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Stereoselective determination of cucurbitine in *Cucurbita* spp. seeds by gas chromatography and gas chromatography–mass spectrometry

E. Schenkel, P. Duez and M. Hanocq

Unité de Chimie Bioanalytique, de Toxicologie et de Chimie Physique Appliquée, Université Libre de Bruxelles, C. P. 205/1, Boulevard du Triomphe, 1050 Brussels (Belgium)

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ABSTRACT

The use of Chirasil-L-Val, a chiral stationary phase, was investigated for the resolution of D,L-cucurbitine as esters of N-trifluoroacetyl derivatives; the influence of the esterifying alcohol on resolution and retention times was studied in comparison with those of different proteic amino acids. The separation mechanism was studied by the thermodynamic analysis of retention data. With flame ionization detection, linear calibration was found between 2 and 40 ng of each isomer detected and the detection limit was *ca.* 1 ng; the internal standard was methyl pentadecanoate. Comparison was made with high-performance thin-layer chromatographic determination. Gas chromatography–mass spectrometry allowed the determination of the structure of synthesized derivatives and positive identification of L-cucurbitine in aqueous extracts of *Cucurbita* seeds.

INTRODUCTION

Cucurbitine [(−)-3-aminopyrrolidine-3-carboxylic acid] is responsible for the anthelmintic, notably taenicidal and schistosomicidal, properties of *Cucurbita* spp. seeds [1,2]; cytotoxicity has recently been demonstrated on *Amoeba proteus* [3]. This amino acid, apparently limited to the *Cucurbita* genus [4], has already been analysed qualitatively by two-dimensional paper chromatography [1,4,5], thin-layer chromatography (TLC) [5], paper electrophoresis [5] and ion-exchange liquid chromatography [5] and also quantitatively by paper [5] and high-performance TLC (HPTLC)–densitometry [6] with post-chromatographic ninhydrin detection and by HPLC on a cyanide column with phenyl isothiocyanate pre-chromatographic derivatization [6].

In this work, stereoselective determination of N-trifluoroacetyl cucurbitine esters was developed using gas chromatography (GC) on a Chirasil-L-Val capillary column with a view to further studies on the preparative resolution of isomers and applications to biological media.

EXPERIMENTAL

Methanol, ethanol, 1- and 2-propanol, 1-, 2- and 3-butanol, isoamyl alcohol and methyl pentadecanoate were analytical-reagent grade reagents supplied by Merck (Darmstadt, Germany). Anhydrous ethyl acetate, acetyl chloride, chloroform and trifluoroacetic anhydride were provided by Aldrich (Milwaukee, WI, USA).

Racemic cucurbitine hydrobromide was synthesized according to Sun *et al.* [7] from 1,3-dicarbethoxy-4-pyrrolidone, obtained by condensation of ethyl acrylate and N-carbethoxyglycine ethyl ester

Correspondence to: E. Schenkel, Unité de Chimie Bioanalytique, de Toxicologie et de Chimie Physique Appliquée, Université Libre de Bruxelles, C.P. 205/1, Boulevard du Triomphe, 1050 Brussels, Belgium.

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[8]; this compound was prepared from ethyl chloroformate and glycine ethyl ester hydrochloride [9].

The identity of the synthesized cucurbitine hydrobromide was checked by melting point determination (decomposition at 286°C), carbon-13 NMR [6] and elemental analysis (calculated: H 5.25, C 28.45, N 13.27, O 15.16, Br 37.86; found: H 5.25, C 28.42, N 13.19, O 15.21, Br 37.92).

Cucurbita moschata seeds were harvested in Burkina Faso (Farako Bâ Agronomical Research Station), reduced to coarse powder and extracted as described previously [6]. The seed powder was defatted with light petroleum (b.p. 40–60°C) and extracted with boiling water; the aqueous extract was adsorbed on Amberlite IR-120 (H^+ form), desorbed with 1 *M* aqueous ammonia and evaporated to dryness under reduced pressure. Weights were corrected for residual water as determined on aliquots by drying in an oven at 105°C.

HPTLC–densitometry

Volumes of 0.5 μl of standard or sample solutions in methanol–water (1:1, v/v) were applied to precoated HPTLC plates of silica gel 60 F_{254} provided by Merck and developed with methanol–chloroform–25% aqueous ammonia–acetic acid (80:10:8.5:1.5, v/v) (saturated tank) to a distance of 90 mm. After drying at 105°C for 1 h and dipping in ninhydrin reagent [80 mg of ninhydrin in a solution of water–acetone–acetic acid (18:80:2, v/v), the plates were left for 10 min at ambient temperature and 15 min at 60°C. Spots were measured with a Shimadzu CS-930 high-speed TLC scanner (linear scan at 370 nm) [6].

