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CAPILLARY GAS CHROMATOGRAPHY–FOURIER TRANSFORM INFRARED SPECTROSCOPY OF PYRROLIZIDINE ALKALOIDS OF *SENECIO INAEQUIDENS* DC.

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SUMMARY

The pyrrolizidine alkaloid (PA) fraction of *Senecio inaequidens* DC. was studied by capillary gas chromatography–Fourier transform infrared spectroscopy. The vapour-phase IR spectra of PAs and the advantages and disadvantages of this combined technique for their structure elucidation in complex mixtures are discussed. Examples of the distinction between necine bases (retronecine and otonecine) and geometric isomers (senecionine and integerrimine) are given.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are well known for their hepatotoxic properties and, to a lesser extent, as inducers of pulmonary arterial hypertension. PAs are present in species belonging to plant families throughout the world, in particular Boraginaceae, Compositae (Senecioneae and Eupatorieae) and Leguminosae (genus *Crotalaria*). Comprehensive reviews and textbooks describing this class of compounds and their chemotaxonomic significance and toxicity are available[1–7].

Senecio inaequidens DC. (Compositae) is a species native to South Africa, naturalized in Italy after the Second World War and now so widely diffused in Eastern Italy as to be considered potentially dangerous, both as a food contaminant and directly for cattle. The composition of the PA fraction of *S. inaequidens* was investigated by Wiedenfeld *et al.*[8], who identified senecionine and retrorsine, and by Bicchi and co-workers[9,10]. In the latter studies, one of the most complex PA fractions ever studied was isolated during an on-going ontogenic study at the beginning of the vegetative period. Nineteen PAs were characterized and 16 identified by capillary gas chromatography (GC) and mass spectrometry (MS) in different ionization modes: electron impact (EI), positive ion chemical ionization (PICI) with ammonia and negative ion chemical ionization (NICI) with ammonia and hydroxyl ions as reagent species[10]. The identified PAs were macrocyclic diesters derived from two necine bases (retronecine and otonecine); in particular senecivernine (**1**) (M.W. 335), senecionine (**2**) (M.W. 335), seneciphylline (**3**) (M.W. 333), spartioidine (**4**) (M.W. 333), integerrimine (**5**) (M.W. 335), retrorsine (**7**) (M.W. 351), usaramine (**10**) (M.W. 351), senkirkine (**6**) (M.W. 365), neosenkirkine (**9**) (M.W. 365), otosenine (**11**) (M.W. 381),

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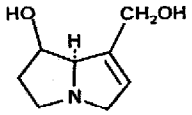
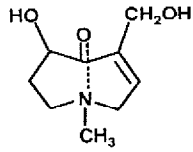
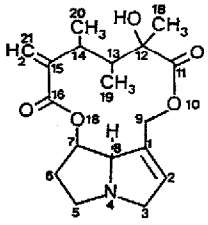
O-acetylsenkirkine (**13**) (M.W. 407), desacetyldoronine (**14**) (M.W. 417), florosenine (**16**) (M.W. 423), floridanine (**17**) (M.W. 441), doronine (**18**) (M.W. 449) and floricaline (**19**) (M.W. 483) were identified. Fig. 1 shows the structures of the identified PAs.



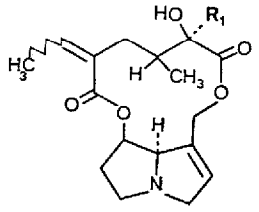
Retronecine



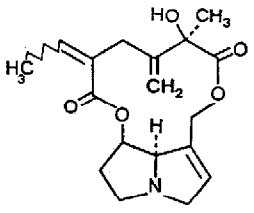
Otonecine



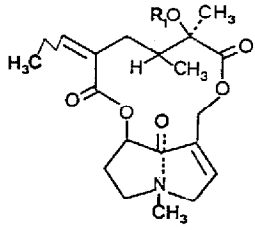
Senecivernine (1)



