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Note

Application of gas chromatography–mass spectrometry to the identification of isoflavonoids in lupine root extracts

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Isoflavonoids present in the genus *Lupinus* have been isolated and many components, derivatives of genistein and 2'-hydroxygenistein, have been identified[1–4]. Because of intensive research on the biological activities of this class of compounds[5,6], it became necessary to find a method for the identification of flavonoids or isoflavonoids in mixtures after isolation from plant material. The separation and identification of this class of compounds by high-performance liquid chromatography (HPLC) have been described by several workers and gas chromatography has also been used[7–10]. This paper describes an attempt to use gas chromatography–mass spectrometry (GC–MS) for the identification of isoflavonoids, isolated from lupine root extracts, as methyl and trimethylsilyl derivatives.

EXPERIMENTAL

Chemicals

Solvents of analytical-reagent grade were obtained from POCh (Gliwice, Poland). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMS) and methyl iodide, used as derivatizing reagents, were obtained from Fluka (Buchs, Switzerland). Silica gel H60 used for column chromatography and plates for thin-layer chromatography (TLC) with silica gel 60 F_{254} were purchased from E. Merck (Darmstadt, F.R.G.).

Gas chromatography–mass spectrometry

GC–MS analyses were carried out with a Hewlett-Packard Model 5890 instrument equipped with an HP-1 fused-silica capillary column (12 m × 0.25 mm I.D.). The carrier gas was helium at a flow-rate of 1.5 ml/min. The column temperature was programmed from 200°C (held for 2 min) at 5°C/min to 280°C, which was held for 10 min. The injector temperature was 250°C. Injections were made in the splitless mode.

Biological samples

Methanolic root extract obtained from 100 g of fresh roots of 3-week-old seedlings of bitter lupine plants (*Lupinus albus* L. *cv.* BAC) was evaporated to dryness,

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giving a brown syrup which was dissolved in 300 ml of water. In order to isolate isoflavonoids, the aqueous solution was extracted five times with the same volume of ethyl acetate. The isoflavonoid fraction, concentrated on a Rotavapor, was adsorbed on silica gel H60 (20 g) and dried. The gel coated with the extract was transferred to the column filled with the same silica gel (50 g). The column was eluted in a stepwise mode with benzene and increasing amounts of ethyl acetate (0, 5, 15, 30, 40, 55, 70 and 100%) in benzene, 75 ml per fraction. Elution of the isoflavonoid fractions was monitored by TLC with chloroform–methanol (50:3) as the developing solvent. Compounds of interest were detected by inspection of the plates under UV radiation of 254 and 360

