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Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent

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Abstract

An isocratic liquid chromatography method using volatile ion pairing agent and evaporative light scattering detection was developed for the analysis of 10 underivatized amino acids (Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala, Pro) which are not sufficiently retained and resolved in the LC system commonly used. Five perfluorinated carboxylic acids (trifluoroacetic, heptafluorobutyric, nonafluoropentanoic, tridecafluoroheptanoic and pentadecafluorooctanoic acid) have been evaluated as ion-pairing agents in various concentrations (0.25–1 mM) using three analytical reversed-phase columns (LiChrospher RP-Select B, Purospher RP-18e and Supelcosil ABZ$^+$ Plus). The quantities adsorbed on the chromatographic supports and the equilibration times of each system are also studied. Excellent separation with satisfactory baseline resolution was achieved on the chromatographic system made up of a Purospher RP-18e and an aqueous mobile phase containing pentadecafluorooctanoic acid at 0.5 mM. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The biological importance of amino acids is well known. Liquid chromatography has become the standard procedure for their separation, but the common amino acids do not fluoresce and, with the exception of the few amino acids that contain an aromatic group, direct detection by UV absorbance is possible only at low wavelengths where sensitivity is poor and interference in background absorption is potentially high [1].

In recent years, the idea of separation of un-

derivatized amino acids has attracted the interest of some investigators as the elimination of derivatization (pre- or post-column) brings the advantages of simplicity, flexibility as well as the desired sensitivity and separation speed. This has become possible thanks to the development of chromatographic columns with increasingly improved performance.

Direct detection by means of electrochemical methods [2–4] and indirect photometric, fluorometric detection [5–8] have been proposed for the analysis of underivatized amino acids. Generally, however, these methods are unsuitable when elution gradient is required. Evaporative light scattering detection (ELSD) can be considered, like refractometry, as a universal detection method but even more sensitive,

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without the drawbacks of base-line drift during elution gradient and with a rapid equilibration time. It has recently been shown that it is a good choice for the analysis of underivatized amino acids [9–11]. In order to obtain a background noise as low as possible and satisfactory detection limits, the LC–ELSD methods require volatile mobile phases.

The separation of the most hydrophobic amino acids can be easily achieved under reversed-phase chromatography [11], whereas polar ones are co-eluted near void volume.

Due to the ionisable nature of free amino acids (cationic, anionic or even zwitterionic), most of the recent work concerning their separation consists in the use of ion-exchange [2–4,12] or ion-pair reversed-phase liquid chromatography (IP-RPLC) [7,13]. In IP-RPLC, ion pairs can be formed between the acidic or amino form of the amino acid and, respectively, a cationic or anionic surfactant. In the latter case, stationary phases often have higher viability. The use of alkylsulfonic acids has already been demonstrated as suitable for the separation of underivatized amino acids [8,13]. In comparison to these surfactants, perfluorinated carboxylic acids have a lower boiling point and are therefore compatible with ELSD [14] and mass spectrometry [15] and suitable for preparative chromatography [16,17]. Moreover, trifluoroacetic acid (TFA) is the most commonly used volatile ion-pairing agent (IPA) in IP-RPLC due to its high purity, water solubility and transparency at 220 nm [18]. Recently, this agent has been used for the separation of underivatized amino acids with ELSD [10,11] but the selectivity towards high polar amino acids remains bad and several coelutions are observed under IP-RPLC conditions [11]. Perfluorinated carboxylic acids with longer *n*-alkyl chains have been already employed as an alternative to TFA for the separation of peptides and proteins [18,19] but never for natural polar amino acids. So the purpose of this work is to evaluate these surfactants as ion pairing agents for the separation of 10 of the most polar amino acids (Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala and Pro) for which some coelutions are generally noticed in reversed-phase (RP or IP-RPLC) chromatographic methods. Three commercially available reversed-phase packings were tested and ELSD was used for detection of underivatized amino acids.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile, methanol and tetrahydrofuran were purchased from J.T. Baker (Noisy le Sec, France). Among the perfluorinated carboxylic acids used in this study only TFA is commercially available in HPLC grade, whereas the higher homologues exist in lower grade (97–99%). TFA, nonafluoropentanoic acid (NFPA) and tridecafluoroheptanoic acid (TDFHA) were obtained from Interchim (Montluçon, France), heptafluorobutyric acid (HFBA) and pentadecafluorooctanoic acid (PDFOA) from Aldrich (St. Quentin Fallavier, France). Butylbenzene, amylbenzene, phenol and caffeine were obtained from Fluka (Buchs, Switzerland). Aspartic acid (Asp), asparagine (Asn), glutamine (Gln), glutamic acid (Glu), serine (Ser), threonine (Thr), cysteine (Cys), glycine (Gly), alanine (Ala), and proline (Pro) were purchased from Sigma (St. Louis, MO, USA). All standard mixtures (100 mg l^{-1} for each amino acid) as well as perfluorinated carboxylic acid solutions were prepared in purified water obtained from an Elgastat UHQ II System (Elga, Antony, France).