Chromatographic analysis

GC analyses were performed either on a Perkin-Elmer Sigma 2000 insrument with flame ionization detection or on a Hewlett-Packard Model 5890 instrument with a Model 5970 quadrupole mass-selective detector interfaced to Model 59940 A Chem Station. The mass-selective detector was operated in the electron impact ionization mode with an ionization potential of 70 eV; the scanning range was *m/z* 40–800.

The chromatographic column was a 25 m × 0.25 mm I.D. WCOT fused-silica column coated with a 0.12-μm Chirasil-L-Val film (Chrompack, Bergen op Zoom, Netherlands). The carrier gas was nitrogen (purified on a high-capacity gas purifier coupled with an OMI-1 indicating purifier (Supelco, Bellefonte, PA, USA)) at 0.5 bar; the oven temperature ranged from 100 to 175°C and the injector and detector temperatures were 220°C. Injection was in split mode with a splitting ratio ranging from 40:1 to 20:1.

Derivatization

Derivatization was carried out in 100 × 14 mm I.D. glass tubes with PTFE-lined screw-caps (Sovirel, Bagneaux-sur-Loing, France). Heating was performed using a block heater (Pierce, Rockford, IL, USA) and an aluminium block in which 35-mm deep holes 16 mm in diameter were drilled. Evaporation was carried out in a 40°C vacuum vortex evaporator (Buchler, Fort Lee, N.J., USA). Samples containing 10–200 μl of amino acid solutions [10–50 μmol/ml in water or methanol–water (1:1, v/v] or *Cucurbita* extracts [extract from 150 mg of seeds dissolved in 1 ml methanol–water (1:1, v/v)] were evaporated, dissolved in 300 μl of the esterifying solution [acetyl chloride–alcohol (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 3-butanol or isoamyl alcohol) (1:2, v/v)], heated for 1 h at 110°C, evaporated to dryness, the residue dissolved in 400 μl of anhydrous chloroform, 200 μl of trifluoroacetic anhydride added, heated for 10 min at 110°C, evaporated to dryness and the residue dissolved in 200 μl of ethyl acetate [10–12] containing 50–100 nl of methyl pentadecanoate (internal standard).

RESULTS AND DISCUSSION

According to previous studies [13–15], N-TFA-isopropyl derivatives of amino acid enantiomers can be readily separated by GC on a Chirasil-L-Val capillary column. Application to cucurbitine of the described derivatization resulted in long retention times and poor enantiomeric resolution. These observations led to further investigations concerning the modification of the esterifying agent and the influence of the chromatographic conditions.

Influence of esterifying alcohol on resolution

In comparison with the 2-propyl derivatives, an increase in retention times with a better resolution was observed with 1-butyl derivatives. However, 2-

TABLE I

EXPERIMENTAL ISOTHERMAL t'_R VALUES OF THE DIFFERENT N-TFA ESTERS OF D-AND L-CUCURBITINE, t_M OF METHANE, NET CAPACITY FACTOR k' OF EACH ENANTIOMER, RESOLUTION FACTOR α AND PEAK RESOLUTION R

Chromatographic conditions: column, Chirasil-L-Val (25 m × 0.25 mm I.D.); injector and detector temperatures, 220°C; carrier gas, nitrogen at 0.5 bar; oven temperature, 145°C; 0.2 μg of racemic mixture injected; splitting ratio, 20:1. N-TFA-1-butyl D- and L-cucurbitine derivatives were not eluted at 125°C. No separation of the N-TFA-2-propyl D- and L-cucurbitine derivatives occurred at 155 and 165°C.

