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DETERMINATION OF PYRROLIZIDINE ALKALOIDS IN *SENECIO INAEQUIDENS* D.C. BY CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A study of the toxic pyrrolizidine alkaloids contained in *Senecio inaequidens* D.C., an infestant species of the *Senecio* genus, widespread in the North East of Italy, is reported. Five of these compounds, senecivernine, senecionine, integerrimine, retrorsine and an analogue of retrorsine, were identified by means of capillary gas chromatography and capillary gas chromatography–mass spectrometry.

INTRODUCTION

Pyrrolizidine alkaloids constitute a class of more than 200 compounds generated through an ornithine biogenetic pathway. Comprehensive reviews and textbooks describing this class of compounds[1–5], their chemotaxonomic significance[6] and general pyrrolizidine chemistry[7] are available.

Pyrrolizidine alkaloids are well known for their toxicological characteristics, inducing serious chronic or acute intoxication in both man and cattle. In fact, these compounds show marked hepatotoxic activity, producing liver necrosis and cirrhosis in animals and man[5,8,9] and, even in small amounts, they can induce pulmonary arterial hypertension[10,11]. Some of them are also hepatocarcinogenic[12–14] and chronic intoxication has been considered responsable for the unusual incidence of cancer of the liver in some geographic areas[15,16]. Sporadic episodes of human intoxication and loss of life from pyrrolizidine alkaloids have been reported[17]. Human contact is generally due either to chance contamination of foods, such as flour, because several *Senecio* or *Heliotropium* species can infest corn and maize crops, or to swallowing herbal "medicines" (generally teas) containing plants in which such compounds are present[18,19]. Pyrrolizidine alkaloids have also been detected in honey[20,21] and in milk[22,23].

The toxicological risks related to pyrrolizidine alkaloids are widespread, because plants containing these compounds occur in all parts of the world. The plant species most frequently responsible for poisoning belong to the genera *Senecio* (Compositae), *Heliotropium* (Boraginaceae) and *Crotalaria* (Leguminosae).

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Owing to the toxicity of these compounds and their chemotaxonomic importance, it is of great importance to have rapid and sensitive methods available for their analysis So far, analytical techniques involving thin-layer chromatography (TLC)[24–26], high-performance liquid chromatography[27–29], gas–liquid chromatography and gas chromatography–mass spectrometry (GC–MS)[24,30,31–33] and NMR spectroscopy[34] have been reported.

In this paper, a capillary gas chromatographic (CGC) method for the determination of pyrrolizidine alkaloids in *Senecio inaequidens* D.C. is described. *S. inaequidens* is a *Senecio* species, native to South Africa, naturalized in Italy after the Second World War, and so widely diffused in Eastern Italy as to be considered potentially dangerous, both indirectly as a food contaminant and directly for cattle. Wiedenfeld *et al.*[32] identified senecionine and retrorsine from a sample of *S. inaequidens*. At least five compounds belonging to this class were detected by CGC and CGC–MS analysis of a sample of *S. inaequidens* from Eastern Italy.

EXPERIMENTAL

Plant material

Flowering plants of *Senecio inaequidens* D.C., collected in June 1984 along the edges of the road on the outskirts of Padua, were utilized.

Reagents

All the chemicals used were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Authentic samples of senecionine and retrorsine were kindly provided by Dr. C. C. J. Culvenor, Parkville, Australia.

Sample preparation

A 25-g amount, exactly weighed, of the aerial parts was extracted in a Soxhlet apparatus with methanol for 4 h. The extract was evaporated to dryness under reduced pressure and the residue was treated with 2.5% hydrochloric acid and washed with diethyl ether and chloroform to remove chlorophylls and lipids, respectively. The aqueous layer was made alkaline with 25% ammonia solution and extracted with dichloromethane. The organic layer (dichloromethane) was again treated with 2.5% hydrochloric acid, 25% ammonia solution and dichloromethane. The resulting solution, containing the free alkaloids, was dried over anydrous sodium sulphate and evaporated to dryness. To investigate the presence of pyrrolizidine alkaloid N-oxides, an aliquot of the solution resulting after washing with diethyl ether and chloroform was reduced with zinc dust overnight, filtered and subsequently treated as described above. The dried residues were weighed on an analytical balance and dissolved in appropriate amounts of dichloromethane to produce suitable concentrations for TLC, CGC and CGC–MS analysis. Decreasing amounts (25, 10, 5, 2 and 1 g) were extracted to investigate the minimum amount of dried plant material necessary to obtain reliable results. The composition of the methanol extract of 25 g of dried plant material after different extraction times (0.5, 1, 2, 4 and 8 h) was also investigated to evaluate the minimum time necessary for complete extract of the pyrrolizidine alkaloids.