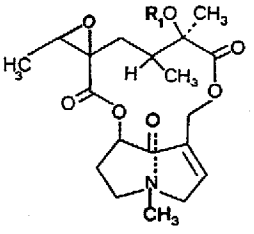
R_1=CH_3 Z Senecionine (2)
E Integerrimine (5)
R_1=CH_2OH Z Retrorsine (7)
E Usaramine (10)



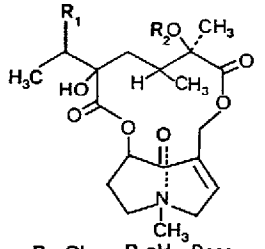
Z Seneciphylline (3)
E Spartioidine (4)



R_1=H Z Senkirkine (6)
E neo-Senkirkine (9)
R_1=Ac O-ac. Senkirkine (13)



R_1=H Otosenine (11)
R_1=Ac Florosenine (16)



R_1=Cl R_2=H Desac. doron. (14)
R_1=Cl R_2=Ac Doronine (18)

Fig. 1. Structures of PAs identified in *S. inaequidens* DC.

The structure elucidation of the components of such a complex mixture through their isolation is, of course, difficult and time consuming; better results can be obtained through the combined techniques [*i.e.*, capillary GC–MS and capillary GC–Fourier transform (FT)-IR].

GC–MS is the most widely used combined technique to identify PAs when a total plant extract is analysed. Several workers[8–16] have applied GC–MS in different ionization modes to identify PAs both as such and as their trimethylsilyl derivatives.

Even if MS is the pre-eminent technique for identifying a component of a complex mixture after a chromatographic separation, it sometimes has drawbacks, such as in the differentiation of structural isomers and in some doubtful or erroneous identifications produced by a library search. In such cases, FT-IR is at present the most useful complementary or alternative technique for characterizing a chromatographic peak. The use of capillary GC–FT-IR can be very helpful in the identification of PAs, considering the complexity of their structure and the number of structural isomers present in this class of compounds. IR spectra of PAs were studied by, among others, Culvenor and co-workers[17,18] and Gupta *et al.*[19], who discussed the IR spectra in the solid and liquid phase with respect to the absorptions of both the necine ring and the ester functions.

This paper describes the results obtained by applying capillary GC–FT-IR to the study of the PA fraction of *S. inaequidens*.

EXPERIMENTAL

Plant material

Plant material was collected in March 1986 from a roadside on the outskirts of Padua (Italy).

Reagents

All chemicals were of analytical-reagent grade (E. Merck, Darmstadt, F.R.G.). PS 264 and PS 122 are commercially available from Petrarch Systems (Bristol, PA, U.S.A.). Authentic samples of senecionine, seneciphylline, integerrimine and retrorsine were kindly provided by Dr. C. C. J. Culvenor (Parkville, Australia).

Sample preparation

A 25-g amount of air-dried plant material was extracted in a Soxhlet apparatus with methanol for 4 h. The extract was evaporated to dryness under vacuum and the residue suspended in 2.5% hydrochloric acid and washed with diethyl ether and chloroform. Half of the aqueous phase was basified with 25% ammonia solution and extracted with dichloromethane. The organic layer was again treated with 2.5% hydrochloric acid then 25% ammonia solution and again extracted with dichloromethane. The resulting solution was dried over anhydrous sodium sulphate and evaporated to dryness. To investigate the presence of PA N-oxides, the second half of the solution (resulting after washing with diethyl ether and chloroform) was reduced with zinc dust overnight, filtered and subsequently treated as described previously. The dried residues were weighed and dissolved in appropriate amounts of dichloromethane to produce suitable concentrations for capillary GC and capillary GC–FT-IR analysis. The sample under investigation contained 69.7 mg of PAs as free bases and 22.6 mg in the form of N-oxides.

Capillary GC analysis

Capillary GC analyses were carried out by introducing 1 μl of the PA extract dissolved in dichloromethane (1:250) into a Carlo Erba Mega 5360 instrument. The following conditions were used: carrier gas, hydrogen; flow-rate, 3 ml/min; injection system, split, with a splitting ratio of 1/30; injector temperature, 300°C; detection, flame ionization (FID); detector temperature, 300°C; column temperature, programmed from 120°C (1 min) to 280°C (20 min) at 3°C/min.

