Off-line Supercritical Fluid Extraction and Capillary Gas Chromatography of Pyrrolizidine Alkaloids in Senecio Species

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OFF-LINE SUPERCRITICAL FLUID EXTRACTION AND CAPILLARY GAS CHROMATOGRAPHY OF PYRROLIZIDINE ALKALOIDS IN *SENECIO* SPECIES

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ABSTRACT.—The pyrrolizidine alkaloid (PA) fraction of two *Senecio* species (*Senecio inaequidens* and *Senecio cordatus*) was extracted by off-line supercritical fluid extraction (sfe) and analyzed by capillary gas chromatography. Sfe was carried out with a home-assembled apparatus, using MeOH/CO_2 as extraction medium, at 55° and 15 MPa. Compared to the classical MeOH Soxhlet extraction, this technique requires a smaller sample amount and provides a quicker extraction, a simplified PA fraction clean-up, and a higher recovery.

Supercritical fluid extraction (sfe) is a very powerful method for the extraction of organic compounds from solid matrices, e.g., flavors and fragrances from natural products, toxic organics from resins, polycyclic aromatic hydrocarbons and polychlorinated biphenyls from environmental solids, etc. The supercritical fluid most often applied is CO_2 because of its low critical temperature (31°) and pressure (7.3 MPa). Sfe therefore can be performed at relatively low temperatures, avoiding decomposition of thermolabile compounds. Sfe can be coupled either on-line or off-line with capillary gas chromatography (gc) (1–3). Several sfe systems have been described: Sandra, Onuska, and co-workers proposed an effective and easy-to-assemble apparatus for off-line sfe/capillary gc (4–6).

Pyrrolizidine alkaloids (PAs) with known hepatotoxic and allelopathic properties (7–9) occur in several worldwide plant families, e.g., Boraginaceae, Compositae (Senecioneae and Eupatorieae tribes), and Leguminosae (genus *Crotalaria*).

The applicability of off-line sfe/capillary gc for the analysis of the PA fraction in plants of the *Senecio* genus [in particular *Senecio inaequidens* DC. and *Senecio cordatus* Koch ex *Senecio alpinus* L. (10)] was investigated and compared to the classical Soxhlet extraction method. Sfe has already been applied to PA extraction by Schaeffer *et al.* (11–13), who investigated in depth the extraction of monocrotaline from the seeds of *Crotalaria spectabilis*.

RESULTS AND DISCUSSION

PAs are classically extracted from a vegetable matrix by MeOH Soxhlet extraction. This operation is relatively time-consuming (4 h) and requires large amounts of solvent and plant material. Sfe can provide very rapid extraction with high recovery using very small sample amounts.

Various densities of supercritical CO_2 were first tried for the extraction of PAs; this was unsuccessful, probably due to the inappropriate polarity or solvent strength of CO_2. Polarity can be increased by adding a modifier. MeOH was chosen for two reasons: it is the usual extraction solvent for PAs, and it is one of the most effective polarity modifiers for supercritical CO_2, since amounts as high as 20% are miscible with CO_2.

The sfe apparatus used was not equipped with two pumps nor a mixing chamber; therefore MeOH had to be added directly in the cartridge containing the plant material before extraction. CO_2 modified with MeOH successfully extracted the PA fraction. The supercritical fluid parameters were investigated, in particular temperature (50°, 55°, 60°), pressure (10, 15 MPa), density, and MeOH percentage (1–5%). The following conditions afforded the highest extraction recovery from 500 mg of *S. inaequidens* and *S. cordatus*: pressure 15 MPa, temperature 55°, density of CO_2 0.65, added MeOH 800 μl. Table 1 reports the concentrations (mg/g of plant material) of senecionine and seneciphylline (two of the main PAs present in both species) under different sfe conditions in comparison to those obtained through a Soxhlet extraction. PA amounts were calculated by capillary gc after adding methyl palmitate as internal standard in the final solution.

