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Series-coupled capillary columns for the separation of N,(0)-trifluoroacetyl isopropyl derivatives of D,L-aspartic acid and L-hydroxyproline by gas chromatography

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ABSTRACT

N-Trifluoroacetyl (TFAc)-D,L-aspartic acid (Asp) isopropyl ester and N,O-TFAc-L-hydroxyproline (Hpr) isopropyl ester, which give overlapping peaks when a single chiral capillary column is used, were separated with a cross-linked polycyanoethyl vinyl siloxane–L-Val–*tert.*-butylamide capillary column coupled in series with either a cross-linked polyethylene glycol 20M capillary column or a wall-coated OV-101 capillary column. A method for calculating the appropriate lengths of the coupled columns was developed. D,L-Asp and L-Hpr in real samples were also separated using the series-coupled column systems.

INTRODUCTION

Most amino acids in nature are of L configuration. However, in tissues such as bone [1,2], tooth [3], eye lens [4] and brain [5], all of them have a slow turnover rate. Therefore, o-amino acids tend to accumulate and increase in concert with ageing [6]. The racemization in living cells, which do not exhibit renewal processes, is of importance in the study of ageing phenomena. The racemization of amino acids in dead, fossilized materials can be employed as a dating parameter [7]. Among the amino acids, aspartic acid (Asp) is the most frequently used for age estimation [8]. Unfortunately, the N-trifluoroacetyl (TFAc) isopropyl ester of Asp is seriously overlapped by the N,O-TFAc isopropyl ester of L-hydroxyproline (Hpr) , a common component in living tissues and fossilized materials, when a cross-linked polycyanoethyl vinyl siloxane–L-Val–*tert.*-butylamide capillary column is used.

Several methods, such as stationary phase tuning, mixed-phase columns and series-coupled columns, for selectivity tuning have been reported [9]. The series-coupled column systems are the most important because they are convenient and can present a general applicable technique for optimizing selectivities when capillary columns are to be used [10]. Selectivity can be obtained with the coupled columns by using different lengths or phase ratios of the columns, by adjusting the flow-rate through each column or by operating each column at different temperatures [11]. This technique has been studied and applied by several groups [9–17]. However, the use of this approach has not been fully investigated in the separation of enantiomers.

In this work, two different capillary columns,

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polyethylene glycol (PEG) 20M and OV-101, were series-coupled to a cross-linked **poly**-cyanoethyl vinyl **siloxane–L-Val–*tert.*-butylamide** capillary. The lengths of the coupled columns needed for the separation of the derivatives of **D,L-Asp** and **L-Hpr** were calculated and optimized.

THEORY

There is general agreement among several workers that the effective capacity factor, $k'(\mathrm{eff})$, for a system of series-coupled columns can be calculated by

$$k'(\mathrm{eff}) = \sum_{i=1}^{n} \phi(i)k'(i) \quad (1)$$

$$\phi(i) = t(mi)/t(m) = t(mi)\Big/\sum_{i=1}^{n} t(mi) \quad (2)$$

$$t(mi) = L(i)/u(i) \quad (3)$$

where $k'(i)$, $t(i)$, $\phi(i)$, $L(i)$ and $u(i)$ are the capacity factor, carrier gas hold-up time, fractional carrier gas hold-up time, length and average carrier gas velocity of the ith column, respectively [10].

For a two-column system:

$$\begin{aligned} k'(\mathrm{eff}) &= \phi(1)k'(1) + \phi(2)k'(2) \\ &= [t(m1)k'(1) + t(m2)k'(2)]/t(m) \\ &= [L(1)k'(1)/\overline{u(1)} + L(2)k'(2)/\overline{u(2)}]/t(m) \end{aligned} \quad (4)$$

The separation factor (α) of a pair of components (a and b) is defined as

$$\alpha = k'(a)/k'(b)$$

$$= \frac{L(1)k'(a1)/u(1) + L(2)k'(a2)/u(2)}{L(1)k'(b1)/u(1) + L(2)k'(b2)/u(2)} \quad (5)$$