The column was a 30 m × 0.32 mm I.D. fused-silica capillary coated with 0.3 μm of PS 264 (polydimethylsiloxane, 7% diphenyl, 1% vinyl). To deactivate the column prior to coating with the stationary phase, the capillary was persilylated at 320°C for 4 h with a solution of polymethylhydrosiloxane (PS 122) in dichloromethane[20].

Capillary GC–FT-IR analysis

A Hewlett-Packard 5965 capillary GC–IR system was used. A 1-μl volume of PA extract solution in dichloromethane (1:100) was injected in the capillary GC–IR system. Capillary GC analysis was carried out on the fused-silica open-tubular column and under the chromatographic conditions given above.

FT-IR spectroscopy was carried out as follows: capillary GC–FT-IR interface, 100-μl volume light-pipe (10 cm × 1.2 mm I.D.); temperature, 280°C; make-up gas, helium at a flow-rate of 0.2 ml/min; time resolution (repetition rate), three scans per second at 8 cm^{-1} resolution; in most instances five interferograms were added in real time, resulting in an effective time slice of about 2 s; FT-IR detector, HgCdTe of narrow band width (4000–800 cm^{-1}).

RESULTS AND DISCUSSION

Fig. 2 shows the capillary GC–FID pattern of the PA extract of *S. inaequidens* and Fig. 3 that section of the capillary GC–FT-IR pattern in which the PAs eluted. As can be seen, capillary GC–FT-IR, is a very powerful tool for PA structure elucidation, despite the FT-IR detection sensitivity being lower than that of either FID of (MS) total ion current detection. The differences between the two chromatographic patterns can mainly be attributed to the detection limit of the FT-IR HgCdTe detector, which can be as much as one order of magnitude lower than that of the FID response; the high IR detector operating temperature (280°C), which involves a decrease in sensitivity; in the authors' experience, sensitivity decreases above 220°C; the method used to reconstruct the IR chromatogram; in fact, the Gram–Schmidt method employed here reconstructs each chromatographic peak through the contribution of each absorption band of the IR spectrum and the intensity of each band depends on its molar absorptivity, which is characteristic of each individual structure; and the chromatographic resolution, which can be lower when using capillary GC FT-IR and it is not always possible to obtain spectra from all the peaks resolved by capillary GC analysis.

Some authors have suggested installing a flame ionization detector in series with the light-pipe to obtain comparable chromatographic results[21].

A short discussion of the absorption bands characterizing the PA vapour-phase FT-IR spectra present in the *S. inaequidens* extract is given below. Table I reports the characteristic IR bands of the significant vapour-phase FT-IR spectra of PAs.

