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CAPILLARY GAS CHROMATOGRAPHY OF LUPIN ALKALOIDS

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SUMMARY

The resolution and identification of twelve lupin alkaloids are demonstrated using capillary gas chromatography and gas chromatography–mass spectrometry. The quantitative capabilities of capillary gas chromatography are illustrated by specific reference to the four major alkaloids of lupinus angustifolius, namely lupanine, 13-hydroxylupanine, augustifoline and α-isolupanine.

INTRODUCTION

With the breeding of sweet lupin varieties suitable for the agricultural conditions of Western Australia[1,2] this crop has gained considerable importance as a stockfood for both local consumption and export. The production forecast for lupinseed in Western Australia for 1982 is estimated at 200,000 tonnes. The industry is based primarily on the white seeded, white flowered narrow leafed lupin, *Lupinus angustifolius*.

The quinolizidine alkaloids comprise the bitter tasting toxic components present in lupins. These alkaloids have caused illness and death in both animals and man[3]. The individual alkaloids vary considerably in toxicity with D-lupanine being the most toxic lupin alkaloid reported and 13-hydroxylupanine only one tenth as toxic[4]. Keeler[5] in 1978 illustrated that certain lupin alkaloids may be teratogenic.

One of the major uses for lupinseed is as a protein source for pig rations. Monitoring of the alkaloid content of lupinseed is required because feed rejection can occur with levels as low as 0.03% alkaloid in the diet[6–8]. In addition the National Health and Medical Research Council has approved the use of lupin flour in bread manufacture provided that the alkaloid content of the lupin flour does not exceed 0.02%[9]. There is therefore a need to identify and quantify the alkaloids present in sweet lupinseeds and products.

Capillary gas chromatography (GC) is a rapid and sensitive technique suited to the analysis of the individual lupin alkaloids. Data are presented in this paper to illustrate the resolution obtained for the more common alkaloids and the suitability of the technique for quantification of low levels of alkaloid.

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EXPERIMENTAL

Apparatus and operating conditions

A Hewlett-Packard 5880A gas chromatograph equipped with a hydrogen flame ionization detector and a level four control terminal was used, with a split ratio of 60:1.

The following operating conditions were employed in conjunction with a 12 m × 0.2 mm I.D. flexible fused-silica OV-101 capillary column (Hewlett-Packard): injection port temperature 250°C; detector temperture 300°C; temperature programme 60°C hold 1 min, increase 30°C/min to 120°C, hold 0.5 min, then increase 10°C/min to 260°C, final hold 2 min; helium carrier gas flow-rate 2 ml/min; helium make up gas flow-rate 28 ml/min; hydrogen flow-rate 30 ml/min; air flow-rate 400 ml/min.

A 5990A Hewlett-Packard gas chromatograph–mass spectrometer with a 5992 terminal was used: injection port temperature 240°C; temperature program 90°C hold 2.5 min, increase 8°C/min to 250°C, hold 5 min.

Reagents and alkaloid standards

Solvents were of analytical grade. Caffeine base was purchased from Fluka (Buchs, Switzerland). The major alkaloids which could not be purchased were separated in these laboratories from the seeds of the bitter cultivar *L. angustifolius* Fest. Column chromatography, high-performance liquid chromatography (HPLC) and liquid–liquid extraction techniques provided gas chromatographically pure angustifoline, D-lupanine perchlorate and 13-hydroxylupanine.

L-Sparteine, α-isolupanine perchlorate, cytisine and 17-hydroxylupanine perchlorate were purchased from Sigma (St. Louis, MO, U.S.A.) and Koch-Light (Colnbrook, Great Britain).

Resolution and identification by capillary GC and gas chromatography–mass spectrometry (GC–MS)

The following alkaloids were incorporated in a mixed standard: epilupinine, sparteine, caffeine (internal standard[10,11]), 13-hydroxysparteine, cytisine, tetrahydrorhombifoline, angustifoline, α-isolupanine, lupanine, 17-hydroxylupanine, multiflorine, 17-oxolupanine and 13-hydroxylupanine. Table I lists the weight of each alkaloid in the standard. Each alkaloid was prepared as a stock solution in ethyl acetate and an appropriate amount added to the mixed standard.

