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Amino Acid Determination in Conophor Nut by Gas–Liquid Chromatography

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ABSTRACT

The amino acids composition of conophor nut was determined by GLC and ion-exchange chromatography.

Conophor nut contains the essential amino acids in adequate amounts for nutrition except methionine. Two unusual amino acids, α-aminobutyric acid and γ-aminobutyric acid, are reported for this seed.

INTRODUCTION

In order to produce a simple, rapid and less costly analysis of amino acids, several workers, including Kaiser *et al.* (1964) Zanetta & Vicendon (1973), Pearce (1977), Mackenzie & Hogge (1974) and Mackenzie & Tenasckuk (1974) have developed methods of making amino acid derivatives and separating them on suitable GLC columns. Heptafluorobutyryl isobutyl esters were separated on a single column packed with SE 30 on Chromosorb Q (Mackenzie & Hogge, 1974; Mackintosh *et al.*, 1977). The identities of the derivatives of the amino acids separated on GLC columns have been established (by mass spectroscopy) by the latter workers. The mass spectra of the heptafluorobutyryl isoamyl esters were reported by (Felker & Bandurski, 1975). In recent years amino acids have been determined by precolumn formation of the *o*-phthaldehyde (OPA) derivatives, followed by reversed phase HPLC separation and fluorimetric detection. The gas chromatograph compares favourably with the HPLC method in rapidity.

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Both separations take between 30 min and 60 min. The sensitivity of the HPLC, however, is greater than that of GLC.

The present work was carried out in order to use GLC to determine the amino acid composition of *Tetracarpidium* conophorum (conophor nut), an oilseed cultivated in Nigeria, and to compare the results with those obtained by ion-exchange chromatography. GC–MS analyses of the amino acid derivatives were carried out to confirm the eluting order of the derivatives and to rule out the presence of artifact during derivative formation. Earlier work by Ogunsua & Adebona (1983) has shown that defatted conophor nut is high in protein content (38%).

MATERIALS AND METHODS

Preparation of reagents

Isobutanol-HCl was prepared by adding 2 ml of acetyl chloride to 85 ml dry isobutanol to yield 3N HCl in isobutanol. All the solvents were dried over calcium chloride.

Lithium buffer solution

This was obtained from BDH Chemicals Ltd. It has the following composition:

Lithium citrate buffer solution pH 2·2

Lithium	0·3M
Citrate	0·09M
Contains 2-methoxy ethanol	1·25% w/v
Polyoxyethylene lauryl ether	0·05% w/w
Sodium octanoate	0·01% w/v

Derivative formation

This is a modification of the method of Mackenzie & Tenasckuk (1975). Aliquots of solution of each standard amino acid were freeze-dried in 1 ml Pierce reactivials (Pierce, Rockford, III, USA). To each was added 100 μl isobutanol–3N HCl. After sonication for 1 min, the mixture was esterified in an oven at 110°C for 30 min. The tubes were cooled to room temperature and the contents were evaporated in a stream of dry nitrogen. 20 μl dry isobutanol was added, and redried. Acylation was carried out by adding 50 μl ethyl acetate/BHT and 20 μl HFBA (heptafluorobutyric anhydride) and heating in an oven at 110°C for 10 min. The samples were cooled, evaporated almost to dryness in a stream of nitrogen and then heated with 30 μl ethyl

acetate and 5 μl EFA (ethoxy formic anhydride) for 5 min at 110°C. The cooled sample was evaporated again and redissolved in 30 μl ethyl acetate.

Gas chromatography

Gas chromatography was carried out in an Erba model gas chromatograph equipped with a hydrogen ionization detector. The conditions of chromatography were as follows:

Nitrogen flow rate	20 ml/min
Injector temperature	275°C
Detector temperature	275°C
Temperature programming: temperature rise 3·13°C/min	95°C isothermal 6 min: 95–240°C
Stationary phase	3% SE 30 on Gas Chrom Q

Retention times, peak areas and relative areas of the curves compared with area of norleucine (internal standard) were obtained by an Infotronics integrator.

Hydrolysis of samples

For the GLC, the ground sample was defatted with petroleum ether. The dried sample was then subjected to hydrolysis and ion-exchange clean-up as described by Kaiser *et al.* (1964). For ion exchange analysis the hydrolysed sample, after the removal of HCl, was dissolved in lithium citrate buffer 0·2N Li pH 2·20 (BDH). It was filtered on a membrane filter (0·2–0·5 μm) prior to analysis. The average concentration of each amino acid was maintained at between 20 and 30 μ moles per 100 μl. The ion-exchange analysis was performed on a Biotronick amino acid analyser, model LC 2000.

GC–Mass spectrometry of the derivatives

The mass spectrometry of the amino acid derivatives was done in a Pye 204 GC/Finnigan mass spectrometer. OV 101 was used as a stationary phase in the packed column. The transfer line was maintained at 250°C. The temperature was programmed from 100 to 240°C with a temperature rise of 8°C/min.