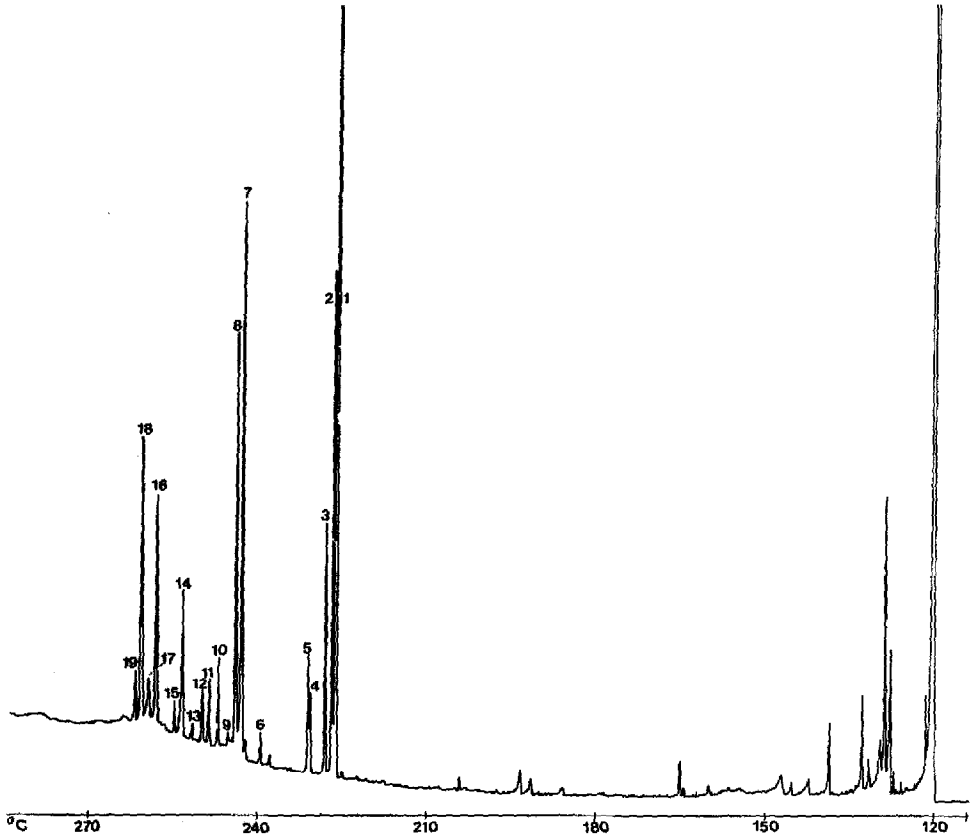


Fig. 2. Capillary GC–FID pattern of PA extract of *S. inaequidens* DC.

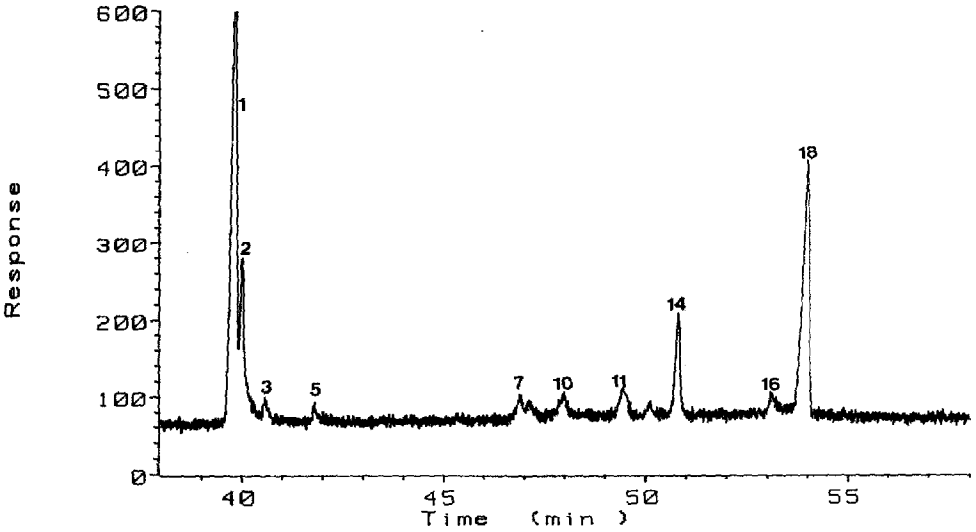


Fig. 3. Section of capillary GC–FT-IR pattern in which PAs eluted.

TABLE I
CHARACTERISTIC IR BANDS OF THE PA COMPOUNDS IDENTIFIED IN *S. INAEQUIDENS* DC.