2.2. Apparatus

Chromatography was carried out using a Beckman (Fullerton, CA, USA) model 128 System gold binary pump, a Rheodyne (Cotati, CA, USA) model 7125 injection valve fitted with a 20-μl loop, a Shimadzu (Kyoto, Japan) C-R6A integrator and a Sedere (Vitry s/Seine, France) Model Sedex 45 evaporative light scattering detector set as follows: drift tube temperature, 60°C; nebulizer gas pressure, 2.2 bar; and photomultiplier, 9.

A Varian (Palo Alto, CA, USA) Model 2550 UV spectrophotometric detector set at 254 nm was used as detection mode for stationary phase characterization.

Conductivity was monitored using a Vydac conductivity meter (Wescan Instrument, Santa Clara, USA) Model 6000 CD.

For each solution, pH was checked on a Beckman pH meter model Φ 10 (Gagny, France).

2.3. Stationary phases and regeneration procedure

The analytical columns used were: Lichrospher RP-Select B 125×4 mm I.D. (Merck, Darmstadt, Germany) Purospher RP-18e 125×4 mm I.D. (Merck) fitted with its precolumn and Supelcosil ABZ$^+$Plus 150×4.6 mm I.D. (Supelco, Bellefonte, PA, USA). Flow-rate is 1 ml min^{-1} and all experiments are done at room temperature.

To ensure that no irreversible surface modification has occurred between each experiment, a regeneration procedure is applied as follows: acetonitrile (50 ml) then methanol (30 ml). For PDFOA, which shows a greater affinity than other homologous shorter alkylchain surfactants, the procedure is: methanol (50 ml), acetonitrile (50 ml), tetrahydrofuran (30 ml) then methanol (50 ml). Columns are then tested with the methodology of Tanaka and co-workers [20] before a new equilibration.

3. Results and discussion

3.1. Choice of the analytical columns

Liquid chromatography is one of the most popular tools in the analytical laboratory and among all available stationary phases, octyl- as well as octadecyl-bonded silica are the most commonly used ones. This fact led the manufacturers to search for new methods for improving the above stationary phases. Moreover, various tests [20,21] have been established providing information which helps chromatographers to make the right column selection.

It is well established that highly polar compounds interact inefficiently with reversed-phase stationary support. Owing to non-specific interactions with the silica gel surface leading to peak tailing, we have to chose columns that reduce the effects of silanol groups through 'end-capping' or a specific treatment of the based-silica support (removal of metal impurities, transformation of silanol groups in siloxane bonds, etc.) or through new methods of bonding the alkyl chain. Two commercially available analytical columns among the new generation of columns based on high-purity silica gel (Purospher RP-18e and Supelcosil ABZ$^+$Plus) and one analytical column (LiChrospher RP-Select B), which is recommended by its supplier for the separation of polar basic compounds, were selected for our study. Their physicochemical parameters are reported in Table 1. For each column, the hydrophobicity and the residual silanols presence were evaluated, respectively, from the retention factor of amylbenzene (k_{AB}), the separation factor α_{CH2} (calculated from the ratio of the retention factor of amylbenzene and the retention factor of butylbenzene) and the separation factor $\alpha_{C/P}$ (calculated from the ratio of the retention factor of caffeine and the retention factor of phenol) according to the methodology of Tanaka co-workers [20].