According& Guiochon and co-workers [12,181 $u(1)$ and $u(2)$ can be written as

$$u(1) = j(1)u(a) \quad (6)$$

$$\overline{u(2)} = j(2)u(o) \quad (7)$$

$$j(1) = \frac{3}{2}\cdot\frac{p^2(i) - p^2(a)}{p^3(i) - p^3(a)}\cdot p(a) \quad (8)$$

$$j(2) = \frac{3}{2}\cdot\frac{p^2(a) - p^2(o)}{p^3(a) - p^3(o)}\cdot p(o) \quad (9)$$

$$p^2(a) = \frac{L(2)p^2(i) + L(1)p^2(o)}{L(1) + L(2)} \quad (10)$$

$$u(a)p(a) = u(o)p(o) \quad (11)$$

Let

$$t = \bar{u}(1)/\bar{u}(2) \quad (12)$$

Combining eqns. 6-12, we have

$$t = \frac{L(1)[p(a) - p(o)]}{L(2)[p(i) - p(a)]} \quad (13)$$

Let

$$x = \frac{t(m2)}{t(m1)} = \frac{L(2)/\overline{u(2)}}{L(1)/u(1)} \quad (14)$$

Then eqn. 5 can be written as

$$\alpha = \frac{k'(a1) + xk'(a2)}{k'(b1) + xk'(b2)} \quad (15)$$

For a certain separation factor and resolution (R), the minimum effective plate number needed can be calculated from

$$N = \left(\frac{4R\alpha}{\alpha - 1}\right)^2 \quad (16)$$

The effective plate number is defined as

$$N = \left(\frac{t(R)}{\sigma}\right)^2 \quad (17)$$

$$\frac{1}{N} = \left(\frac{\sigma}{t(R)}\right)^2 \quad (18)$$

$$\begin{aligned} t(R) &= t(1) + t(2) \\ &= \frac{k'(1)L(1)}{u(1)} + \frac{k'(2)L(2)}{u(2)} \end{aligned} \quad (19)$$

If the extra-column effect can be ignored, then σ^2 can be written as

$$\begin{aligned} \sigma^2 &= \sigma^2(1) + \sigma^2(2) \\ &= \left(\frac{\sigma(1)t(1)}{t(1)}\right)^2 + \left(\frac{\sigma(2)t(2)}{t(2)}\right)^2 \end{aligned}$$

$$= \frac{[t(1)]^2}{N(1)} + \frac{[t(2)]^2}{N(2)} \quad (20)$$

$$\mathbf{N(i)} = L(i)n(i) \quad (21)$$

Combining eqns. 14, 18, 19 and 20 yields

$$\frac{1}{N} = \frac{[k'^2(1)/N(1) + x^2k'^2(2)/N(2)]}{[k'(1) + xk'(2)]^2} \quad (22)$$

The ranges of x, L(1) and L(2) values can be calculated by an iterative technique by combining eqns. 12, 14, 15 and 22. In the first step, the effect of the velocity gradient along the column was ignored, and the columns used in our experiments had the same inner diameter (0.25 mm), hence $u(1)=u(2)$

$$x = \frac{L(2)/u(2)}{L(1)/u(1)} = \frac{L(2)}{L(1)} \quad (23)$$

After obtaining the ranges of ***L,*** L(1) and L(2) values, the approximate inlet pressure [p(i)] can be easily presumed for a practical average carrier gas velocity. Substituting these values into eqns. 10 and 13, the t value $[u(1)/u(2)]$ can be calculated. Using this t value, more concise values of ***L, L(1)*** and L(2) can be obtained by combining eqns. 14, 15 and 22. Even more concise values of ***L,*** L(1) and L(2) can be reached by iteration oft several times. From a practical point of view, iteration of t twice is sufficient.