No.	*Compound*	*IR band positions (cm^{-1})*[a]
1	Senecivernine	3557, 2981(m), 2945(m), 2910(m), 2876(m), 2849(m), 1736(s), 1631, 1453, 1420, 1376, 1358, 1253(s), 1165(s), 1132(s), 945(m), 820
2	Senecionine	3563, 2982(m), 2941(m), 2915(m), 2875(m), 2843(m), 1735(s), 1645, 1450, 1373, 1325, 1224(s), 1176(s), 1156(s), 955, 820
3	Seneciphylline	3550, 2981(m), 2942(m), 2918(m), 2873(m), 2838(m), 1736(s), 1640, 1447, 1364, 1229(s), 1145(s), 955, 820
5	Integerrimine	3561, 2981(m), 2941(m), 2915(m), 2875(m), 2840(m), 1735(s), 1654, 1452, 1380, 1268(s), 1211(s), 1178(s), 1163(s), 1110(m), 1084(m), 1025, 950, 822
7	Retrorsine	3630, 3541, 2980(m), 2960(m), 2942(m), 2875(m), 2830(m), 1735(s), 1640, 1450, 1380, 1348, 1221(s), 1154(s), 1079(m), 1004, 948, 821
10	Usaramine	3636, 3543, 2979(m), 2962(m), 2940(m), 2870(m), 2830(m), 1735(s), 1640, 1444, 1375, 1260(s), 1218(s), 1155(s), 1059, 950
11	Otosenine	2988(m), 2961(m), 2940(m), 2870(m), 2840(m), 2814, 1745(s), 1679(m), 1450, 1370(m), 1231(s), 1185(s), 1150(s), 1111(s), 950
14	Desacetyldoronine	3560(m), 2990(m), 2969(m), 2945(m), 2887(m), 2855(m), 2813, 1747(s), 1674(s), 1454, 1379, 1330, 1210(s), 1178(s), 1150(s), 1090, 941
16	Florosenine	2991(m), 2966(m), 2930(m), 2890(m), 2850(m), 2814, 1766(s), 1678(m), 1454, 1371, 1232(s), 1185(m), 1145(s), 1105(s), 1060, 954
18	Doronine	3557, 2990(m), 2958(m), 2885(m), 2865(m), 2914, 1753(s), 1676(m), 1449, 1371(m), 1231(s), 1188(s), 1147(s), 1105(s), 1015(m), 952

[a] m = Medium; s = strong.

The absorption band in the 3630–3540 cm^{-1} range, weak where present, is due to OH stretching. The lack of hydrogen bonds in the vapour phase produces a lower intensity band than in the spectra in the solid and liquid phases.

The necine bases are characterized by a group of absorptions in the 3000–2800 cm^{-1} range which correspond to CH_2 symmetric and asymmetric stretching. In particular, retronecine derivatives show a series of absorptions near 2950 cm^{-1} (characteristic of all the macrocyclic esters), near 2975 and 2915 cm^{-1} (CH_2 asymmetric stretching), near 2870 and 2850 cm^{-1} (CH_2 symmetric stretching) and near 2825 cm^{-1} (symmetric stretching of CH_2 bound to the nitrogen atom in the heterocyclic ring). These data are in agreement with those reported by Gupta *et al.*[19]. The otonecine derivatives exhibit absorptions of the same intensity, normally falling at a slightly higher frequency (*ca.* 10 cm^{-1}), together with a characteristic absorption at 2815 cm^{-1} which can be attributed to the symmetric stretching of CH_3 on the nitrogen atom of the heterocyclic ring. The macrocyclic PA ester functions give rise to a medium to strong absorption band in the 1770–1730 cm^{-1} range corresponding to C=O stretching. The α,β-unsaturated esters also show a medium to weak absorption in the 1640 cm^{-1} range, due to C=C, which is stronger for the asymmetrically substituted compounds [*i.e.*, senecivernine (**1**), seneciphylline (**3**) and integerrimine (**5**)]. In this range of frequency, otonecine derivatives exhibit an important and distinctive medium-intensity band near 1675 cm^{-1}, attributed to the C=O group of the otonecine ring. As already reported for the IR spectra in the solid and liquid phases[22], the unusually low-frequency absorption can be correlated to the strong transanular interaction between the C=O group and the nitrogen atom[23,24].