TABLE 1. Concentrations (mg/g of plant material) of Senecionine and Seneciphylline under Different Supercritical Fluid Extraction (sfe) Conditions after the First and Second Extraction Steps in Comparison to Those Obtained with a Soxhlet Extraction.

Compound	Soxhlet extraction	10 MPa 55°		15 MPa 55°		15 MPa 50°		15 MPa 60°	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd
Senecio inaequidens									
Senecionine	0.58	0.50	0.24	0.64	0.19	0.52	0.21	0.62	0.19
Seneciphylline	0.16	0.15	0.06	0.20	0.06	0.16	0.06	0.19	0.06
Senecio cordatus									
Senecionine	0.38	0.31	0.13	0.37	0.10	0.33	0.11	0.36	0.10
Seneciphylline	2.01	2.43	0.78	2.87	0.62	2.59	0.76	2.80	0.60

Figure 1 shows the capillary gc/fid pattern of the PA fraction of *S. inaequidens* after sfe. The composition of this PA fraction has already been studied in detail (14–17). Figure 2 shows the capillary gc/fid pattern of the PA fraction of *S. cordatus* extracted by sfe;

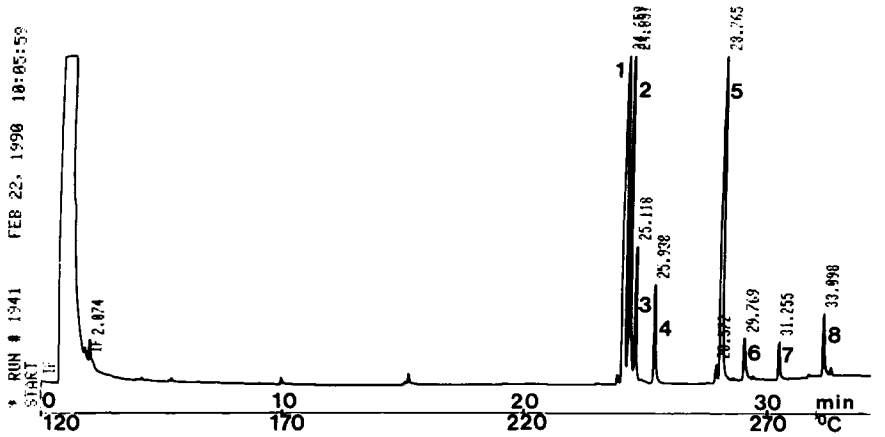

FIGURE 1. Capillary gc/fid pattern of *Senecio inaequidens* pyrrolizidine alkaloid fraction extracted by off-line supercritical fluid extraction. 1, Senecivernine; 2, senecionine; 3, seneciphylline; 4, integerrimine; 5, retrorsine; 6, usaramine; 7, desacetyl doronine; 8, doronine.

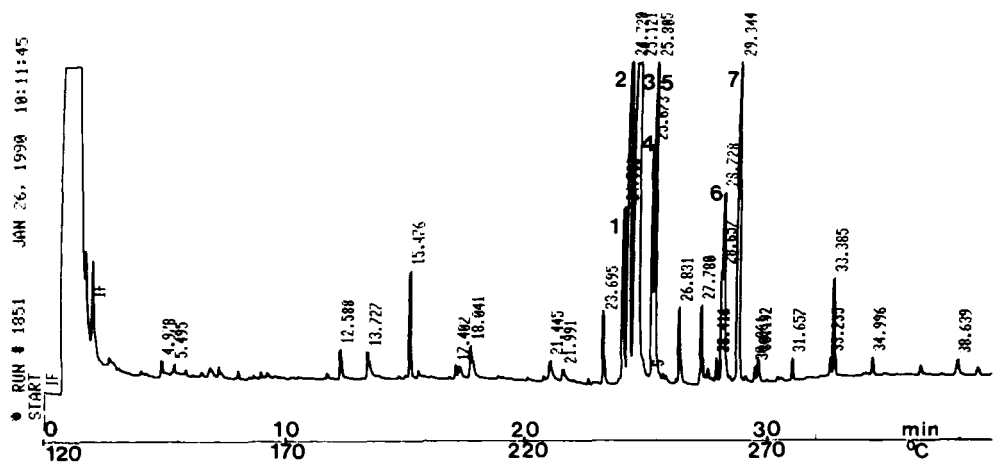

FIGURE 2. Capillary gc/fid pattern of *Senecio cordatus* pyrrolizidine alkaloid fraction extracted by off-line supercritical fluid extraction. 1, Senecivernine; 2, senecionine; 3, seneciphylline; 4, spartioidine; 5, integerrimine; 6, jacobine; 7, jacozine.