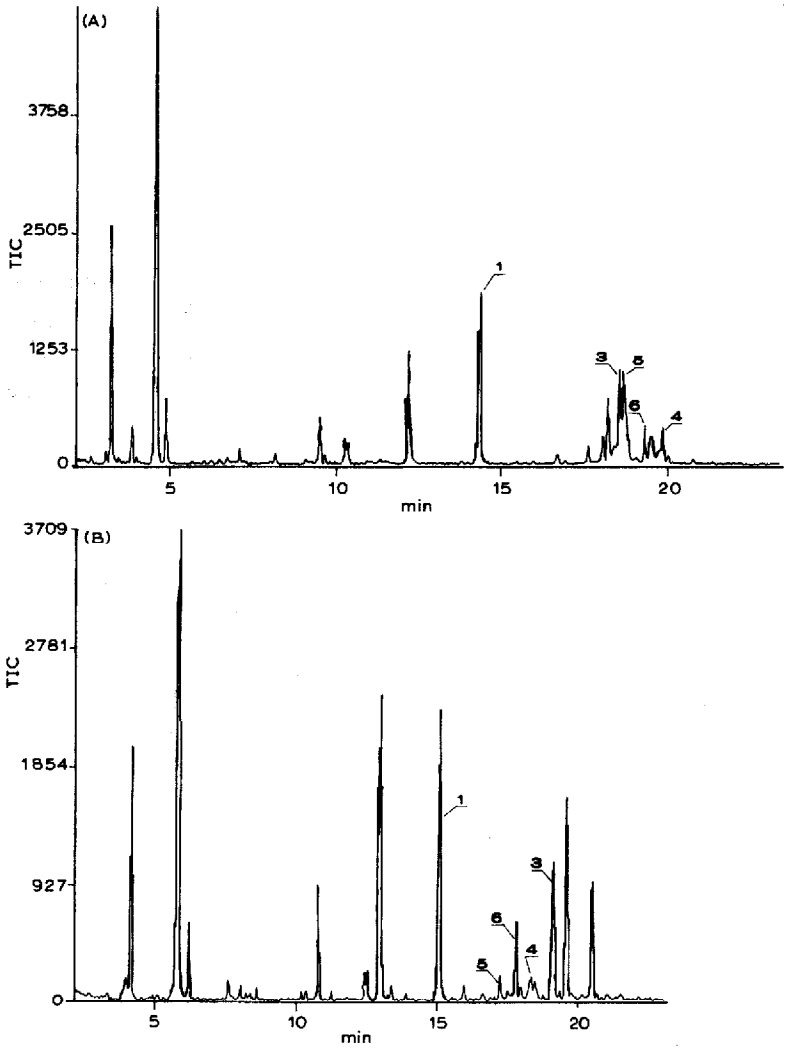

Fig. 1. TIC chromatograms of (A) methyl and (B) trimethylsilyl derivatives of fraction 1. For conditions, see Experimental. Peaks: 1 = genistein; 3 = wighteone; 4 = luteone; 5 = lupinisoflavone A; 6 = parvisoflavone B.

nm and by the characteristic colours (blue and violet) formed with Gibbs reagent[11].

Isoflavonoids were eluted from the column with solvent mixtures containing from 0 to 40% of ethyl acetate in benzene. Fractions with similar spot patterns of isoflavonoids were pooled into three solutions and, after derivatization, analysed by GC–MS.

Derivatization of samples for GC–MS analysis

Isoflavonoids were methylated with methyl iodide in dimethyl sulphoxide following the procedure of Ciucianu and Kerek[12]. Samples for methylation (2–4 mg)

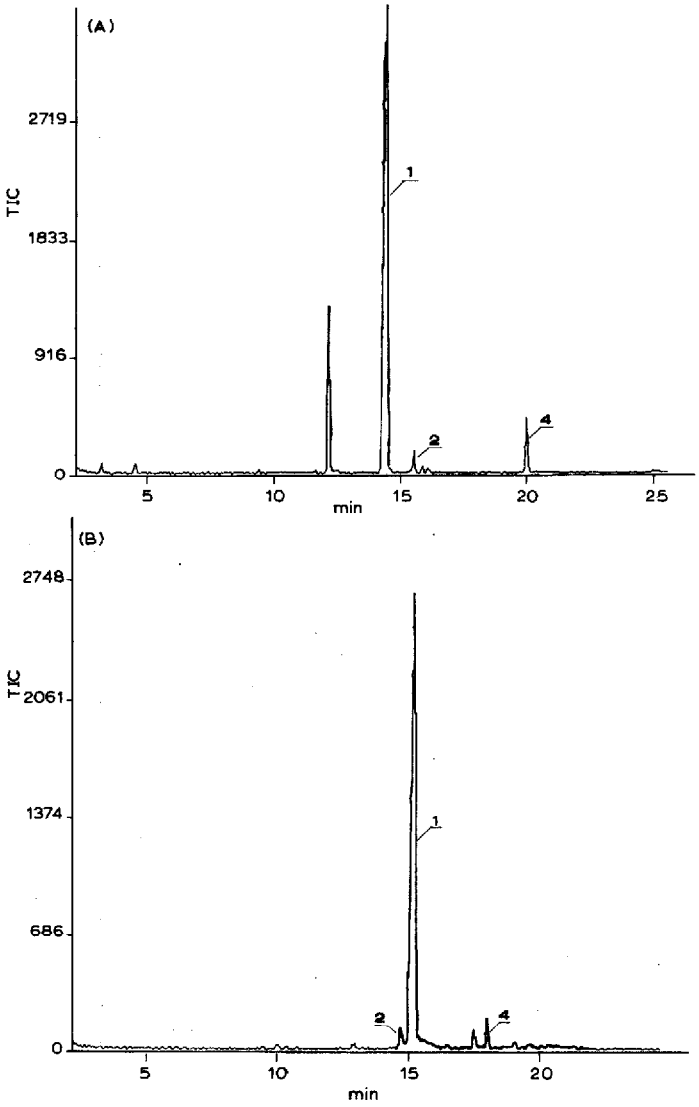

Fig. 2. TIC chromatograms of (A) methyl and (B) trimethylsilyl derivatives of fraction II. For conditions, see Experimental. Peaks: 1 = genistein; 2 = 2′-hydroxygenistein; 4 = luteone.

were transferred into test vials, evaporated to dryness in a stream of nitrogen and dried over P_2O_5 overnight. The dried samples were dissolved in 1 ml of dimethyl sulphoxide and 70 mg of powdered potassium hydroxide and 200 μl of methyliodide were added. The vials were vortex mixed for 5 min, then the reaction was stopped with 2 ml of water. The reaction mixtures were extracted three times with 2 ml of chloroform. The combined organic layers were extracted twice with 3 ml of water. The chloroform solutions were evaporated to dryness and the residue was dissolved in 200 μl of methylene chloride. The solution obtained was used for GC–MS analysis.