EXPERIMENTAL

Chromatographic conditions

The preparations of the fused-silica capillary columns, cross-linked polycyanoethyl vinyl **siloxane–L-Val–*tert.*-butylamide** (column C), **cross-linked** PEG 20M (column P) and wall-coated OV-101 (column 0), have been described previously [19–21].

Fused-silica tubing (2 cm ***x 0.53*** mm I.D.) and two silicone rubber septa were used to connect columns in the two-column system (Fig. 1). The chromatographic separations were carried out with a GC **R1A** gas **chromatograph** equipped with a split injector and a flame ionization detector.

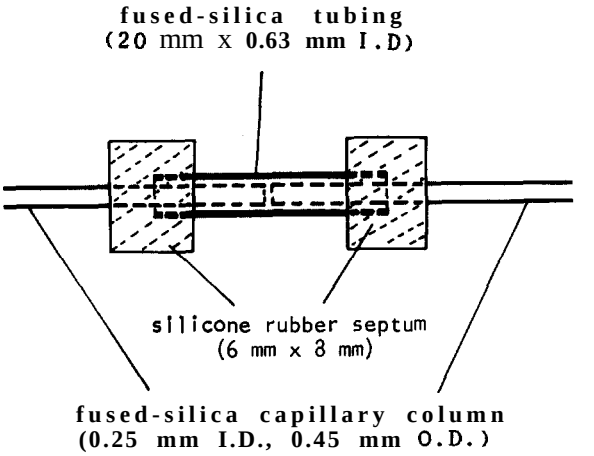

Fig. 1. Connection of the series-coupled columns.

Samples and derivatization

Aspartic acid (Asp) was obtained from Sigma. **L-Hydroxyproline** (Hpr) was kindly supplied by Professor X. Xu (Shanghai Institute of Materia **Medica,** Academia **Sinica,** Shanghai, China) and the tooth and bone samples by Ms. Y. Shun (Shanghai Institute of Expert Testimony, Shanghai, China). According to Wang ***et al.*** [8], the samples were hydrolysed in 6 ***M*** **HCl** at 110°C for 24 h and the hydrolysis products were treated with a column of Dowex AG **50W-X8,** 100-200 mesh (Bio-Rad Labs., Munich, Germany), in the H^+ form, eluted with 2 ***M*** aqueous ammonia. The amino acids were derivatized as N,(O)-trifluoroacetyl **(TFAc)** isopropyl esters according to McKenzie and Tenaschuk [22].

Calculations

An IBM-AT compatible microcomputer and software written in Quick-Basic Ver. 4.0 (Microsoft) were used for the calculations.

RESULTS AND DISCUSSION

The capacity factors (k') and separation factors **(α)** of the solutes are given in Tables I-III.

The D/L ratio of Asp is a widely used value for age estimation of living cells and fossilized materials. However, the peak of **TFAc-L-Asp** isopropyl ester is seriously overlapped by that of **N,O-TFAc-L-Hpr** isopropyl ester when using a cross-linked polycyanoethyl vinyl **siloxane–L-**

TABLE I

k' AND a VALUES OF THE SOLUTES ON CROSS-LINKED POLYCYANOETHYL VINYL SILOXANE–L-VAL–*TERT.*-BUTYLAMIDE COLUMN

Solute	120°C		130°C		140°C		150°C	
	k'	*α*	*k'*	*a*	*k'*	*a*	*k'*	*α*
D-Asp	11.98		7.18		4.48		2.84	
		1.051		1.040		1.031		1.025
L-Asp	12.59		7.47		4.62		2.91	
L-Hpr	12.72		7.50		4.64		2.91	

TABLE II

k' VALUES OF THE SOLUTES ON CROSS-LINKED PEG 20M COLUMN

Solute	120°C	130°C	140°C	150°C
D,L-Asp	15.84	9.36	5.63	3.50
L-Hpr	13.06	7.68	4.60	2.83

TABLE III

k' VALUES OF THE SOLUTES ON WALL-COATED 0%101 COLUMN

Solute	120°C	130°C	140°C	150°C
D,L-Asp	10.41	6.66	4.43	3.02
L-Hpr	7.82	5.04	3.37	2.30

Val–*tert.*- butylamide column (column C), and this situation cannot be improved simply by changing the column temperature (Table I). The use of series-coupled columns is the method of choice for selectivity tuning. The derivatives of Asp and L-Hpr can be readily separated with either a PEG 20M (column P) or an 0%101 column (column 0) (Tables II and III). Therefore, it is possible that the derivatives of D,L-Asp and L-Hpr might be separated with column P or column 0 series-coupled to column C.

