analyses[14]. It is clear that there is still a great need for a rapid and sensitive analytical method that treats PAs gently and preferably in the absence of solvents which may react with them. We describe here, for the first time, the application of capillary supercritical fluid chromatography (SFC) to the analysis of the PAs from the common plant, *Senecio anonymus Wood.*

Capillary SFC has emerged within the last five years as an increasingly popular analytical technique[15]. The supercritical mobile phase allows separations at low temperatures and with high chromatographic efficiency. Density- or pressure-programming control the solvating power of the mobile phase and provide the same advantages as gradient elution in HPLC. Detection is achieved by the highly sensitive and universal flame-ionization detector as well as by mass spectrometry. Most applications have dealt with the analysis of non-polar samples, such as polymers, polycyclic aromatic hydrocarbons, fuel samples and lipids[16–18]. More recently, the technique has been expanded to the separation of more polar compounds by the addition of modifiers to the carbon dioxide mobile phase[19]. We demonstrate here that pyrrolizidine alkaloids of the retronecine and otonecine type can be separated by pressure-programmed conventional capillary SFC.

## EXPERIMENTAL

### *Plant material*

Flowering specimens of *Senecio anonymus* were collected along the edges of a highway near downtown Atlanta.

### *Sample preparation*

The fresh plant material was extracted with ethanol, and the extract was partitioned between chloroform and water. Pyrrolizidine alkaloid N-oxides in the aqueous phase were reduced with zinc dust in acidic medium. After filtration, the solution was made alkaline with ammonia and extracted with chloroform. After removal of the solvent, an aliquot of the extract was redissolved in chloroform to produce a suitable concentration for SFC analysis.

The pure alkaloids were isolated in preparative amounts from the extract by HPLC and droplet counter-current chromatography. A more detailed description of the sample preparation and isolation of PAs will be presented in a forthcoming publication[20]. Standard solutions of PAs, isolated from *S. anonymus*, were prepared by dissolving the pure compounds in chloroform to give concentrations of 0.1–0.5 mg/ml.

### *SFC analysis*

The capillary SFC apparatus consisted of a Model 501 high-pressure syringe pump (Lee Scientific, Salt Lake City, UT, U.S.A.) controlled by an IBM PC XT (IBM, Boca Raton, FL, U.S.A.) with expanded memory, I/O Mini (AST Research, Irvine, CA, U.S.A.) and a HP 5980A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a flame ionization detector. A 0.1-μl Model C14W manual injector valve (Valco Instruments, Houston, TX, U.S.A.) operated at room temperature without inlet splitter, was used for sample introduction. SFC separation of the PAs was achieved on a 10 m × 50 μm I.D. fused-silica capillary, coated with a 0.25-μm film of cross-linked methylpolysiloxane or a 10 m × 50 μm I.D. fused-silica capillary, coated with a 0.25-μm film of SB-Biphenyl-30 (both from Lee Scientific). Restrictors were fabricated by fusing the capillary end with a microtorch and then opening a small orifice by wet polishing[21].

The column exit was placed several several millimeters below the top of the flame ionization detector jet. The flame ionization detector was operated at 340°C, and the column temperature was kept constant during analysis. The chromatographic system was equipped with a HP 3392A recorder/integrator. The mobile phase was carbon dioxide (SFC grade, Scott Specialty Gases, Plumsteadville, PA, U.S.A.) supplied in cylinders with a dip tube. A linear velocity of 1.84 cm/s was measured under the initial chromatographic conditions of 130°C and 100 atm. Linear pressure programming was performed from 100 to 280 atm at 3 atm/min, after an initial isobaric period of 20 min.

## RESULTS AND DISCUSSION

We have isolated twelve-membered macrocyclic diesters of both the retronecine and otonecine type from *S. anonymus*. Experimental details and structural data will