The preparation of the samples for trimethylsilylation was identical with that for methylation. The dried samples were dissolved in 150 μl of pyridine and mixed with 150

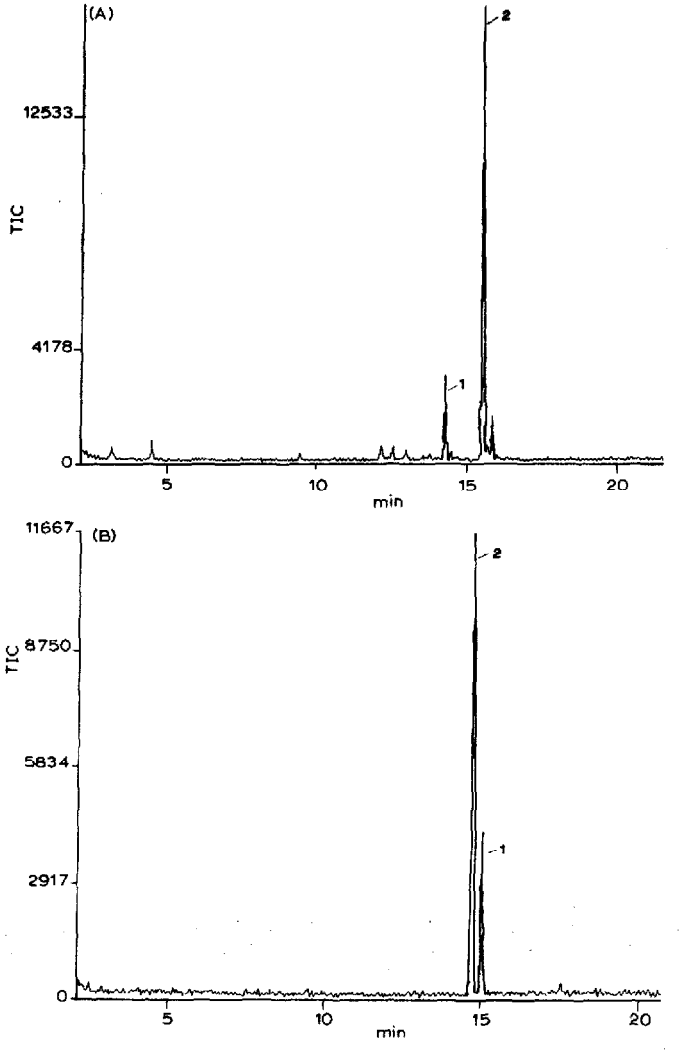

Fig. 3. TIC chromatograms of (A) methyl and (B) trimethylsilyl derivatives of fraction III. For conditions see Experimental. Peaks: 1 = genistein; 2 = 2'-hydroxygenistein.

TABLE I

GC RETENTION TIMES AND FRAGMENT IONS IN MASS SPECTRA OF METHYL DERIVATIVES OF ISOFLAVONOIDS IDENTIFIED IN FRACTIONS ISOLATED FROM LUPINE ROOT EXTRACTS

Ion	Isoflavonoid[a]											
	1 *(14.38)*[b]		*2* *(15.55)*[b]		*3* *(18.52)*[b]		*4* *(19.85)*[b]		*5* *(18.55)*[b]		*6* *(19.33)*[b]	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
M^+	312	100	342	100	380	72	410	75	394	25	394	100
$M-CH_3$	297	5	327	8	365	66	395	21	379	100	379	8
$M-OCH_3$	281	20	311	58	349	22	379	100	363	23	363	45
$M-(OCH_3+CH_3)$	266	25	296	13	–	–	–	–	–	–	–	–
M−41	–	–	–	–	–	–	–	–	353	18	–	–
M−68	–	–	–	–	–	–	–	–	–	–	326	10
M−69	–	–	–	–	311	100	341	38	–	–	–	–
$M-(69+OCH_3)$	–	–	–	–	281	11	311	6	–	–	–	–
M−83	–	–	–	–	–	–	–	–	311	40	–	–
M−108	204	12	–	–	–	–	–	–	–	–	–	–
M−138	–	–	204	6	–	–	–	–	–	–	–	–
M−161	–	–	–	–	–	–	–	–	–	–	233	7
$C_9H_8O_4$	180	6	180	17	–	–	–	–	–	–	–	–
$C_{10}H_{10}O_2$	–	–	162	19	162	16	–	–	162	11	162	16
$C_8H_9O_2$	137	17	137	10	–	–	137	6	137	7	137	4
C_9H_8O	132	33	–	–	132	12	–	–	–	–	–	–
C_7H_7O	107	5	–	–	107	8	–	–	–	–	–	–

[a] 1 = Genistein; 2 = 2′-hydroxygenistein; 3 = wighteone; 4 = luteone; 5 = lupinisoflavone A; 6 = parvisoflavone B.