It is well known that direct cbiral separations can only be carried out with chiral columns. The coupling of an achiral column will decrease the enantioselectivity of a chiral column (eqn. 15). Asp is an amino acid with low a values when using most diamide chiral stationary phases (CSPs) [23]. Therefore, the lengths of the coupled columns must be carefully calculated and optimized.

The plots of a *versus* x $[L(2)\overline{u(1)}/L(1)\overline{u(2)}]$ at 130°C are shown in Fig. 2. With a certain a value, the range of x values can be easily calculated from eqn. 15.

In order to obtain a satisfactory resolution between N-TFAc-D,L-Asp isopropyl ester and N,O-TFAc-L-Hpr isopropyl ester with reasonable column lengths, the minimum **a** values were set as 1.025 and the R values as 1.5. By an iterative technique and combining eqns. 13, 15 and 22, the range of x values, the column length ratios $[L(2)/L(1)]$ and the minimum column lengths (L) can be obtained. Some results are given in Table IV

Fig. 3 shows how $u(1)/u(2)$ changes with the column length ratio $[L(2)/L(1)]$. It is found that, with values of $p(i)/p(o) < 2.0$, the variation of $u(1)/u(2)$ is not significant over a wide range of $L(2)/L(1)$. In most open-tubular column

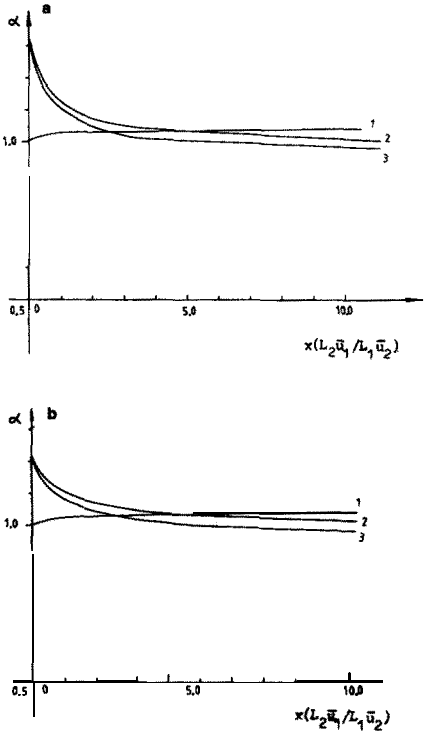

Fig. 2. Plots of 4 *versus x* **at 130°C. (a) Column O-column C; (b) column P-column C. 1= L-Asp/D-Asp; 2 = L-Asp/L-Hpr; 3 = D-Asp/L-Hpr.**

TABLE IV

RANGES OF x, α AND $L(2)/L(1)$ OF THE SERIES-COUPLED COLUMN SYSTEMS UNDER ISOTHERMAL CONDITIONS [p(i) = 0.15 MPa, p(o) = 0.10 MPa]