A great deal of information can be drawn from the absorptions in the 1500–900 cm^{-1} range. The medium–weak absorptions near 1450 and 1360 cm ^{1} can be attributed to the CH_2 bending and twisting, respectively. The medium–strong absorptions, in contrast, in the 1265–1245 and 1230–1210 cm^{-1} ranges are related to C–O stretching of the ester group and to CH_2 wagging, respectively. All the PAs with a tertiary hydroxyl group in their structure give rise to a strong absorption near 1150 cm^{-1} corresponding to the C–O stretching. Finally, an absorption related to the ring deformation modes in the 960–940 cm^{-1} range is present for both the retronecine and otonecine derivatives in all instances.

Fig. 4 reports the vapour-phase FT-IR spectra of senecivernine (**1**) and desacetyldoronine (**14**), which is a member of the PA class identified for the first time in

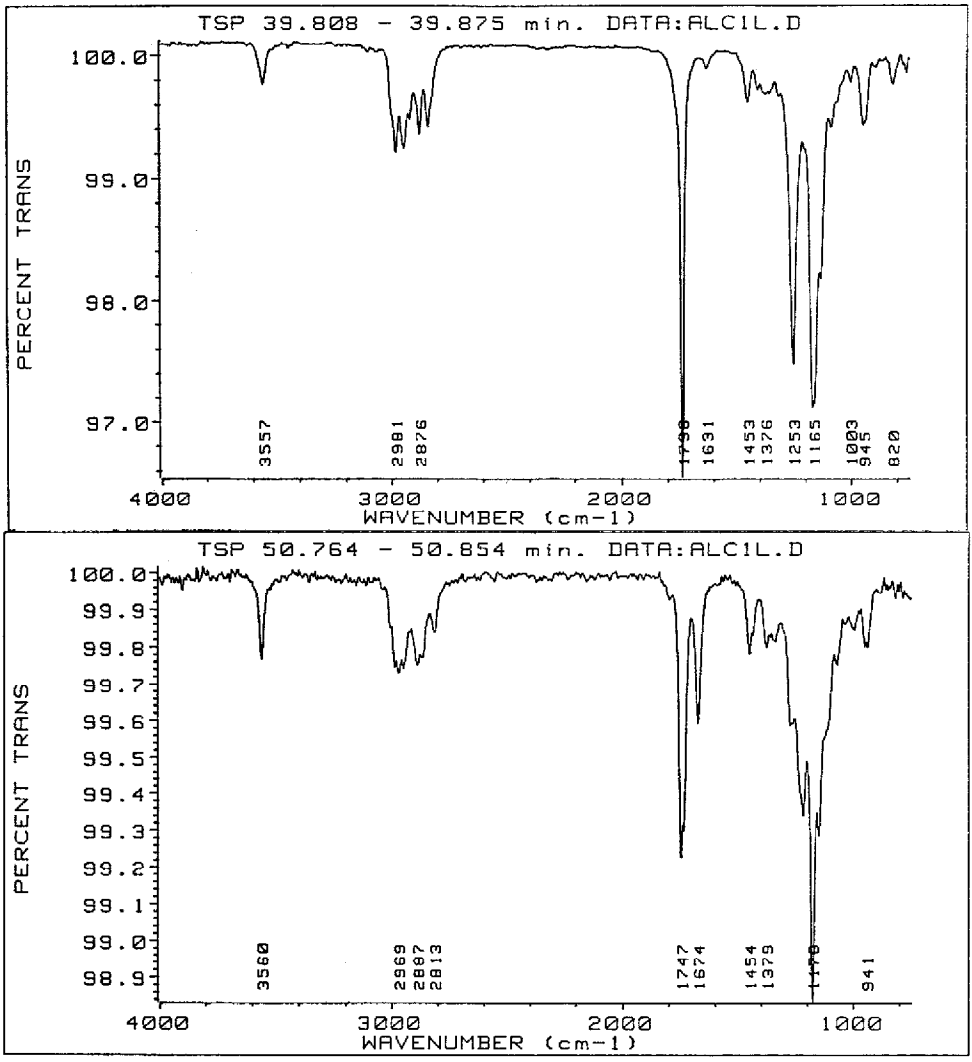


Fig. 4. Vapour-phase FT-IR spectra of senecivernine (**1**) and desacetyldoronine (**14**).