[b] Retention times (min) in parentheses.

TABLE II

GC RETENTION TIMES AND FRAGMENT IONS IN MASS SPECTRA OF TRIMETHYLSILYL DERIVATIVES OF ISOFLAVONOIDS IDENTIFIED IN FRACTIONS ISOLATED FROM LUPINE ROOT EXTRACTS

Ion	Isoflavonoid[a]											
	1 *(15.12)*[b]		*2* *(14.70)*[b]		*3* *(19.15)*[b]		*4* *(18.30)*[b]		*5* *(17.22)*[b]		*6* *(17.80)*[b]	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
M^+	486	4	574	3	–	–	642	4	568	9	–	–
$M-CH_3$	471	100	559	45	539	15	627	25	553	72	553	74
$M-(CH_3+TMS)$	399	10	487	5	467	15	–	–	481	13	481	3
$M-(CH_3+2TMS)$	327	3	415	3	–	–	483	10	–	–	–	–
$C_6H_{15}OSi_2$	147	3	147	8	147	26	147	8	147	7	147	15
TMS	73	23	73	100	73	100	73	100	73	100	73	100

[a] 1 = Genistein; 2 = 2′-hydroxygenistein; 3 = wighteone; 4 = luteone; 5 = lupinisoflavone A; 6 = parvisoflavone B.

[b] Retention times (min) in parentheses.

μl of BSTFA containing 1% TMS. Derivatization was conducted for 1 h at 80°C to give samples for GC–MS analysis.

RESULTS AND DISCUSSION

All samples were subjected to GC–MS analysis. Total ion current (TIC) chromatograms of the methyl and trimethylsilyl derivatives are shown in Fig. 1–3. Peaks of isoflavonoids and of other substances, probably phenolics related to the analysed class of compounds, were observed. Mass spectral data for particular isoflavonoid peaks in the TIC chromatograms of the three fractions studied are given in Tables I and II. In the TIC chromatograms of the trimethylsilyl and methyl derivatives, the peaks related to isoflavonoids were clearly resolved. The only exception was the TIC chromatogram of the methyl derivatives of fraction I, where the peaks of two isoflavonoids (wighteone and lupinisoflavone A) were very close. In the studied samples, six isoflavonoids were recognized and were identified on the basis of their molecular weights (MW) and mass fragmentation pathways.