Column coupling	Parameter[a]	120°C	130°C	140°C[b]	150°C[b]
C-P	x	1.3-2.3	2.2–2.9	–	–
	α(L/D-Asp)	1.025-1.032	1.025-1.028	–	–
	α(D-Asp/L-Hpr)	1.061–1.025	1.040-1.026	–	
	First iteration				
	$L(2)/L(1)$	1.3-2.3	*2.2-2.9*		
	$t[u(1)/u(2)]$	0.82-0.83	*0.83-0.83*		
	Second iteration				
	$L(2)/L(1)$	1.6-2.8	*2.7-3.5*		
	$t[u(1)/u(2)]$	0.83-0.83	*OH-O.83*		
	Third iteration				
	$L(2)/L(1)$	1.6-2.8	*2.7-3.5*		
C–O	x	0.9-2.2	1.6-2.9		–
	α(L/D-Asp)	1.026-1.036	1.026-1.031	–	–
	α(D-Asp/L-Hpr)	1.100-1.027	1.065–1.026	–	–
	First iteration				
	$L(2)/L(1)$	*0.9-2.2*	1.6-2.9		
	$t[u(1)/u(2)]$	*0.82-0.83*	0.83–0.83		
	Second iteration				
	$L(2)/L(1)$	1.1-2.7	1.9-3.5		
	$t[u(1)/u(2)]$	0.83-0.83	0.83–0.83		
	Third iteration				
	$L(2)/L(1)$	1.1-2.7	1.9-3.5		

[a] x is the ratio $L(2)\overline{u(1)}/L(1)\overline{u(2)}$.
[b] Dashes indicate no suitable values.

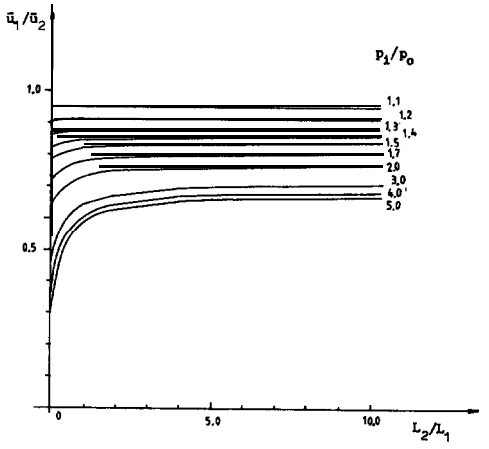

Fig. 3. Variation of $\overline{u(1)}/\overline{u(2)}$ with $L(2)/L(1)$.

systems, the values of $p(i)/p(o)$ are <2.0 because of their high permeability. Hence for calculating the ranges of $L(2)/L(1)$, L(1) and L(2), iteration of t twice is sufficient in most instances.

From Table IV, it can be seen clearly that at column temperatures of 120 and 130°C, the requirement of the α values can be met with certain ranges of x values with both series-coupled column systems, P-C and O-C. The achiral columns were used as the first column. Considering the analysis speed and the carrier gas velocity gradient, a total column length L = 21.3 m, p(i)/p(o) = 1.5, x = 2.5 [$L(2)/L(1)$ = 3] and a column temperature of 130°C were selected for both systems. The α values of the systems were calculated and compared with the experimental results (Table V).

The chromatograms of N-TFAc-D,L-Asp isopropyl ester and N,O-TFAc-L-Hpr isopropyl ester obtained using column C and the series-coupled column systems are shown in Fig. 4. With the optimized lengths of the coupled columns, both pairs of solutes, Asp and Hpr and D,L-Asp, can be baseline separated. Fig. 5 shows

TABLE V

COMPARISON OF α VALUES FROM CALCULATION AND EXPERIMENT

L(1) = 5.3 m, L(2) = 16 m, temperature = 130°C, p(i) = 0.15 MPa, p(o) = 0.10 MPa.

Solutes	Column O-column C		Column P-column C	
	Experimental	Calculated	Experimental	Calculated
D-Asp/~-Hpr	1.036	1.034	1.034	1.033
~-Asp/~-Asp	1.030	1.029	1.028	1.027

typical chromatograms of amino acids in tooth and bone samples obtained using column C and the series-coupled column systems.

CONCLUSIONS

A method for calculating the appropriate lengths of series-coupled columns was developed. With this method, good results for the separation of N-TFAc-D,L-Asp isopropyl ester and N,O-TFAc-L-Hpr isopropyl ester were obtained with two systems of series-coupled columns.