3.2. Choice of perfluorinated carboxylic acids as ion-pairing agents

In our study, different perfluorinated carboxylic acids were evaluated as ion pairing agents for the analysis of amino acids with ELSD. These perfluorinated surfactants have been previously used for the separation of peptides [18,19] with a UV detection mode, but under these conditions baseline drifts and UV spikes have been observed due to UV-absorbing

Table 1
Characteristics of the three studied columns and evaluation of their hydrophobic properties by the retention factor of amylbenzene (k_{AB}) and the separation factor between amylbenzene and butylbenzene (α_{CH2}) as described in the tests of Tanaka and co-workers

Column	Characteristics							
	Dimensions (mm)	Particle size (μm)	Pore size (Å)	Surface area (m^2 g^{-1})	Carbon load (%)	k_{AB}	α_{CH2}	$\alpha_{C/P}$
Supelcosil ABZ$^+$Plus	150×4.6	5	100	170	12	3.86	1.40	0.28
Purospher RP-18e	125×4	5	80	500		9.30	1.52	0.51
LiChrospher RP-Select B	125×4	5	60	360	12	3.05	1.38	0.63

Residual silanols presence is estimated by the separation factor between caffeine and phenol ($\alpha_{C/P}$).

contaminants with possibly high molar absorbance coefficient ϵ. In addition, it can be assumed that during column equilibration, accumulation of these contaminants may take place and, as a result, can give additional peaks during elution gradient. In order to obtain a background noise as low as possible and satisfactory detection limits, ELSD requires volatile mobile phases. The ELSD response is in direct relation to the quantities of injected compounds [22], regardless of UV absorbance. Therefore, if these contaminants of perfluorinated surfactants are volatile, they cannot be detected. TFA, HFBA and NFPA are liquid at ambient temperature and TDFHA becomes liquid above 25°C; consequently, they are easily evaporable. Using these perfluorinated acids at low concentration in the mobile phase (≤ 1 mM) would carry out steady baseline even in elution gradient mode. PDFOA which is solid at room temperature proves to be volatile enough to be used with ELSD without an increase in background noise.

3.2.1. Adsorbed quantity of surfactant and LC equilibration time

The nature (type and chain length of the hydrophobic group) of the ion pairing agent is considered to be an important parameter in the retention of charged solutes in reversed-phase ion-pair chromatography. The adsorption of the different perfluorinated carboxylic acids on the stationary phase was determined by measuring the breakthrough [23] with a conductivity meter. Before these measurements,

each stationary phase was regenerated as described in the experimental part to obtain a reference state. We have worked without salt or pH control, as buffers lead to less volatile salts which lead to an increase in the baseline when ELSD is used. Working without salt control should be advantageous as has been shown for alkylsulfonate surfactants [24]: selectivity differences between solutes are to be expected when the alkylchain of a homologous series of surfactant changes.

Table 2 shows that adsorbed quantities of surfactant on Purospher RP-18e increase with surfactant concentration in the mobile phase as well as with the increase of the surfactant alkylchain length. The same behavior has been observed for the other two columns but is not reported. The ion-pairing added to mobile phase, when flowing in isocratic conditions induces a dynamic modification of the surface of the reversed-phase packing material. Under these chromatographic conditions, hydrophobic interactions between hydrophilic amino acids and the stationary phase can occur only through a concerted mechanism involving the stationary phase, the ion-pairing agent and the solute [24,25]. In our study, as previously reported by Patthy [26], systems containing TFA as mobile phase have an equilibration time corresponding to that of the system void volume and no modification of the original packing material is observed with these systems. Aqueous TFA mobile phase is ineffective in promoting retention and selectivities for hydrophilic amino acids on reversed-phase support, as reported in Ref. [11].