CGC and CGC–MS analysis

CGC analyses were performed by introducing 1 μl of pyrrolizidine alkaloid extract into a glass capillary column, installed in a Carlo Erba 4160 instrument, equipped with a flame-ionization detector. The following conditions were used: carrier gas, hydrogen; flow-rate, 3 ml/min; injection system, splitting ratio 1:30; injector temperature, 250°C; detector temperature, 280°C; column temperature, programmed from 120°C (1 min) to 230°C (20 min) at 5°C/min; columns, 20 m × 0.32 mm I.D. soda-lime and Duran-50 glass capillary columns, pre-treated by high-temperature silylation, coated with OV-1, then immobilized (film thickness 0.1 μm).

Quantitation of the alkaloids was carried out with respect to a suitable amount of C_{24} hydrocarbon in hexane solution as internal standard, added to the alkaloid solution. The peak areas were calculated with a Carlo Erba Mega 2 integrator.

CGC–MS analyses were carried out on a Finnigan-MAT Model 4021 GC–MS system, equipped with a Data General Nova 3 computer with helium as carrier gas. The CGC conditions were the same as above. Identification was based on comparison of the mass spectral data with data obtained from the literature, and on comparison with retention data and mass spectra of pure compounds.

TLC analyses were performed by applying the pyrrolizidine alkaloid extract to silica gel pre-coated plates (DC Alufolien Kieselgel 60 F254; Merck). The plates were developed with dichloromethane–methanol–25% ammonia solution (85:14:1) for a distance of approximately 15 cm. The plates were dried and either observed under UV light at 254 nm or sprayed with Dragendorff reagent and heated for 1 min.

RESULTS AND DISCUSSION

Fig. 1 shows the GC pattern of the fraction containing pyrrolizidine alkaloids of *Senecio inaequidens*. CGC–MS analysis showed the presence of five different alkaloids of this class. In particular, senecivernine (a), senecionine (b), integerrimine (c), retrorsine (d) and a retrorsine analogue (e), molecular weight (MW) 351, were separated and identified. Two other peaks, not yet identified and with probable MW 354 (f) and 396 (g) were detected. Fig. 2 shows the structures of senecivernine, senecionine, integerrimine and retrorsine. Dried samples of *Senecio inaequidens* gave a yield of total alkaloids (free base plus N-oxides) in the range 0.3–0.4% of the dry weight.

In Table I, molecular weights, percentage compositions and standard deviations for five analyses of each compound present in the sample are reported.

The identification of senecionine (b) and retrorsine (d) was carried out by comparing the retention times, mass spectra and TLC R_F values of the peaks of the sample with those of pure standards. The mass spectra of senecivernine and integerrimine reported in the literature[31,33] were found to be identical with those of peaks a and c of the sample. The mass spectrum of the retrorsine analogue (e) is reported in Fig. 3. Compounds f and g have presumably even molecular ions (354 and 396 a.m.u., respectively); the very similar fragmentation patterns and intensities of the peaks in their mass spectra led us to conclude that compound g is an acetyl derivative of f. The elucidation of the structures of f and g is in progress.