The perchlorate salts of lupanine, α-isolupanine and 17-hydroxylupanine were first dissolved in a basic solution (pH 14)[11,12]. The free bases were then extracted with dichloromethane and made to volume. One microlitre of mixed standard was injected into the gas chromatograph and the peaks were identified by injection of the individual alkaloids. Further identification and confirmation were obtained using GC–MS.

Quantitative aspects

The composition of the total free alkaloids in six sweet cultivars and one bitter cultivar of *L. angustifolius* was established by injection of 1 μl of alkaloid extracts obtained from lupinseed as follows. One gram of finely milled lupinseed was extracted overnight with ethanol–water (70:30, v/v). After centrifugation and washing with

TABLE I

RETENTION TIMES OF LUPIN ALKALOIDS

Retention times relative to 200 μg caffeine (9.11 min).

Alkaloid	*Volume of stock solution added (μl)*	*Concentration of final stock solution (mg/ml)*	*Weight of alkaloid in standard (μg)*	*Retention time (min)*
Epilupinine	50	2	100	−4.10
Sparteine	50	5	250	−0.50
13-Hydroxysparteine	500	0.2	100	1.83
Cytisine	100	2	200	2.00
Tetrahydrorhombifoline	50	5	250	2.30
Angustifoline	50	3	150	2.65
α-Isolupanine	150	2	300	2.78
Lupanine	50	4	200	3.32
17-Hydroxylupanine	50	1	50	3.57
Multiflorine	100	2	200	4.71
17-Oxolupanine	50	5	250	5.05
13-Hydroxylupanine	50	4	200	5.71

ethanol–water (70:30, v/v), the combined supernatants were rotary evaporated (50°C) and the resulting residue made up to 20 ml with water. The lipid material was removed by extraction at pH 2 with diethyl ether (two 30-ml portions). Alkaloid esters were removed by extraction with dichloromethane (two 20-ml portions) at pH 2. The aqueous extract was then made alkaline (pH 14)[11,12] and extraction with dichloromethane (three 20-ml portions) yielded the free alkaloids which were diluted with ethyl acetate to a volume of 1 ml. Two hundred microlitres of this solution were added to 50 μg of caffeine. One microlitre of this mixture was injected into the gas chromatograph.

Six independently prepared standards containing varying amounts of the four alkaloids angustifoline, α-isolupanine, lupanine and 13-hydroxylupanine, and 50 μg of the internal standard caffeine were used to calibrate the gas chromatograph. Compositional data of these standards are presented in Table II.

TABLE II

ALKALOID WEIGHT RESPONSE FACTORS

Weight response factor (WRF) = $\frac{\text{Area caffeine}}{\text{Area alkaloid}} \times \frac{\text{Weight alkaloid}}{\text{Weight caffeine}}$. C.V. is the coefficient of variation.

Alkaloid	*Weight of alkaloid (μg) in standard solution*						*Mean WRF*	*S.D.*	*C.V. (%)*
	1	*2*	*3*	*4*	*5*	*6*			
Angustifoline	15	30	75	150	225	300	0.496	0.009	1.68
α-Isolupanine	7.1	14.2	35.6	71.3	106.9	142.5	0.489	0.013	2.65
Lupanine	17.8	35.6	89.1	178.2	267.2	356.3	0.508	0.017	3.38
13-Hydroxy-lupanine	25	50	125	250	375	500	0.634	0.022	3.44

RESULTS AND DISCUSSION

Fig. 1 illustrates the resolution obtained between twelve quinolizidine alkaloids and the internal standard caffeine. The run time is 15 min and can be decreased to 9.5 min with an alternate oven programme without a significant loss in resolution. The retention times of the alkaloids relative to caffeine are listed in Table I. The tailing which is present in Fig. 1 for epilupinine and 13-hydroxylupanine is due to the hydroxyl functions present in these compounds. Structural confirmation of the alkaloids is illustrated by Table III. The MS data correlate well with that presented by Cho and Martin[13].