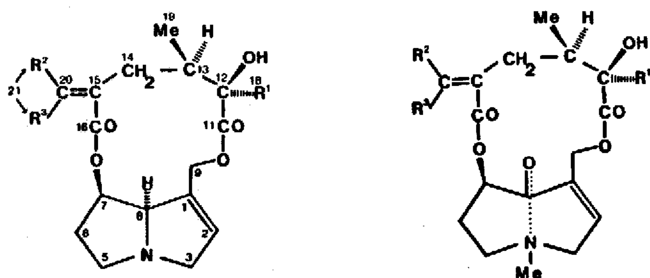

Fig. 1. Structures of pyrrolizidine alkaloids isolated from *S. anonymus*. Anonamine is the trival name given to a new alkaloid, isolated in our laboratory. Retrosine type (left): senecionine: $R^1$ = $CH_3$, $R^2$ = H, $R^3$ = $CH_3$; integerrimine: $R^1$ = $CH_3$, $R^2$ = $CH_3$, $R^3$ = H; retrorsine: $R^1$ = $CH_2OH$, $R^2$ = H, $R^3$ = $CH_3$. Otoserine type (right): senkirkine: $R^1$ = $CH_3$, $R^2$ = H, $R^3$ = $CH_3$; neosenkirkine: $R^1$ = $CH_3$, $R^2$ = $CH_3$, $R^3$ = H; otosenine: $R^1$ = $CH_3$, $R^2$ = H, $R^3$ = $CH_3$, epoxide (15S, 20S) in place of $\Delta^{15}$; hydroxysenkirkine: $R^1$ = $CH_2OH$, $R^2$ = H, $R^3$ = $CH_3$; anonamine: $R^1$ = $CH_3$, $R^2$ = $CH_2OH$, $R^3$ = H.

be the subject of a forthcoming publication[20]. The structures of the PAs are shown in Fig. 1. Anonamine is the trivial name given to a new alkaloid isolated in our laboratory. Structures of all alkaloids mentioned in this communication have been established by single-crystal X-ray and confirmed by high-field $^1H$ NMR and $^{13}C$ NMR analyses and comparison with authentic samples for previously reported alkaloids.

The separation of a standard mixture of PAs consisting of eight pyrrolizidine alkaloids, isolated from *S. anonymus* was investigated on two stationary phases of different polarities. The elution pattern shown in Fig. 2 was obtained with a non-polar column, coated with methylpolysiloxane. The concentration of PAs in the standard ranged from 10 to 30 $\mu$g per ml chloroform, which corresponds to 1–3 ng PAs per injection. The chromatogram shown in Fig. 3 was obtained with a SB-Biphenyl-30 stationary phase and represents the actual distribution of the PAs in *S. anonymus*. Prior to injection, the pyrrolizidine N-oxides were reduced to the corresponding PAs. The compounds were identified by chromatography in mixture with known alkaloids. The two alkaloids which eluted before and after senecionine and the group of compounds emerging between 160 atm and 180 atm remain to be identified.

The polarity of supercritical carbon dioxide was sufficient to solvate these molecules. An examination of the peak shapes shows only minor tailing, indicating that the stationary phase was inert. At operating temperatures of 100—140°C, the PAs were found to be stable; no thermal decomposition was noticed.

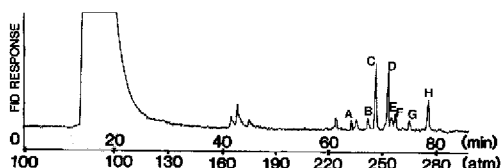


Fig. 2. SFC of senecionine (A), integerrimine (B), senkirkine (C), neosenkirkine (D), retrosine (E), otosenine (F), hydroxysenkirkine (G) and anonamine (H). Column, 10 m × 50 $\mu$m I.D., methylpolysiloxane; pressure program, 100 atm carbon dioxide (20 min) to 280 atm at 3 atm/min; column temperature, 130°C.

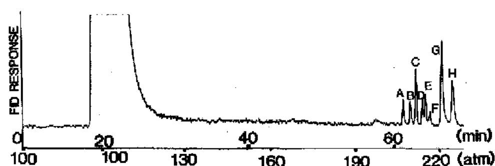


Fig. 3. SFC separation of pyrrolizidine alkaloids isolated from *S. anonymus*. Column, 10 m × 50 μm I.D., SB-Biphenyl-30; pressure program, 100 atm carbon dioxide (20 min) to 280 atm at 3 atm/min; column temperature, 130°C. Peaks as in Fig. 2.

The SB-Biphenyl-30 has a 30% biphenyl content, which makes it slightly more polar than a methylpolysiloxane coating. Senecionine and integerrimine, which differ only in the orientation of the methyl group attached to C-20 (*trans*- and *cis*-, respectively) are completely separated on both columns. The corresponding *trans/cis*-pair of the otonecine type, senkirkine and neosenkirkine are equally well resolved. As expected, the PAs are retained longer on the more polar biphenyl column, where neosenkirkine, retrorsine and otosenine are completely separated. These three compounds are only partially resolved on the less polar methylpolysiloxane column. Increased operating temperatures (up to 145°C) resulted in longer retention times and increased resolution. At 145°C and a pressure gradient of 2.5 atm/min, compounds neosenkirkine, retrorsine and otosenine showed the best separation on the non-polar column. Higher pressure gradients can be used to shorten the analysis time, depending on the complexity of the PA composition.

Our studies have demonstrated the ability of SFC to separate alkaloids of the pyrrolizidine type. The polarity of carbon dioxide is sufficient to solubilize PAs of the macrocyclic diester structure. The analysis time is somewhat longer than in HPLC, but it can be shortened, depending on the complexity of the PA sample. With a detection limit of 1 ng at a signal-to-noise ratio of 3, the sensitivity of the method is excellent.

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