The CGC pattern of the aliquot treated with zinc dust was very similar to that of the untreated sample; therefore, it may be concluded that only small amounts of

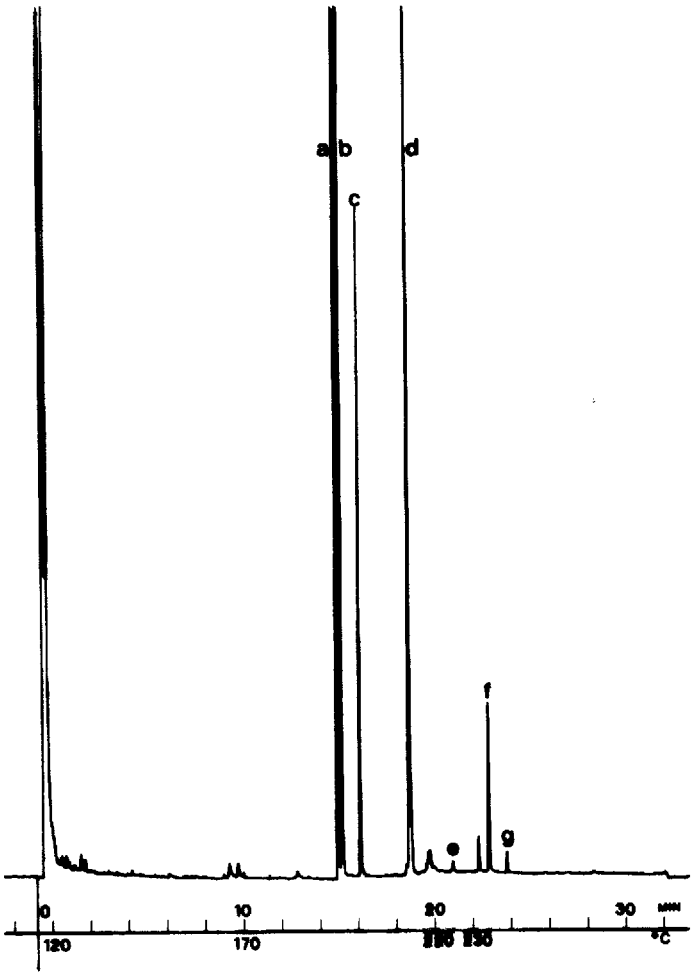

Fig. 1. GC pattern of pyrrolizidine alkaloid fraction of *Senecio inaequidens* D.C.

pyrrolizidine alkaloid N-oxides are present. Pyrrolizidine alkaloid N-oxides are thermally labile and partially decomposed under CGC temperature conditions, hence this technique will provide only qualitative information on the free base/N-oxide ratio in a sample[33]; these results were confirmed by TLC analysis.

The experiments designed to evaluate the minimum amount of plant material required showed that 1 g is sufficient to obtain a reliable and significant GC pattern of the pyrrolizidine alkaloid fraction. Parallel tests to check the yield showed that more than 85% of each pyrrolizidine alkaloid was extracted after extraction for 4 h with methanol in the Soxhlet apparatus.

TLC data were used to confirm the results obtained by CGC. In fact, the separation of the MW 335 and 351 analogues was not as good as that in CGC. The R_F values of senecionine and retrorsine (0.62 and 0.29, respectively) agreed with those reported in the literature[33].

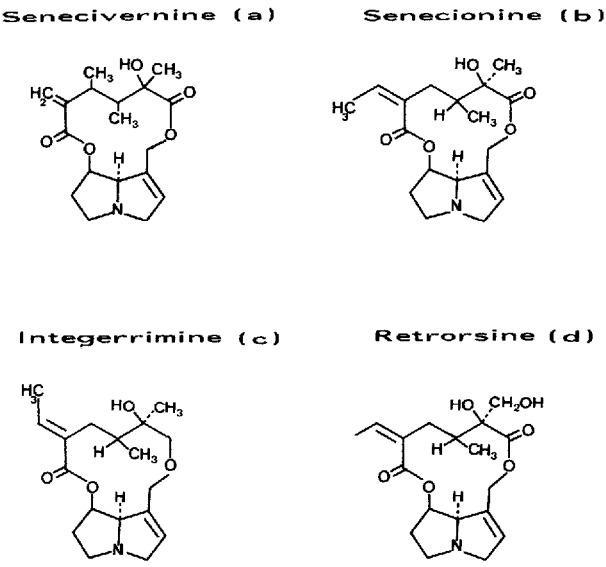

Fig. 2. Structures of the identified pyrrolizidine alkaloids: senecivernine, senecionine, integerrimine and retrorsine.