the composition of this PA fraction is in agreement with the data reported by Luthy and co-workers (18,19). The PAs were identified by capillary gc-ms.

Exhaustive extraction of the PA fraction contained in 500 mg of plant material required 80 ml of MeOH/CO_2, and the sfe pump used has a reservoir capacity of only 20 ml; therefore four extraction steps were necessary. The flow rate was between 250 and 350 μl/min; as a consequence about one hour was required for each extraction step. For both *Senecio* species investigated, at 15 MPa and 55°, about 65% of the PA fraction is extracted in the first step and 15–25% in the second, while the remaining 10 to 15% is recovered in the last two steps.

Sfe applied to PAs is highly reliable and reproducible: under constant pressure and temperature, both the yield at each extraction step and the PA ratio were constant. This is of importance in control analyses, e.g., for ontogenetic studies, since the total PA amount can be calculated from the results of a single extraction step by applying a correction factor. The individual PAs were quantitatively determined by capillary gc through the internal standard method using methyl palmitate as reference compound. Table 2 shows the mean composition after the first extraction step, based on five experiments, of the PA fractions of the two *Senecio* species.

Sfe produces a higher recovery of PAs than classical Soxhlet extraction. Three samples from the same lot of both *Senecio* species were submitted to the two different extraction processes. The PA fraction obtained by sfe from 500 mg of *S. inaequidens* was 0.36% of the dried plant material (mean value), whereas by Soxhlet extraction carried out on 10 g of dried plant material, the PA fraction represented only 0.28%. For *S. cordatus* PA fraction obtained by sfe from 500 mg of dried plant material represented 0.48%, whereas by Soxhlet extraction carried out on 10 g of dried plant material, this fraction was 0.37%. Even more evident are the differences when the single PA concentration is considered (Table 1). Senecionine and seneciphylline recovery from the first extraction at 15 MPa is always higher than the total extracted amount via Soxhlet; at 10 MPa a recovery of the same order as the total amount extracted via Soxhlet is obtained. At the second sfe step, recoveries are about 60% above those of Soxhlet extraction.

Sfe seems to be more selective and, as shown in Table 1, provides a "cleaner" extract. The sfe extract contains smaller amounts of by-products co-extracted with the PAs than does the Soxhlet extract. Moreover, the extract can be collected directly in HCl, which avoids evaporating the MeOH and subsequently redissolving the extract in

TABLE 2. Relative Percentage Composition and Standard Deviations (based on five experiments) of the *Senecio inaequidens* and *Senecio cordatus* Pyrrolizidine Alkaloid Fractions after the First Step (1 h) of Off-line Supercritical Fluid Extraction (sfe)/Gc Analysis.

Compound	*S. inaequidens*			*S. cordatus*		
	Soxhlet extraction		Sfe	Soxhlet extraction		Sfe
	Mean (%)	Mean (%)	SD	Mean (%)	Mean (%)	SD
Senecivernine	23.6	24.4	1.8	0.5	0.7	0.1
Senecionine	25.3	24.8	2.1	10.5	9.9	0.8
Seneciphylline	7.0	7.4	0.8	79.5	77.3	3.2
Spartioidine				0.4	0.7	0.2
Integerrimine	6.2	6.7	0.6	4.2	5.2	1.0
Retrorsine	25.0	23.9	2.4			
Usaramine	3.9	4.3	0.7			
Desacetyl doronine	3.5	2.3	0.4			
Doronine	5.3	4.5	0.4			
Jacobine				0.3	0.4	0.1
Jacozine				1.9	1.9	0.2

2.5% HCl. The sample can also be purified in a single step, avoiding the repurification necessary with Soxhlet extracts. Therefore, considering the time saved with sfe and in sample clean-up (HCl extraction and repurification), the total time required for sample preparation from dried plant material to the capillary gc analysis can be reduced from the 8 h required for Soxhlet extraction to 3 h for sfe. This obviously excludes the reduction of *N*-oxides with Zn dust which takes at least 4 h in either case.