Compound	T(°C)	t_M (min)	$t'_{R(D)}$ (min)	$t'_{R(L)}$ (min)	k'_D	k'_L	α	R
Methyl ester	125	2.23	77.17	80.97	34.61	36.31	1.0491	1.38
	135	2.27	45.03	47.13	19.84	20.76	1.0464	1.49
	145	2.32	26.28	27.38	11.33	11.80	1.0415	1.24
	155	2.35	15.35	15.95	6.53	6.79	1.0398	1.05
	165	2.38	10.02	10.42	4.21	4.38	1.0404	0.89
Ethyl ester	125	2.23	84.57	87.67	37.92	39.31	1.0367	1.22
	135	2.27	51.23	52.93	22.57	23.32	1.0332	1.20
	145	2.32	27.68	28.68	11.93	12.36	1.0360	1.15
	155	2.35	17.15	17.65	7.30	7.51	1.0288	0.98
	165	2.38	10.92	11.22	4.59	4.71	1.0261	0.67
1-Propyl ester	125	2.23	132.67	136.37	59.49	61.15	1.0279	1.03
	135	2.27	75.13	77.13	33.10	33.98	1.0266	1.16
	145	2.32	42.28	43.38	18.22	18.70	1.0263	1.14
	155	2.35	25.05	25.75	10.66	10.96	1.0281	0.99
	165	2.38	15.52	15.82	6.52	6.65	1.0199	0.62
2-Propyl ester	125	2.23	82.47	83.85	36.98	37.60	1.0168	0.30
	135	2.27	48.53	49.23	21.38	21.69	1.0145	0.29
	145	2.32	27.58	27.88	11.89	12.02	1.0109	0.28
	155	2.35	16.85	16.85	7.17	7.17	1.000	
	165	2.38	10.72	10.72	4.50	4.50	1.000	
1-Butyl ester	125	2.23						
	135	2.27	113.63	115.83	50.06	51.03	1.0194	1.14
	145	2.32	60.88	61.98	26.24	26.72	1.0183	1.05
	155	2.35	36.35	37.05	15.47	15.77	1.0194	0.84
	165	2.38	22.72	23.02	9.55	9.67	1.0126	0.56

butyl, 3-butyl and isoamyl derivatives could not be eluted under experimental conditions. From *n*-propyl to methyl derivatives the retention times were found to decrease and the resolution to increase at all tested temperatures with a maximum resolution at the lower temperatures (Table I).

Thermodynamic analysis of retention data is an important source of information about the chiral discrimination process in the separation of enantiomers on chiral stationary phases [16,17]. The interpolation and extrapolation of the retention data such as the net retention time, t'_R (Table II) allow the different thermodynamic properties of each derivative (Table III) and the best chromatographic conditions to be established. The thermodynamic treatment of retention data could be done according to Koppenhoefer and co-workers [16,17] using nomograms [ln k' or ln t'_R (min) *versus* $1/T$ (K^{-1}) and ln α *versus* $1/T$]. We used an original algorithm (FADHA) which permitted the fitting of $1/T$–t'_R or $1/T$–α curves by optimizing a non-linear cost function by the simplex method and allowed the best fit from our experimental data to be assessed statistically [18]; the equation used was $y = e^{a+bx}$, where y is t'_R, x is $1/T$ and b is $(-\Delta H + C_1)/R$, with $C_1 = -0.50$ kcal mol^{-1} for 0.5 bar N_2,

TABLE II

CALCULATED ISOTHERMAL t'_R and α VALUES FOR METHYL, ETHYL AND 1-PROPYL D- AND L-CUCURBITINE DERIVATIVES

Chromatographic conditions as in Table I.

T (°C)	Methyl ester			Ethyl ester			1-Propyl ester		
	$t'_{R(D)}$ (min)	$t'_{R(L)}$ (min)	α	$t'_{R(D)}$ (min)	$t'_{R(L)}$ (min)	α	$t'_{R(D)}$ (min)	$t'_{R(L)}$ (min)	α
50	16 075	17 374	1.0786	18 450	19 230	1.0631	39 339	41 311	1.0548
	± 119	± 125	± 1.0123	± 150	± 170	± 0.0104	± 357	± 378	± 0.0104
	0.7%	0.7%	1.1%	0.8%	0.9%	1.0%	0.9%	0.9%	1.0%
75	2119	2266	1.0675	2403	2539	1.0528	4571	4763	1.0457
	± 15	± 16	± 0.0114	± 20	± 21	± 0.0101	± 42	± 43	± 0.0099
	0.7%	0.7%	1.1%	0.8%	0.8%	1.0%	0.9%	0.9%	0.9%
100	366.6	388.2	1.0572	411.3	430.1	1.0440	708.6	733.7	1.0378
	± 2.8	± 2.8	± 0.0113	± 3.4	± 3.5	± 0.0095	± 6.5	± 6.7	± 0.0095
	0.8%	0.7%	1.1%	0.8%	0.8%	0.9%	0.9%	0.9%	0.9%
145	26.45	27.60	1.0426	29.20	30.07	1.0308	43.37	44.48	1.0261
	± 0.20	± 0.20	± 0.0106	± 0.24	± .25	± 0.0088	± 0.39	± 0.41	± 0.0088
	0.8%	0.7%	1.0%	0.8%	0.8%	0.9%	0.9%	0.9%	0.9%
190	3.180	3.281	1.0310	3.466	3.526	1.0204	4.569	4.649	1.0168
	± 0.024	± 0.024	± 0.0101	± 0.029	± 0.029	± 0.0083	± 0.041	± 0.042	± 0.0083
	0.8%	0.7%	1.0%	0.8%	0.8%	0.8%	0.9%	0.9%	0.8%

or where y is α, x is $1/T$, b is $-\Delta\Delta H/R$ and a is $\Delta\Delta S/R$.