The results reported here show that CGC furnishes complete and reliable fingerprints of the pyrrolizidine alkaloid fraction. CGC allowed us to separate and identify at least five compounds of this class in *Senecio inaequidens*, compared with the two compounds previously identified by packed-column GLC and TLC[33]. The use of thin-film capillary columns (0.1 μm) allowed a more rapid analysis than packed columns. The presence of a basic "active" centre in the structure of pyrrolizidine alkaloids requires the injection port and column to be as inactive as possible in order to avoid both loss of compounds through absorption and severe tailing of the peaks. Probably for the same reasons, the CGC analyses carried out on a soda-lime glass column gave less tailing than those on a column prepared with Duran-50 glass.

TABLE I

COMPOSITION OF THE PYRROLIZIDINE ALKALOID FRACTION OF *SENECIO INAEQUIDENS* D.C.

Compound identification	*Name*	*MW*	*Concentration (%)*	*Standard deviation (%)*
a	Senecivernine	335	16.4	1.4
b	Senecionine	335	21.3	1.5
c	Integerrimine	335	4.7	0.8
d	Retrorsine	351	27.6	1.1
e	Retrorsine analogue	351	1.2	0.3
f	Unidentified	354*	2.9	0.4
g	Unidentified	396*	1.4	0.5

* Assumed.

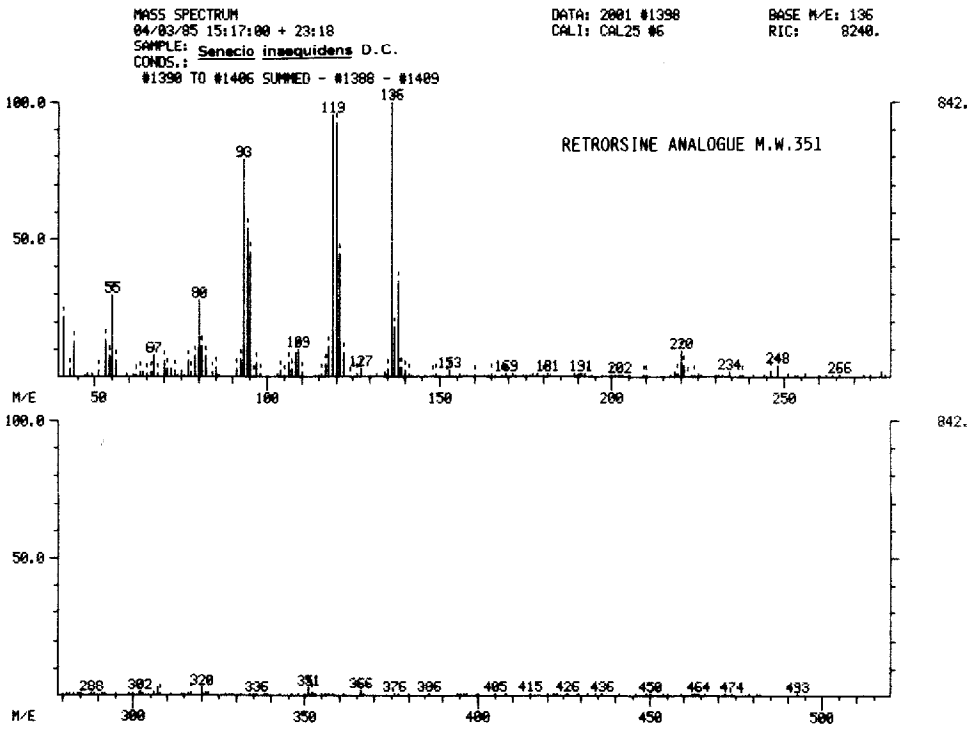

Fig. 3. Mass spectrum of the retrorsine analogue of MW 351 (e).

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