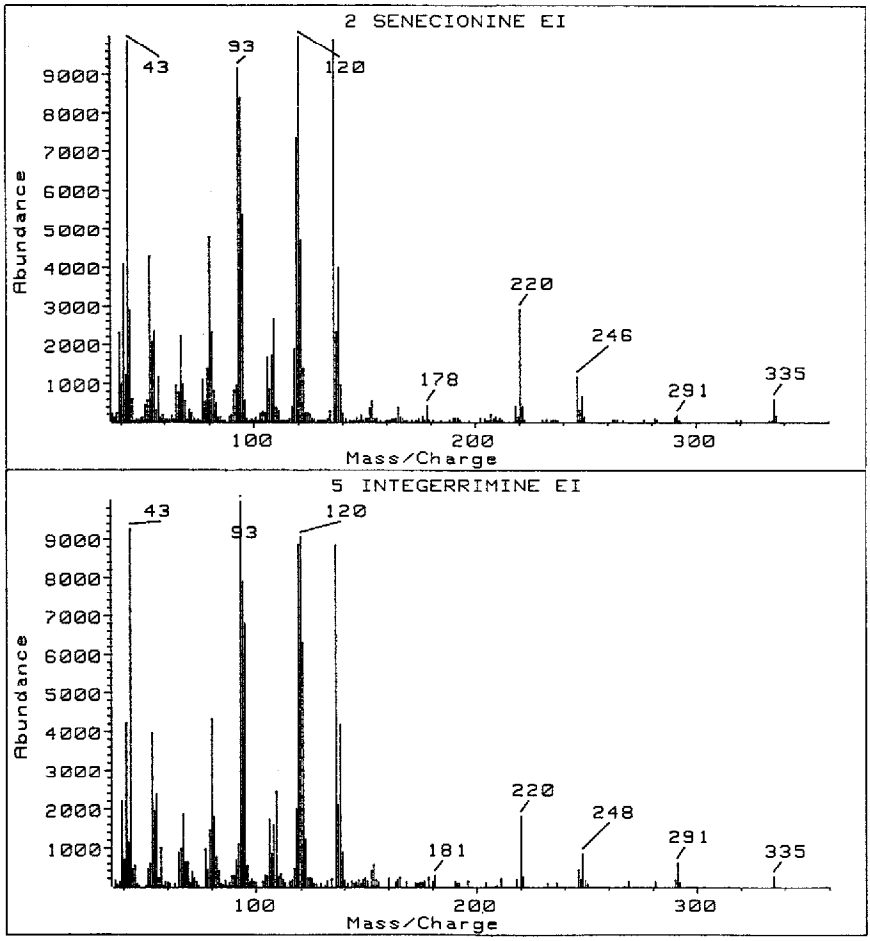


Fig. 5. EI mass spectra of senecionine (**2**) and integerrimine (**5**).

S. inaequidens[10]. Fig. 4 clearly demonstrates that vapour-phase FT-IR spectra can be very helpful in distinguishing the PA necine base through the different absorptions of a retronecine derivative, senecivernine, at 2981, 2945, 2910, 2876, 2849 and 2820 cm^{-1}, and an otonecine derivative, desacetyldoronine, at 2990, 2969, 2945, 2886, 2855, 2815 and, significantly, 1674 cm^{-1}.

Vapour-phase FT-IR spectra of PAs can also provide an unambiguous distinction between geometric isomers, which is not always directly possible through their mass spectra. The sample of *S. inaequidens* under analysis is characterized by the presence of four pairs of geometric isomers: senecionine–integerrimine, seneciphylline–spartioidine, retrorsine–usaramine and senkirkine–neosenkirkine. The EI mass spectra of senecionine and integerrimine (Fig. 5) clearly demonstrate how difficult it is to distinguish between them unequivocally, even when standard samples are available. The small differences in the intensities of the peaks at *m/z* 220, 246, 248, 291 and 335 are not sufficiently pronounced as to allow a correct identification, as they could also be