The three enantiomeric pairs show similar thermodynamic properties (Table III); the $\Delta\Delta S$ values are very similar but the $-\Delta\Delta H$ values are different, with methyl > ethyl > 1-propyl. This is represented when plotting ln α *versus* $1/T$ as three straight lines, which do not intersect. Therefore, whatever the temperature, the methyl ester derivatives will always show a greater resolution than the other derivatives.

Structure of cucurbitine derivatives

Owing to the two amino functions of cucurbitine, and depending on reagent concentration, the possibility arises in the course of N-trifluoroacetylation of the formation of two different monoacyl derivatives, a diacyl derivative or even a mixture of these compounds [19]. However, with the various acetylation reagents tested (33.3%, 50%, 66.6% and 83.3% TFAA in anhydrous chloroform), no differences were observed in the retention times and peak shapes of the end product derivatives and no further peak occurred.

The GC–MS analysis, initially undertaken to confirm peak identity in chromatograms of plant extracts, allowed the exact structure of the derivatives formed to be specified. A series of proteic racemic amino acids (D,L-alanine, D,L-proline, L-leucine, D,L-aspartic acid, D,L-methionine, D,L-glutamic acid, L-arginine and D,L-tryptophan) was similarly derivatized (N-TFA acylation and methyl, ethyl or 1-propyl esterification) and analysed (Fig. 1). Molecular ions were generally not detected but individual mass spectra of the different N-TFA esters allowed similar fragmentation patterns to be deduced (Fig. 2) irrespective of the esterifying alcohol.

The mass spectra of the N-TFA esters of cucurbitine (Table IV) suggest strong evidence for the formation of a diacyl (di-TFA) compound under the conditions of the derivatization reaction; the observed ions cannot be satisfactorily explained by a monoacyl cucurbitine derivative even when rearrangement reactions are considered.

TABLE III

THERMODYNAMIC PROPERTIES OF METHYL, ETHYL AND 1-PROPYL D- AND L-CUCURBITINE DERIVATIVES

Chromatographic conditions as in Table I.

Property		Methyl ester			Ethyl ester			1-Propyl ester		
		Specified units		SI units[a]	Specified units		SI units[a]	Specified units		SI units[a]
$-\Delta H_{(D)}$	(kcal mol^{-1})	17.60 ±0.01	0.06%[b]	73.57 ±0.04	17.71 ±0.01	0.06%[b]	74.03 ±0.04	18.73 ±0.01	0.05%[b]	78.29 ±0.04
$-\Delta H_{(L)}$	(kcal mol^{-1})	17.70 ±0.01	0.06%	73.99 ±0.04	17.82 ±0.01	0.06%	74.49 ±0.04	18.80 ±0.01	0.05%	78.58 ±0.04
$-\Delta\Delta H$	(cal mol^{-1})	95.9 ±3.6	3.8%	400.9 ±15.0	87.1 ±3.6	4.1%	364.1 ±15.0	77.7 ±3.6	4.6%	324.8 ±15.0
$\Delta\Delta S$	(cal K^{-1} mol^{-1})	−0.147 ±0.012	8.0%	−0.614 ±0.050	−0.148 ±0.009	6.1%	−0.619 ±0.038	−0.135 ±0.009	6.7%	−0.564 ±0.038
$-\Delta\Delta G^{323}$	(cal mol^{-1})	48.4 ±0.6	1.2%	202.3 ±2.5	39.3 ±0.7	1.8%	164.3 ±2.9	34.1 ±1.4	4.4%	142.5 ±5.9
$-\Delta\Delta G^{373}$	(cal mol^{-1})	41.1 ±1.8	4.4%	171.8 ±7.5	31.9 ±0.3	0.9%	133.3 ±1.3	27.3 ±0.5	1.8%	114.1 ±2.1
$-\Delta\Delta G^{418}$	(cal mol^{-1})	34.4 ±2.8	8%	144.2 ±11.7	25.2 ±0.2	0.8%	105.3 ±0.8	21.3 ±0.3	1.4%	89.0 ±1.3
T_s	(°C)		382 ±29 8.0%			316 ±12 3.8%			303 ±12 4.0%	

[a] SI units: kJ mol^{-1} or J mol^{-1}.
[b] Relative error.