Determination of tryptophan

Tryptophan was determined spectrophotometrically by the method of Concon (1975). The measurement was carried out at 570 nm using an authentic tryptophan standard (Sigma Co) to prepare the standard curve.

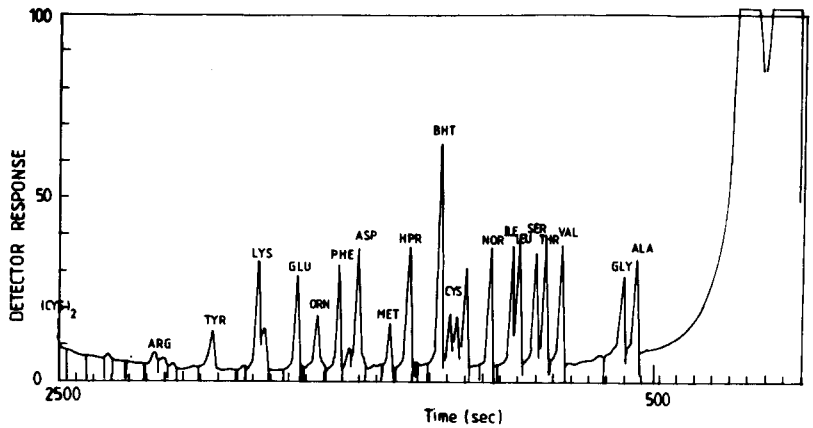

Fig. 1. Gas chromatography of N(O) HFB isobutyl amino acids on SE 30 column.

RESULTS AND DISCUSSION

GLC of standard amino acids

Figure 1 shows the chromatography of the standard amino acid derivatives Although different columns and programmes were used for GC and GC–MS, the orders of elution of the derivatives are the same.

Each amino acid was distinctly separated. The relative molar response (RMR) values are presented in Table 1. Quantitation of histidine, cystine, cysteine and tryptophan cannot be reliably measured by the method. Similar

TABLE 1
Relative Molar Responses (RMR) of Standard Amino Acids[a]

Amino acid	*RMR*	*% SD*	*Amino acid*	*RMR*	*% SD*
Ala	0·624	1·0	Met	0·84	3·5
Gly	0·340	1·2	Asp	1·091	1·9
Val	0·828	13·7	Phen	1·309	1·3
Thr	0·930	6·5	Orn	0·823	2·8
Ser	0·779	4·2	Glu	1·144	4·9
Leu	1·123	4·3	Lys	0·794	2·2
Ile	0·844	6·1	Tyr	1·497	1·1
Norl	—		Arg	0·864	17·8
Prol	0·870	1·0	His	0·410	22·7
$(CYS)_1$	0·473	6·2	$(CYS)_2$	0·983	7·1
Hypro	1·026	2·1	Tryp	0·512	12·3

[a] Six replicates were analysed.

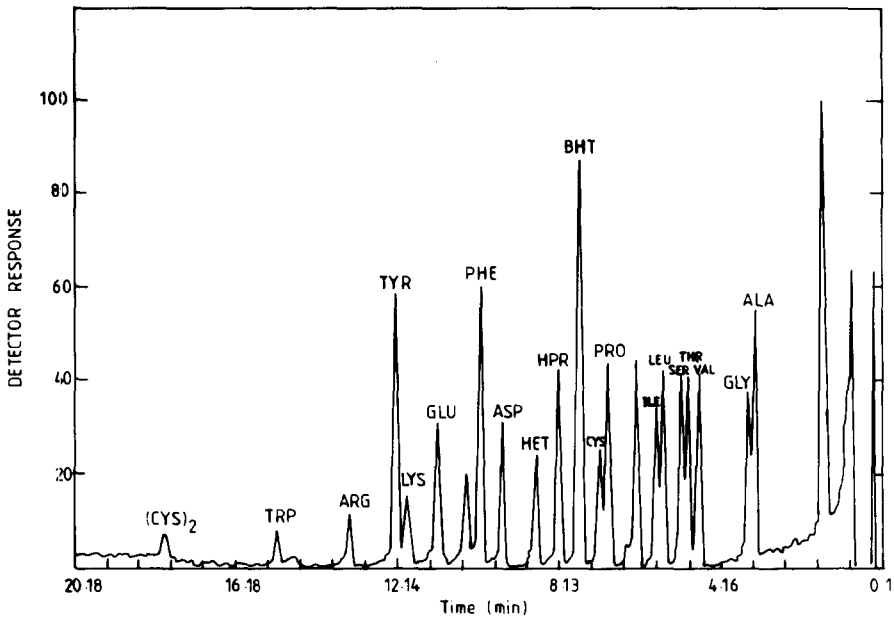

Fig. 2. Gas chromatography of N(O) HFB isobutylamino acids on OV-101 column in GC–MS apparatus.

observations were made by Felker & Bandurski (1975) in their work with isoamyl-heptafluoroisobutyl amino derivatives.