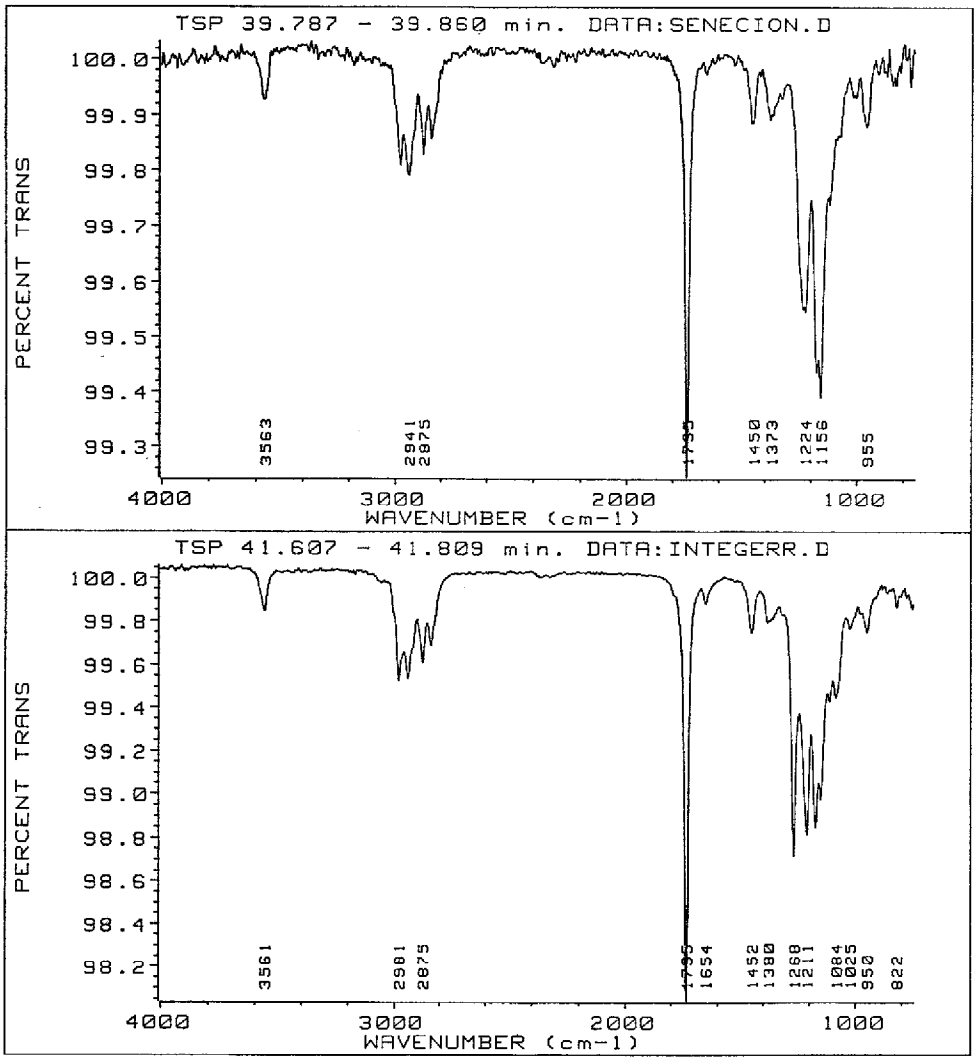


Fig. 6. Vapour-phase FT-IR spectra of senecionine (**2**) and integerrimine (**5**).

influenced by the operating conditions of the mass spectrometer ion source, which can vary slightly from instrument to instrument. Vapour-phase FT-IR spectra (Fig. 6), in contrast, afford a clear distinction between the two isomers in the 1300–1100 cm^{-1} range. In fact, whereas integerrimine absorbs at 1268, 1211, 1178 and 1163 cm^{-1}, senecionine absorbs at 1247, 1224, 1176 and 1156 cm^{-1}, at different intensities. These results were also confirmed with the retrorsine–usaramine pair.

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