EXPERIMENTAL

PLANT MATERIAL.—*S. inaequidens* was collected in 1989 from a roadside on the outskirts of Padua, Italy. *S. cordatus* was collected from the surroundings of Tovel Lake, Trento, Italy. Both species were identified by Dr. R. Caniato of the Dipartimento di Biologia Vegetale, Università di Padova, Padova, Italy. Voucher specimens (DTA 6122) are deposited at the Dipartimento di Biologia Vegetale, Università di Padova, Padova, Italy.

REAGENTS.—All the chemicals used were analytical-reagent grade (E. Merck, Darmstadt, Germany).

SFE APPARATUS.—The apparatus was constructed according to Onuska and Terry (4) and Sandra and co-workers (5,6). It consists of a Carlo Erba Phoenix 20 pump (reservoir capacity 20 ml) connected to an extraction cell placed in a thermostated oven. The extracting fluid is preheated at the extraction temperature in the oven and enters the cartridge filled with plant material via a three-way valve. The other side of the extraction cartridge is connected to an outlet restrictor. The restrictor is introduced in 1 to 2 ml of collecting solvent. The vessel is maintained at the extraction temperature to avoid obstruction of the restrictor.

SAMPLE PREPARATION.—Air-dried plant material (0.5 g) was submitted to sfe in a 4.6 mm i.d. × 12.5 cm cartridge. MeOH (800 µl) was added to the plant material. The cartridge was sealed and assembled in the apparatus. The extraction was carried out at different temperatures (50°, 55°, 60°), pressures (10, 15 MPa), density, and MeOH percentages (1–5%). Supercritical pressure was maintained in the cartridge by using an outlet restrictor of 25 µm i.d. (Chrompack, The Netherlands). The restrictor length (15 cm) was selected in order to maintain a flow rate in the range of 250 to 300 µl/min under the extraction conditions cited above. The extract was collected in 2 ml of 2.5% HCl. The resulting extract was then washed with Et_2O and $CHCl_3$. Half of the aqueous phase was basified with 25% NH_3 solution and extracted with CH_2Cl_2. The resulting CH_2Cl_2 solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. To investigate the presence of PA *N*-oxides, the second aliquot of the aqueous phase was reduced with Zn dust overnight, filtered, and treated as above. The dried residues were weighed and dissolved in suitable amounts of CH_2Cl_2 for capillary gc and capillary gc-ms analysis.

Soxhlet liquid/solid extraction was carried out with 10-g samples of the same plant material. Sample preparation is described in detail elsewhere (15–17).

CAPILLARY GC ANALYSIS.—Capillary gc analyses were performed on a Carlo Erba Mega 5360 instrument by introducing 1 μl PA extract under the following conditions: carrier gas H_2; flow rate 3 ml/min; injection system split, split ratio 1/30; injector temperature 300°; detector fid, temperature 300°; column temperature program 120° (1 min) −280° at 5°/min, and isothermal at 280° for 20 min. A 30 m × 0.25 mm i.d. fused-silica capillary column coated with 0.5 μm of OV-1 was used (16). Chromatographic data were processed by a Hewlett Packard 3393A integrator.

CAPILLARY GC-MS ANALYSIS.—Capillary gc-ms analyses were carried out on a Hewlett Packard 5970B gc/mass selective detector system. The same column as for capillary gc analysis was used. Capillary gc conditions were as above. Carrier gas He; flow rate 1.6 ml/min. PAs were identified by comparison with both spectra of pure samples and spectra reported in the literature. All mass spectra were recorded on-line to the capillary gc separation.

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