Table 2
Influence of concentration and chain length (C$_n$) of the perfluoroalkyl carboxylic acids on the adsorbed quantities (μmol) on the three stationary phases

Columns	Adsorbed quantities (μmol) of perfluoroalkyl carboxylic acid				
	Concentration in mobile phase (mM)	PDFOA (C$_7$)	TDFHA (C$_6$)	NFPA (C$_4$)	HBFA (C$_3$)	TFA (C$_1$)
Purospher RP-18e	0.25	45 (190)	16.5 (76)	1.75 (9)	0.75 (5)	≈ 0
	0.5	94 (198)	32 (74)	3.5 (9)	1.5 (5)	≈ 0
	0.75	127 (179)	47 (73)	5.25 (9)	2.25 (5)	≈ 0
	1	173 (183)	65 (75)	6 (8)	3 (5)	≈ 0
Supelcosil ABZ$^+$	1	175 (185)	70 (80)	11 (13)	10 (12)	≈ 0
LiChrospher RP-Select B	1	165 (175)	51 (61)	11 (13)	3 (5)	≈ 0

Equilibration times (min) are given in the brakets.

It has also been observed that whatever the nature of the support and the column dimensions, equilibration times are almost the same for a given surfactant at a given concentration (1 mM in Table 2) and, as a result, the total adsorbed quantities of surfactant are similar on the three analytical columns. Moreover, for one given perfluorinated carboxylic acid, the equilibration time seems to be independent of the surfactant concentration in the mobile phase.

3.2.2. Retention and selectivity

In this paper, we have chosen to study the separation of 10 amino acids among the most polar ones (Gly, Asp, Asn, Glu, Gln, Cys, Ser, Thr, Ala and Pro). These solutes have low retention and cannot be separated under the chromatographic

conditions described previously by Chaves das Neves [11], i.e. a step gradient elution with a water–acetonitrile mixture containing TFA as mobile phase on RP-8 Spherisorb column and ELSD.

Fig. 1 shows dependence of the retention factor k on the concentration of PDFOA in the mobile phase on Purospher RP-18e support and suggests better selectivities at high ion pairing concentrations. On the other hand, as expected in IP-RPLC, the analysis time is significantly increased when the ion pairing agent concentration in the mobile phase is increased. The same behavior is observed for the other two columns.

Whatever the columns and the surfactant concentration, aspartic acid is always the least retained while proline is the most retained. Table 3 indicates
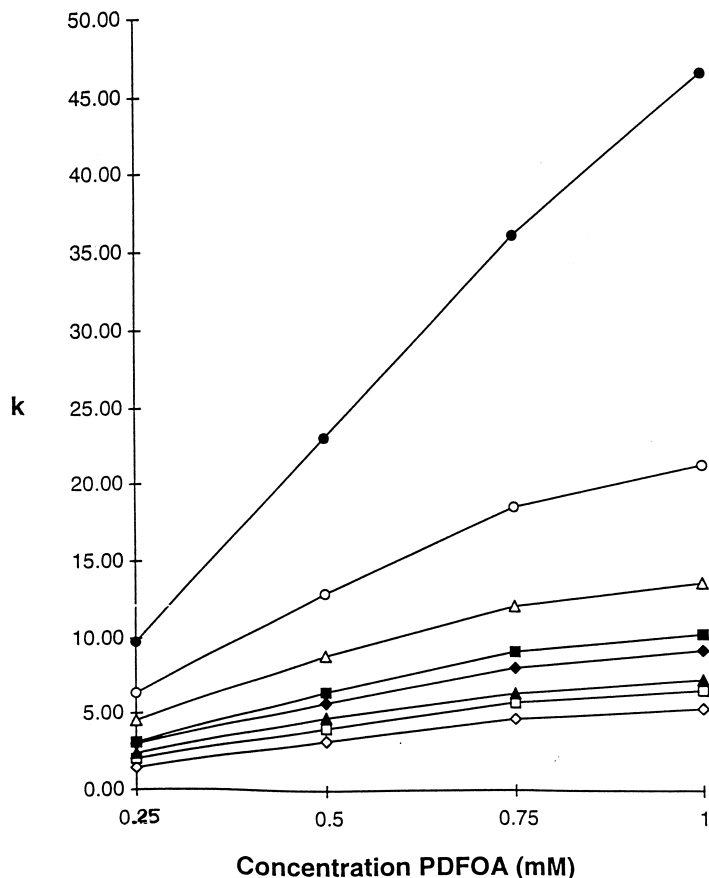