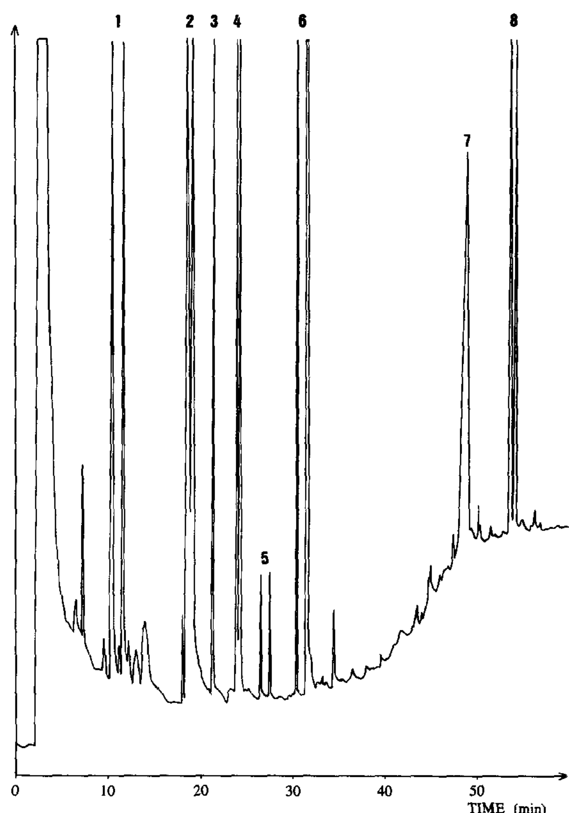

Fig. 1. GC separation of the enantiomers of several amino acids. Chromatographic conditions: column, Chirasil-L-Val (25 m × 0.25 mm I.D.); injector and detector temperatures, 220°C; carrier gas, nitrogen at 0.5 bar; temperature programme, 60°C for 3 min, then increased at 3°C/min to 190°C, held for 3 min. Peaks: 1 = D,L-alanine; 2 = D,L-proline; 3 = L-leucine; 4 = D,L-aspartic acid; 5 = D,L-methionine; 6 = D,L-glutamic acid; 7 = L-arginine; 8 = D,L-tryptophan. In all instances the D-enantiomer was eluted prior to the L-enantiomer.

For all the investigated esters of proteic amino acids, the principal ion (100% relative abundance) was the fragmentation product resulting from the loss of the –COOR radical (R = methyl, ethyl or propyl) even in the case of diacids (glutamic and aspartic acid). However, when applied to cucurbitine derivatives, this rule was found to be invalid; in this instance, the principal ion (100% relative abundance) resulted from the loss of one $-NHCOCF_3$ radical. This can probably be explained by steric factors.

Fig. 3 shows the corresponding mass spectra of N-TFA-methyl, -ethyl and -1-propyl esters of L-cucurbitine derivatives and the possible fragmentation patterns with principal ions.

For every amino acid investigated, including cucurbitine, the mass spectra were similar for both enantiomers.

Quantitative limits of the method

The N-TFA-methyl ester was the derivative used for quantitative work; isocratic elution at 145°C allowed perfect resolution of the two cucurbitine isomer peaks.

It is widely accepted that on Chirasil-L-Val, the elution order of amino acid enantiomers is correlated with stereochemistry; in chromatograms of *Cucurbita* seeds, a single cucurbitine isomer, which was assigned the L stereochemistry from its retention time (Fig. 4), could be detected. This is in accordance with previous stereochemical studies [1]. Assays also permitted the absence of racemization during the derivatization process to be confirmed, as can be seen by the exceedingly high enantiomeric purity of L-cucurbitine from *Cucurbita* seeds (Fig. 4) [20]. Quantitative work was developed with the flame ionization detector; methyl pentadecanoate was used as the internal standard and the splitting ratio was fixed at 20:1.

The method was found to give a linear response for each isomer between 2 and 40 ng; the detection limit was *ca.* 1 ng [the equation of the regression line was $y = 0.00262x - 0.01234$, where y is the peak area (cm^2) and x is the amount of L-cucurbitine injected (ng); $r = 0.9860$; $n = 21$].

The assay was applied to different samples of *Cucurtine* spp. seeds cultivated in Burkina Faso and compared with HPTLC determination. GC appeared to give good accuracy when compared with HPTLC, but with a tenfold better precision (Table V) and also a 10–100-fold greater sensitivity.

Recovery was calculated by the standard addition method, which requires the determination of a calibration line for a particular sample. Results obtained with the standard addition method were compared with those obtained by using direct determination [the equation of the regression line for the standard addition method was $y = 0.00268x + 0.3338$, where y is the peak area (cm^2) and x the amount of L-cucurbitine injected (ng); $r = 0.9985$; $n = 22$]. The mean recovery was 106.8% ($n = 3$).