The major alkaloids of *L. angustifolius* are angustifoline, α-isolupanine, lupanine and 13-hydroxylupanine. These account for greater than 95% of the free

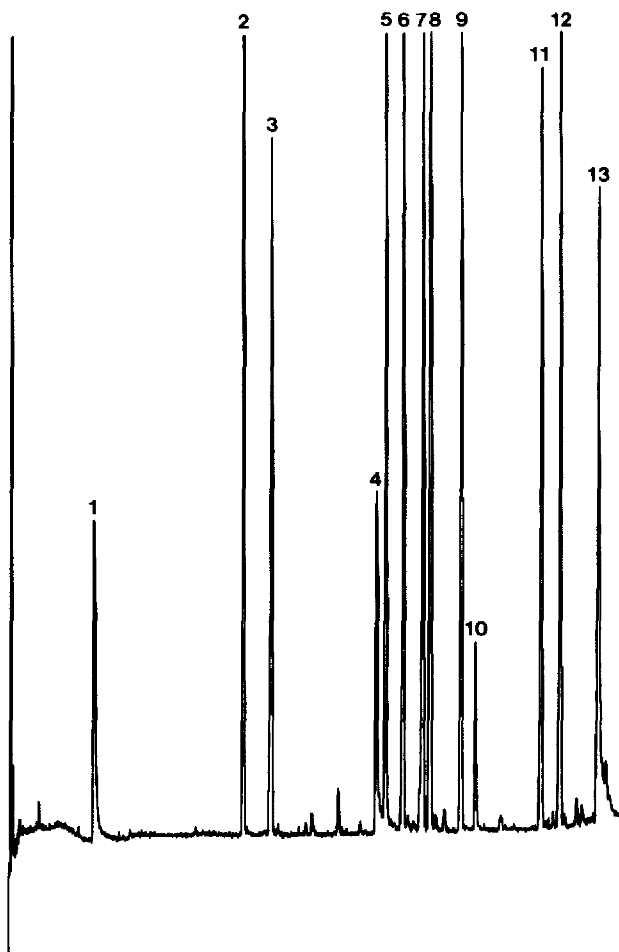

Fig. 1. Capillary GC trace of twelve lupin alkaloids. Peaks: 1 = epilupinine; 2 = sparteine; 3 = caffeine (internal standard); 4 = 13-hydroxysparteine; 5 = cytisine; 6 = tetrahydrorhombifoline; 7 = angustifoline; 8 = α-isolupanine; 9 = lupanine; 10 = 17-hydroxylupanine; 11 = multiflorine; 12 = 17-oxolupanine; 13 = 13-hydroxylupanine.

TABLE III

MASS SPECTROSCOPY DATA

Alkaloid	*M+*	*Five most abundant ions*				
Epilupinine	169	152	168	169	138	97
Sparteine	234	137	98	193	136	234
13-Hydroxysparteine	250	137	98	136	209	250
Cytisine	190	146	147	190	134	160
Tetrahydrorhombifoline	248	207	58	112	55	108
Angustifoline	234	193	112	55	150	194
α-Isolupanine	248	136	55	149	98	150
Lupanine	248	136	149	248	150	247
17-Hydroxylupanine	264	134	55	246	112	136
Multiflorine	246	134	55	246	67	82
17-Oxolupanine	262	55	150	110	96	262
13-Hydroxylupanine	264	152	55	134	112	246