There was a complete resolution of all the amino acids on the column (Fig. 2). The mass spectra data are presented in Tables 2–8. The fragmentation patterns agree with those published by Mackenzie *et al.* (1974; 1977). However, in several cases the base ions are different as well as the relative amounts of the ions. The fragmentation patterns are consistent with the earlier published studies. The amino derivatives are produced without the formation of artefacts. Only in three cases were the molecular ions obtained for these derivatives—methionine, phenylalanine and proline. Mackenzie & Hogge (1977) also observed a molecular ion for proline whereas Felker & Bandurski (1975) obtained a molecular ion for phenylalanine, methionine and proline as well as a few other amino acids possibly due to slightly lower molecular weights of the derivatives. The identity of the amino acid derivative of the sample was established by comparison with the mass spectra of authentic amino acids.

Relative amounts of amino acids in T. conophorum

The relative amounts, in millimoles, of amino acids are shown in Table 2. There was a general agreement between the GLC and the ion-exchange

TABLE 2
Relative Amounts of Amino Acids in Conophor Nut Flour (mmoles)

Amino acid	*By ion-exchange*	*By GLC*
Ala	9·7	10·4
Gly	27·9	27·9
Val	11·5	12·6
Thr	11·2	11·2
Ser	13·7	14·0
Leu	13·1	11·0
Ile	9·0	10·1
Pro	10·4	9·6
$(CYS)_1$		6·9[a]
Hypro		2·1
Met	1·8	2·8[a]
Asp	21·8	23·3
Phen	4·6	4·4
Glu	17·9	14·3
Lys	7·6	3·6[a]
Tyr	6·4	7·5
Arg	14·0	11·1
His	2·9[a]	—
Tryp	—	—
$(CYS)_2$	2·4[a]	—

[a] Average of triplicate analyses.

analysis. In the GLC, a lower result was obtained for lysine than by ion-exchange. Higher values were obtained for methionine by GLC. The reason for a lower value for cystine might be the apparent instability of the derivative of this amino acid as detected by GLC (Felker & Bandurski, 1975). Analysis by ion-exchange gave a value for cystine (2·8 mmoles) but no value for cysteine. On the other hand, GLC gave a value for cysteine (6·9 mmoles) but no value for cystine. Oxidation conditions in both methods of analysis may be partly responsible for the difference although BHT, an antioxidant, was employed in the GLC analysis. Two minor unusual amino acids, α-aminobutyric acid (α-ABA) and γ-aminobutyric acid (γ-ABA), were determined by ion-exchange. These were present at 0·6 and 0·5 mmoles, respectively. Two unidentified minor peaks were present in the GLC. These may be α-ABA and γ-ABA.

The essential amino acid composition of the flour (ion-exchange values) is shown in Table 3. Values for FAO/WHO reference patterns for essential

TABLE 3
Essential Amino Acid Analysis of Conophor Flour (mg/gN)

Amino acid	*FAO/WHO reference*	*Conophor nut flour*
Try	90	184
Phe	180	194
Met	140	68+
Lys	270	354
Thr	180	343
Leu	300	441
Isoleu	270	304
Val	270	345

TABLE 4
Aliphatic Amino Acid 70 eV Fragmentation Patterns

	Glycine			*Alanine*	
	m/e	%		*m/e*	%
Molecular ion	327	—	Molecular ion	341	—
Base ion	57 (C_4H_9)	100	Base ion	240	100
M-$OCOC_4H_9$	226	73·0	M-C_4H_7	286	73
M-C_4H_7	272	7·9	M-($C_3F_7CO + OC_4H_8$)	72	9·5
			C_4H_7	57	349
M-($C_5H_9O_2 + C_4H_8$)	170	17·5			
CF_3	69	14·3			

	Valine		*Leucine*		*Norleucine*	
	m/e	%	*m/e*	%	*m/e*	%
Molecular ion	369	—	383	—	383	—
Base ion	268 (M-$OCOC_4H_9$)	100	69 (CF_3)	100	282	100
M-C_4H_7	314	3·2	327	7·1	327	3·2
M-$OCOC_4H_9$			282	6·2		
M-$C_5H_8O_2 + C_3H_7$			240	69·0		
M-$C_5H_9O_2 + C_4H_8$	212	9·5			226	25·4
M-C_3F_7					214	14·3
C_4H_7 (55)		36·5				
C_4H_9 (57)		42·9		83·3		