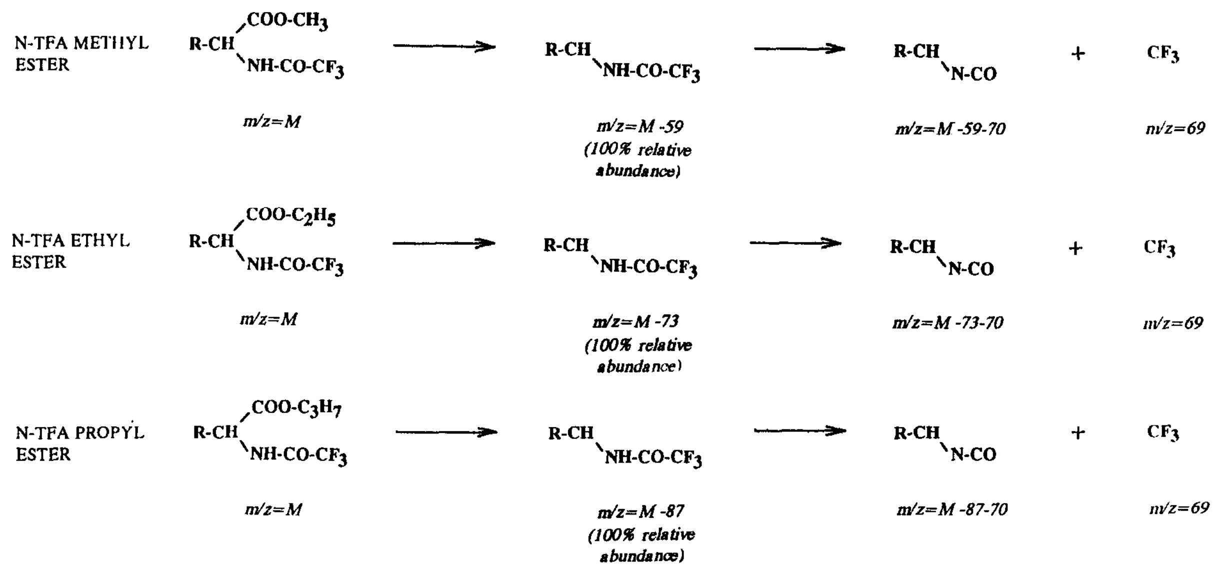

Fig. 2. Deduced fragmentation patterns of N-TFA-methyl, -ethyl and -1-propyl esters of proteic racemic amino acids. Chromatographic conditions as in Fig. 1. Quadrupole mass-selective detector; electron impact ionization potential, 70 eV; scanning from m/z 40 to 800.

TABLE IV

PRINCIPAL IONS OF THE MASS SPECTRA OF THE DIFFERENT N-TFA ESTERS OF SYNTHETIC D- AND L-CUCURBITINE

m/z of observed fragments[a]											
N-TFA-methyl ester (molecular mass of di-N-TFA-methyl ester: 336)				N-TFA-ethyl ester (molecular mass of di-N--TFA-ethyl ester: 350)				N-TFA-1-propyl ester (molecular mass of di-N--TFA-1-propyl ester: 364)			
D-Isomer		L-Isomer		D-Isomer		L-Isomer		D-Isomer		L-Isomer	
267	(12)	267	(8)	281	(9)	281	(7)				
277	(23)	277	(22)	277	(23)	277	(19)	277	(20)	277	(27)
		224	(11)	238	(9)	238	(9)			252	(9)
223	(100)	223	(100)	237	(100)	237	(100)	251	(58)	251	(73)
				209	(27)	209	(17)	209	(100)	209	(100)
				208	(32)	208	(30)	208	(10)	208	(17)
207	(9)	207	(6)	207	(14)	207	(9)	207	(12)	207	(7)
193	(7)	193	(6)	193	(9)	193	(4)	193	(10)		
192	(37)	192	(20)	192	(53)	192	(32)	192	(27)	192	(37)
165	(9)	165	(10)	165	(24)	165	(15)	165	(10)	165	(21)
164	(48)	164	(28)	164	(41)	164	(36)	164	(40)	164	(44)
163	(30)	163	(30)	163	(14)	163	(16)	163	(14)	163	(16)
				112	(10)	112	(12)	112	(25)	112	(23)
96	(13)	96	(4)	96	(16)	96	(8)	96	(13)	96	(18)
94	(13)	94	(10)	94	(19)	94	(15)	94	(17)	94	(14)
83	(15)	83	(7)	83	(14)	83	(10)	83	(8)	83	(9)
69	(65)	69	(44)	69	(50)	69	(41)	69	(29)	69	(36)
						68	(11)	68	(14)	68	(10)
67	(13)	67	(7)	67	(12)	67	(8)	67	(13)	67	(14)

[a] Relative abundances (%) in parentheses.