In the mass spectra of methylated compounds, very abundant molecular ions

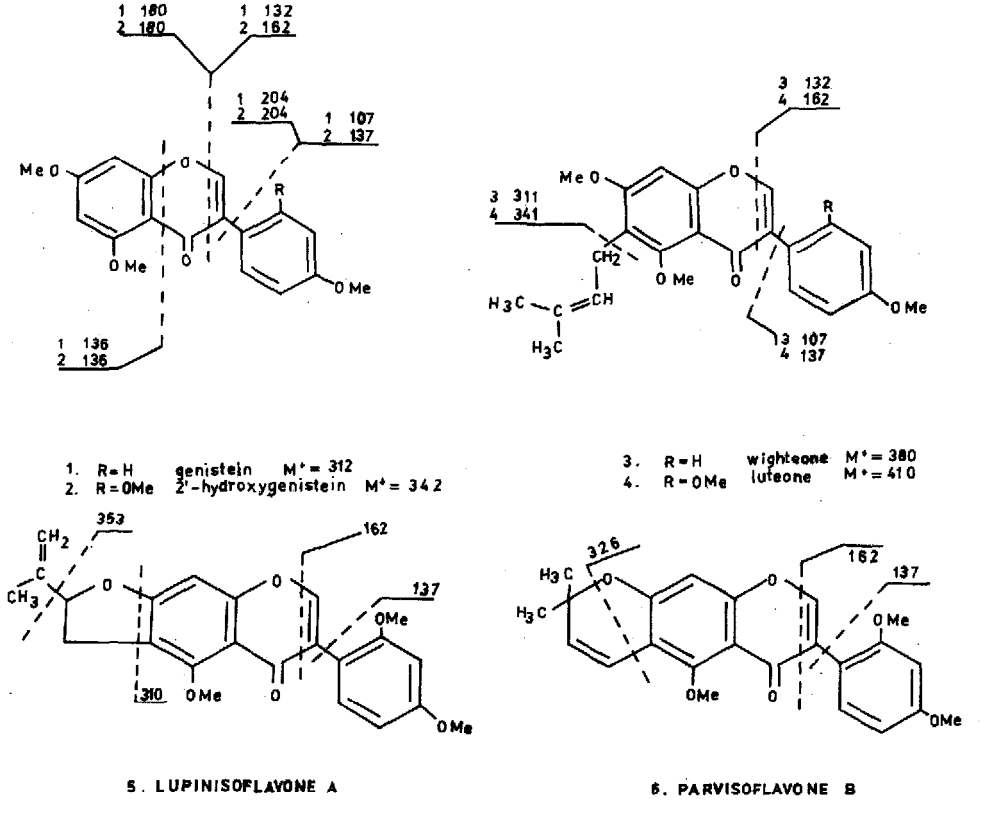

Fig. 4. Characteristic fragmentation pathways of methyl derivatives of isoflavonoids identified in lupine root extracts. Me = Methyl.

(M^+) and fragment ions were observed, giving structural information about the various isoflavonoids present in the studied extracts. On the other hand, in the mass spectra of TMS derivatives small molecular ions were observed for genistein, 2′-hydroxygenistein, luteone and lupinisoflavone A. The main ions observed were those created during elimination of the methyl radical from molecular ions. These mass spectra were dominated by fragment ions created during fragmentation of trimethylsilyl substituents at *m/z* 73 and 147, so apart from information about the molecular weights of compounds from molecular ions or [M − 15] ions there was no structural information.

Genistein, wighteone, lupinisoflavone A, parvisoflavone B and luteone were identified in fraction I, genistein, 2′-hydroxygenistein and luteone in fraction II and genistein and 2′-hydroxygenistein in fraction III. Identification of the genistein and 2′-hydroxygenistein peaks in the TIC chromatogram of methylated samples was based on the characteristic fragment ions present in the mass spectra created during cleavage of the C ring of the isoflavonoid core (see Fig. 4)[13]. In the mass spectrum of genistein these were ions at *m/z* 137 and 132, whereas for 2′-hydroxygenistein these were ions at *m/z* 137 and 161.

In the mass spectra of wighteone and luteone, fragment ions (M − 69) were observed at *m/z* 311 and 341, respectively, due to cleavage of an isoprene substituent from the molecular ions. Additionally, the presence of fragment ions at *m/z* 132 (wighteone) and *m/z* 161 (luteone) in the mass spectra of these compounds and a lack of ions at *m/z* 137 indicated that the isoprene group is attached at C-6 of the isoflavonoid core (Fig. 4).

The last two compounds, lupinisoflavone A and parvisoflavone B, have an additional furan or pyran ring owing to cyclization of the isoprene group at C-6 with the hydroxyl group at C-7 in the isoflavonoid moieties. The establishment of the substitution sites of the dihydrofuran or pyran ring in the molecules of these two compounds was possible because of the presence of the fragment ions at *m/z* 161 and a lack of fragments at *m/z* 137 and 180 (Fig. 4). Differentiation between lupinisoflavone A and parvisoflavone B was based on the observation of the fragment ion at *m/z* 353 in the mass spectrum of lupinisoflavone A. This ion is created by the cleavage of the isoprene radical from the dihydrofuran ring of the molecular ion. The ion mentioned above was absent from the mass spectrum of parvisoflavone B.

Genistein and 2′-hydroxygenistein were two main components among all the isoflavonoids present in the lupine root extract. Genistein was present in all three fractions studied, but in fraction III it occurred in only a relatively small amount. 2′-Hydroxygenistein was identified in fractions II and III. In the latter, 2′-hydroxygenistein was the basic component. The remaining four isoflavonoids were identified only in fraction I, except for luteone, which was found also in fraction II.

CONCLUSIONS

GC–MS is useful for the study of isoflavonoid mixtures, where it is recommended that both methyl and trimethylsilyl derivatives are analysed. Application of methyl derivatives makes it possible to obtain better structural information from mass spectral data than with TMS derivatives. However, the latter derivatives give a better resolution of the TIC chromatograms. In studies of methoxylated isoflavonoids

present in plant materials, utilization of both types of derivative for structural determination would give complementary information.

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