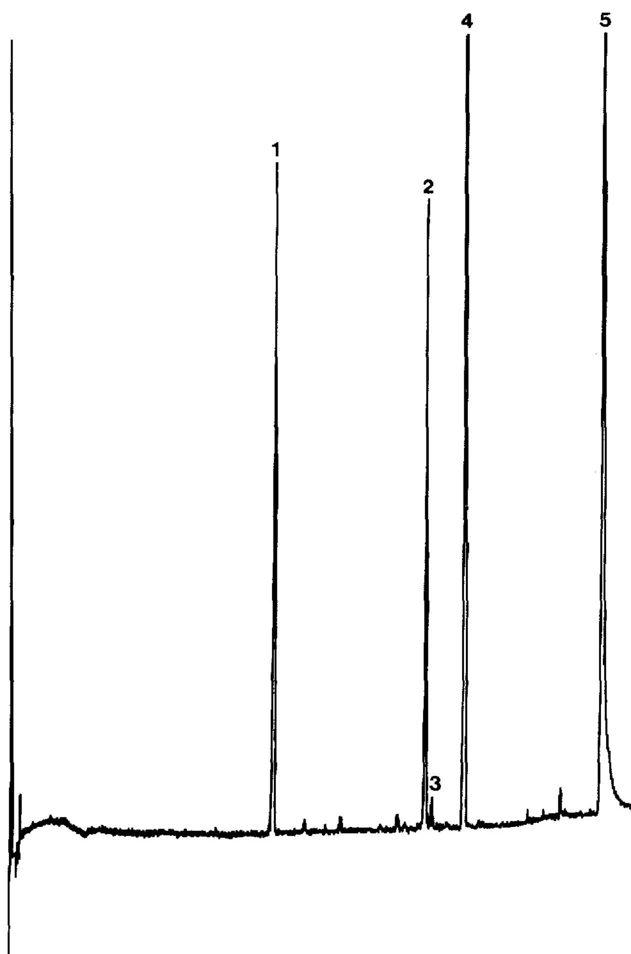

Fig. 2. Capillary GC trace of alkaloid extract of *L. angustifolius* cv. Fest. Peaks: 1 = caffeine (internal standard); 2 = angustifoline; 3 = α-isolupanine; 4 = lupanine; 5 = 13-hydroxylupanine.

TABLE IV

ALKALOID COMPOSITION OF *L. ANGUSTIFOLIUS* CULTIVARS

L. angustifolius cultivar	*Alkaloid as per cent of total alkaloid*			
	Angustifoline	*α-Isolupanine*	*Lupanine*	*13-Hydroxylupanine*
Marri	15	6	42	37
Yandee	8	3	57	32
Illyarrie	10	8	50	32
Unicrop	7	10	59	24
Uniharvest	11	15	50	24
Uniwhite	9	2	44	45
Fest (bitter cultivar)	13	1	52	34

alkaloid material in this variety. The percentage composition using the procedure described is shown in Fig. 2 and Table IV. Traces of the alkaloids tetrahydrorhombifoline and 17-oxolupanine have been identified in the course of alkaloid chromatographic separations[11].

In the bitter variety *L. angustifolius* Fest. the alkaloid content can be as high as 2%[12]. Typically the sweet cultivars of *L. angustifolius*, namely marri, illyarrie, yandee and unicrop have less than 0.02% alkaloid[1,10,12]. However it has been observed in these laboratories that the sweet cultivars may vary in alkaloid content up to 0.2%. Climatic stress or nutrient availability may be factors which contribute to this phenomenon. Scibor-Marchocka[14], for example, describes a relative potassium deficiency causing a rise of approximately 20% in alkaloid content of the sweet varieties.

Accuracy and precision

The correlation coefficients, r, obtained by comparing the area ratio (alkaloid/caffeine) *versus* the amount of alkaloid in the mixture were: angustifoline $r = 0.9997$, α-isolupanine $r = 0.9996$, lupanine $r = 0.9990$ and 13-hydroxylupanine $r = 0.9998$. These demonstrate the excellent linearity obtained over a 20-fold concentration range.

The mean weight response factors (WRF) derived for each alkaloid, over a range of concentrations, together with the precision data shown in Table II, also confirm that the WRF is independent of concentration over the working ranges studied. Alkaloid content of lupinseed extracts examined to date fall within these ranges.

CONCLUSION

The sensitivity and speed of capillary GC provides an efficient technique for quantitative analysis of the individual quinolizidine alkaloids after extraction from lupinmeals, feedstuffs and animal tissues.

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