CONCLUSIONS

GC–MS analysis confirmed the formation of a di-N-TFA cucurbitine derivative. The thermodynamic analysis results show that the methyl ester derivative is preferred because of its greater resolution and its shorter retention times. Chirasil-L-Val is a valuable stationary phase for the separation of optical isomers of cucurbitine. The method allows the identification and determination (nanograms level) of optical isomers of cucurbitine and the efficient resolution of L-cucurbitine in *Cucurbita* seed extracts, and has better precision and sensitivity than HPTLC.

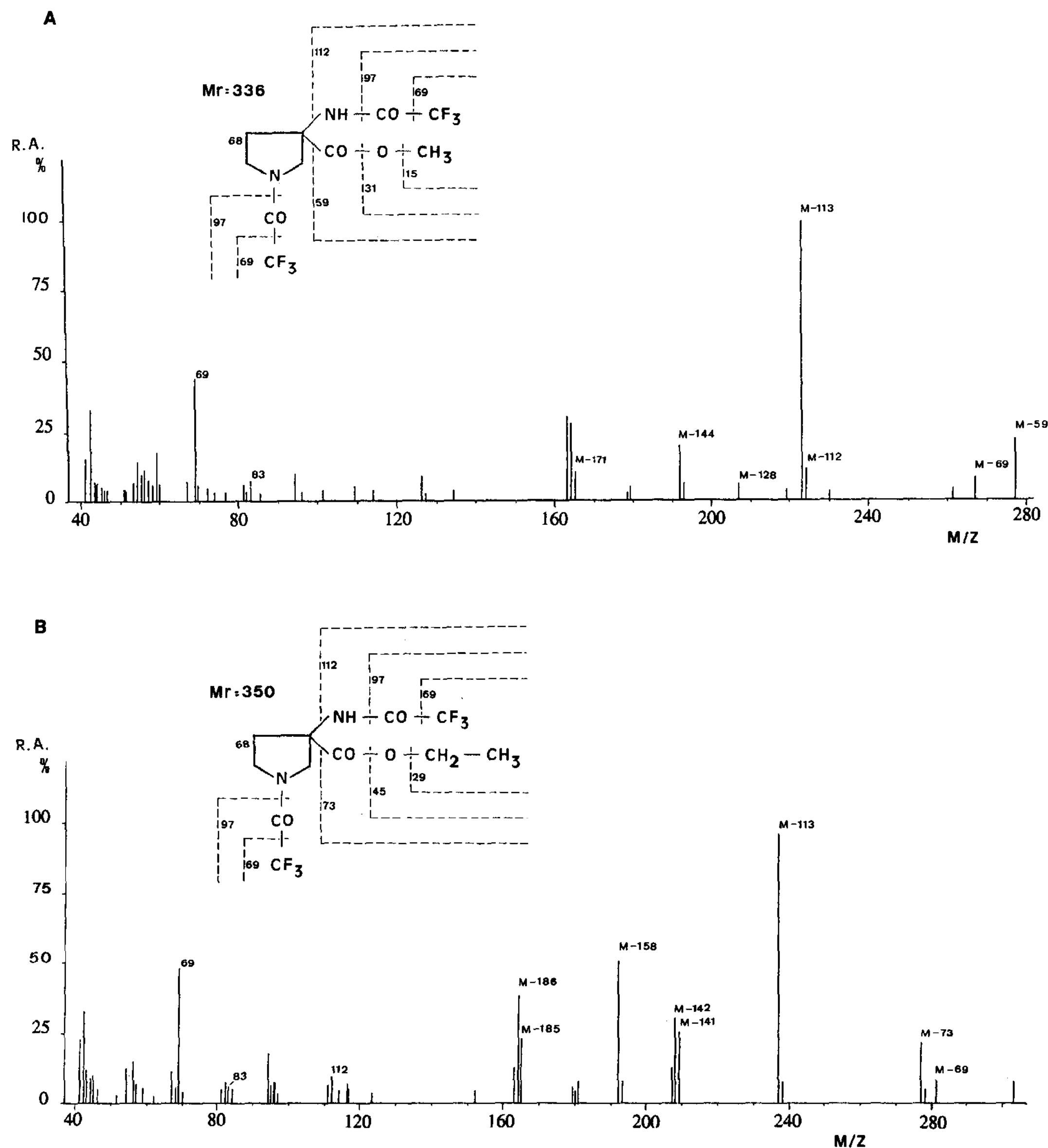

Fig. 3.