TABLE 5
Dicarboxylic and Basic Amino Acid 70 eV Fragmentation Patterns

Ion	*Asp*		*Glu*		*Lys*		*Arg*	
	m/e	%	*m/e*	%	*m/e*	%	*m/e*	%
Molecular ion	441	—	455	—	594	—	818	—
Base ion	57	100	57	100	288	100	57	100
M-C_4H_7	386	0·8						
M-OC_4H_9	340	18·1	354	7·9	520	4·7		
M-($OC_4H_9 + C_4H_8$)	312	13·4	326	14·2	494	11·0		
M-($OCOC_4H_9 + C_4H_8$)	284	26·8	298	44·9			492	20·5
M-($OCOC_4H_9 + OC_4H_{10}$)			280	26·0				
M-($OCOC_4H_9 + OCOC_4H_9$)	239	15·7						
M-($OCOC_4H_9 + OCOC_4H_{10}$)			250	33·1				
$C_3F_7CONHCNNCH_2$			81	22			266	61·4
CF_3					69	15·7		
C_4H_7					57	29·9		
$C_3F_7CONHCNHNCOC_3F_7CCH_2'CH_2$							492	

TABLE 6

70 eV Fragmentation Pattern of *N-O*-HFB Isobutylester of Serine, Threonine, Hydroxyproline and Proline

Ion	*Serine*		*Threonine*		*Hydroxyproline*		*Proline*	
	m/e	%	*m/e*	%	*m/e*	%	*m/e*	%
Molecular ion	553	—	567	—	579	—	367	4·7
Base ion	57	100	56	100	264	100	266	100
M-C_4H_7	512							
M-OC_4H_9	484	3·0			504	1·5		
M-($OCOC_4H_9$)	452	3·0	468	3·5	478	79		
M-($COC_3F_7 + OC_4H_8$)	284	4·0	280	5·5				
M-($COC_3F_7 + OCOC_4H_9$)	239	48·8	253	58·3				
M-$OCOC_3F_7 + OCOC_4H_9 + H$			252	36·2				
CF_3 (69)	69	17·5	69	14·2	69	134		
CF_3CF_2	119	4·5	119	15·7				
$CF_3CF_2CF_2$	169	16·5	169	5·5	169	5·5	169	7·7
C_4H_9 (57)					57	33·1	57	26·8

TABLE 7
Aromatic Amino Acid 70 ev Fragmentation Patterns

Ion	*Phe*		*Tryp*		*Tyr*	
	m/e	%	*m/e*	%	*m/e*	%
Molecular ion	147	0·7	52	—	629	—
Base ion	148	100	326	100	360 ($M-OCOC_3F_7 + OC_4H_8$)	100
$M-OC_4H_9$	316	17·3	551	3·9	528	11·8
$M-OCOC_3F_7$	304	22·0	439	18·4	416	15·0
$M-(OCOC_3F_7 + OC_4H_8)$	148	100	383	142		
C_4H_9	57	19·7	57	7·9	57	47·2
$C_6H_5CH_2$	91	91·4				
$C_6H_4C_2NHCH_2$			129	15·0		
$C_6H_5CHCHCO$	131	18·9				
$M-(CH_3)_2CHCH_2O_2C-C_3F_7CONH_2$					343	15·7
$M-(CH_3)_2CH_2CHO + C_3F_7CONH_2$					303	64·6

TABLE 8
70 eV Fragmentation Pattern of S-Containing Amino Acids

Ion	*Amino acid*					
	Methionine		*Cystine*		*Cysteine*	
	m/e	%	*m/e*	%	*m/e*	%
Molecular ion	401	8·6	744	—	569	—
Base ion (C_4H_9)	57	100	57	100	57	100
$M-OC_4H_9$	327	21·3				
$M-(C_4H_7 + CH_3SCH_2CH_2)$	271	29·7			468	17·3
$M-(OCOC_4H_9 + SCH_3)$	253	33·1				
$C_3F_7CONHCHCHCH_2$			265	20·5	266	20·5
$C_3F_7CONHCHCH_2$					159	12·6
$OC.SCH_2CH(CNHCO)COH$			238	14·2		
$S-CH_2-CH_2CO_2$			103	18·9	103	12·6
C_4H_9	75	29·1				
$CF_3CF_2CF_2$					169	11·02
$C_3F_7CONHCHCH_2$					240	29·9
CH_3SCH_2	61					

amino acids are given for comparison. All the essential amino acids except methionine (68 mg/gN vs 140 mg/gN) are adequate for human nutrition.

CONCLUSIONS

This study shows that GLC is a reliable method for amino acid analysis comparable to analysis by conventional ion-exchange. The identity of each amino acid derivative was established by GLC–mass spectrometry. For biological materials a more detailed study should be made of the method of ion-exchange clean-up as unwanted precipitates sometimes occur which may interfere with the analysis. Conophor nut contains all the essential amino acids in adequate levels for nutrition except methionine.

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