SYMBOLS

i	Column index
$j(i)$	James-Martin pressure correction factor (eqns. 6-9)
k(eff)	Effective capacity factor (eqn. 1)
$k(ai)$	Capacity factor of solute a on ith column
$k(bi)$	Capacity factor of solute b on ith column
L	Total column length of the series-coupled system
$L(i)$	Length of ith column
N	Effective plate number of the series-coupled system
$N(i)$	Effective plate number of ith column
n	Effective plate number per metre of the system
$n(i)$	Effective plate number per metre of ith column
$P(a)$	Intermediate pressure
$P(i)$	Inlet pressure
p(o)	Outlet pressure
R	Resolution
t	Relative average velocity $\bar{u}(1)/\bar{u}(2)$ of the carrier gas
$t(i)$	Adjusted retention time for a retained solute in ith column

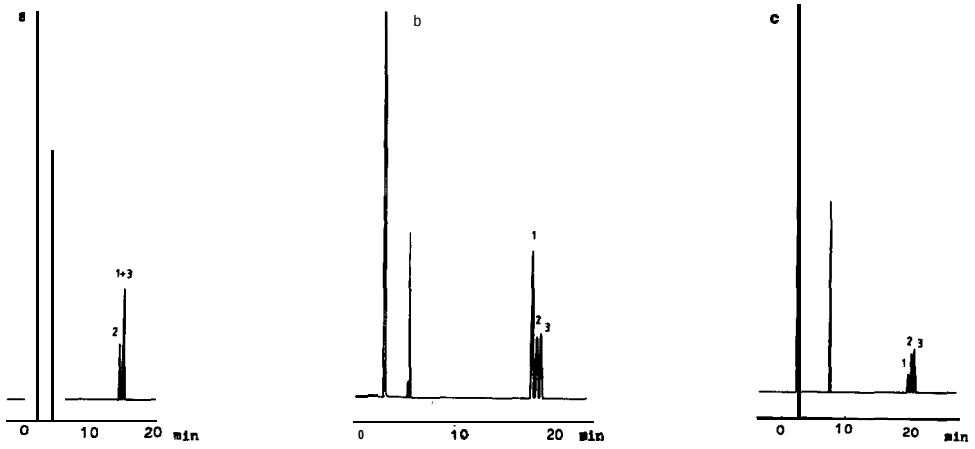

Fig. 4. Chromatograms of D,L-Asp and L-Hpr. Columns: (a) column C (16 m x 0.25 mm I.D.); (b) column 0 (5.3 m x 0.25 mm I.D.)-column C (16 m x 0.25 mm I.D.) [p(i) = 0.15 MPa, p(o) = 0.10 MPa]; (c) column P (5.3 m x 0.25 mm I.D.)-column C (16 m x 0.25 mm I.D.) [p(i) = 0.15 MPa, p(o) = 0.10 MPa]. Temperature, 130°C; carrier gas, nitrogen; detector, flame ionization. Peaks: 1 = L-Hpr; 2 = D-Asp; 3 = L-Asp.