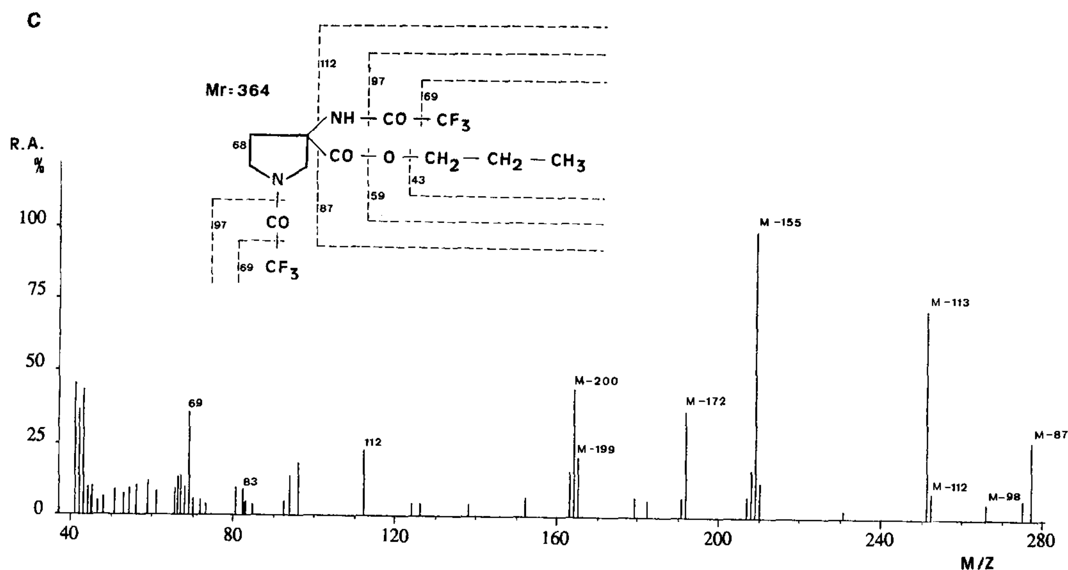

Fig. 3. Mass spectra of N-TFA (A) methyl, (B) ethyl and (C) 1-propyl esters of L-cucurbitine with possible fragmentation patterns explaining principal ions. Mass spectra were similar for both enantiomers. R.A. = Relative abundance.

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TABLE V

GC AND HPTLC DETERMINATION OF DIFFERENT *CUCURBITA PEPO* AND *MOSCHATA* SEED EXTRACTS

Cucurbita seed studied		L-Cucurbitine in defatted seed powder (%)			
Species	Line	GC (n = 5)		HPTLC (n = 11)	
		Mean	S.D.	Mean	S.D.
pepo	L 34	0.201	0.007	0.15	0.04[a]
pepo	L 5	0.441	0.004	0.43	0.03
pepo	L 149	0.776	0.004	0.76	0.01[b]
moschata	B 7 6b	0.292	0.006	0.27	0.04
moschata	E 3	0.462	0.007	0.48	0.05
moschata	B 7 3b	0.457	0.008	0.48	0.05

[a] Cochran statistical test allowed a statistical significant difference to be concluded only between the two means of *Cucurbita pepo* L34.
[b] n = 3.

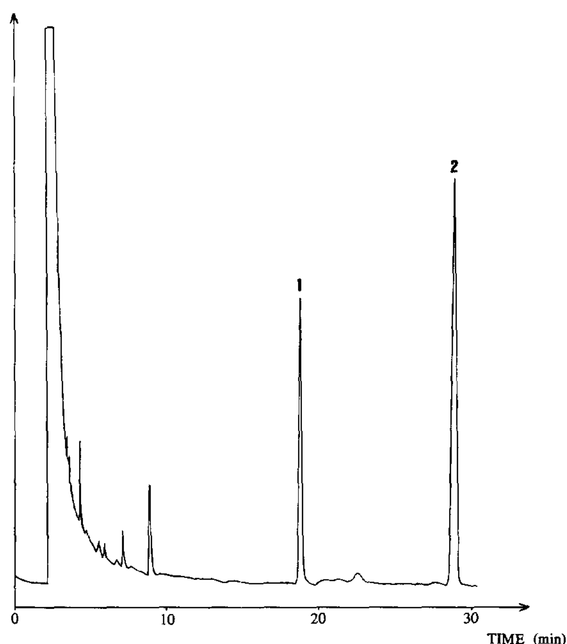

Fig. 4. Chromatogram of *Cucurbita* seed extract. Chromatographic conditions as in Fig. 1. Oven temperature, 145°C. Peaks: 1 = methyl pentadecanoate; 2 = L-cucurbitine.

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