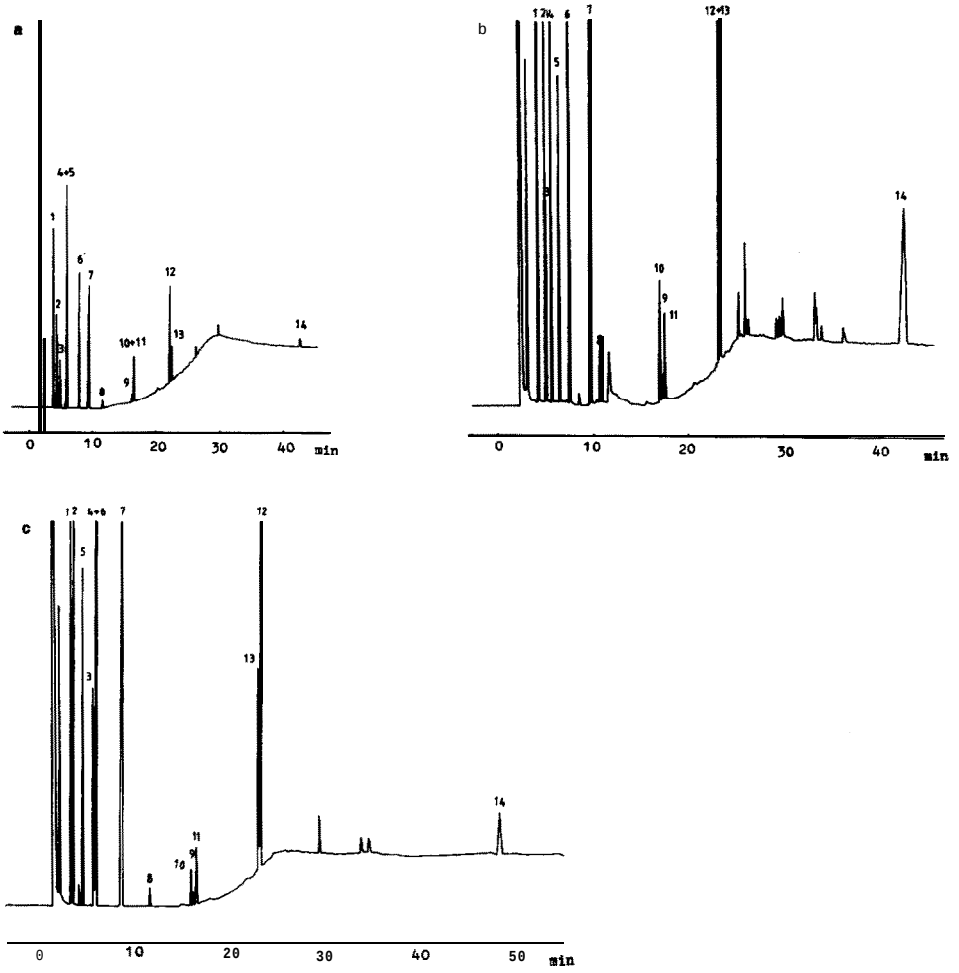

Fig. 5. Chromatograms of amino acids in real samples. (a) Sample, bone; column, C (16 m x 0.25 mm I.D.); temperature, 120°C (10 min), then increased at 4°C/min to 190°C. (b) Sample, tooth; column, 0 (5.3 m x 0.25 mm I.D.)-C (16 m x 0.25 mm I.D.) [p(i) = 0.15 MPa, p(o) = 0.10 MPa]; temperature, 130°C (15 min), then increased at 6°C/min to 190°C. (c) Sample, bone; column, P (5.3 m x 0.25 mm I.D.)-C (16 m x 0.25 mm I.D.) [p(i) = 0.15 MPa, p(o) = 0.10 MPa]; temperature, 130°C (15 mm), then increased at 6°C/min to 190°C. In all instances the carrier gas was nitrogen and a flame ionization detector was used. Peaks: 1 = L-Ala; 2 = L-Val; 3 = L-Thr; 4 = Gly; 5 = L-Ile; 6 = L-Leu; 7 = L-Pro; 8 = L-Ser, 9 = D-Asp; 10 = L-Hpr; 11 = L-Asp; 12 = L-Glu; 13 = L-Phe; 14 = L-Lys.

$t(m)$	Total carrier gas hold-up time
$t(mi)$	Carrier gas hold-up time of ith column
$t(\mathrm{R})$	Adjusted retention time for a retained solute in the system
$u(i)$	Average carrier gas velocity of ith column
$u(\mathrm{a})$	Carrier gas velocity at the connection point of the system
$u(\mathrm{o})$	Carrier gas velocity at the outlet of the system
x	Ratio of carrier gas hold-up time, $t(m2)/t(m1)$
α	Separation factor
$\phi(i)$	Fractional carrier gas hold-up tune of ith column
σ	Standard deviation of the system
$\sigma(i)$	Standard deviation of ith column

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