Fig. 1. Influence of the pentadecafluorooctanoic acid concentration (PDFOA) in an aqueous mobile phase on the retention factor k of seven underivatized amino acids (◇) Asp; (□) Asn; (▲) Ser; (♦) Gly; (■) Gln; (△) Thr; (○) Ala; (●) Pro. Column: Purospher RP-18e (125×4 mm I.D.).

Table 3
Retention factors k and separation factor between the least retained (Asp) and the most retained (Pro) amino acid depending from the chain length of the ion pairing agent at 1 mM in the mobile phase

Perfluoroalkyl carboxylic acid	Columns								
	LiChrospher RP Select B			Supelcosil ABZ$^+$Plus			Purospher RP-18e		
	k_{Asp}	k_{Pro}	$\alpha_{Asp-Pro}$	k_{Asp}	k_{Pro}	$\alpha_{Asp-Pro}$	k_{Asp}	k_{Pro}	$\alpha_{Asp-Pro}$
HFBA (C$_3$)	0.32	0.75	3.75	0.29	0.68	2.34	0.16	0.58	3.63
NFPA (C$_4$)	0.60	1.57	2.61	0.54	1.11	2.05	0.43	1.37	3.18
TDFHA (C$_6$)	1.95	8.49	4.35	1.96	7.27	3.70	2.28	11.66	5.11
PDFOA (C$_7$)	5.15	34.53	6.70	3.63	24.72	6.80	5.18	44.53	8.59

the retention factors of Asp and Pro depending on the nature of the ion pairing agent present at 1 mM in the mobile phase. In accordance with an ion interaction chromatographic method [25], the retention of amino acids is increased with the increase of the perfluorinated chain but it appears that the two surfactants (HFBA and NFPA) with the shortest side chains (C$_3$ and C$_4$) are not efficient enough to separate the 10 amino acids. Too weak a selectivity is observed between Asp and Pro with these ion pairing agents. Surfactant with a longer side chain (PDFOA) leads to higher selectivities. The values can reach two to three times the values observed for surfactant with shorter side chains when PDFOA is used. So TDFHA and PDFOA proved to be the two most suitable ion pairing agents and were selected for further studies.

Whatever the columns and the concentration of PDFOA, the elution order observed is always the same. First, Asp is eluted then Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala and finally Pro. When TDFHA is employed, the elution order is also the same at low ion pairing agent concentration (0.25 mM) but, from

0.5 to 1 mM, we can observe one inversion in the elution order for two amino acid pairs. As shown in Table 4, when TDFHA is used at 1 mM, threonine is eluted before glutamic acid. We can also see from Table 4, which reports the separation factor of amino acid pairs that are difficult to separate, that for the Gln–Cys pair, selectivity is better with TDFHA than with PDFOA but the reverse is true for Glu–Thr pair. Similarly, for a given surfactant (PDFOA), selectivity can be sometimes better at low concentration between some amino acids (Gln–Cys) but worse between the others (Glu–Thr). The same behavior is observed for the three columns for these amino acid pairs. In fact, the best separation will be obtained as a compromise. Indeed, there are no simple rules to select the most suitable surfactant and its optimal concentration. The selectivities offered by one column in comparison with an other are also not equivalent. For example, selectivity between Gln and Cys is better on Purospher RP-18e, while Cys–Glu selectivity is better on LiChrospher RP-Select B and Supelcosil ABZ$^+$Plus. Nevertheless, it must be also mentioned that when the selectivity of two consecu-

Table 4
Comparison between separation factors of amino acid pairs that are difficult to separate on three different supports

Columns	Perfluoroalkyl carboxylic acid	Concentration (mM)	Separation factors				
			$\alpha_{Asn-Ser}$	$\alpha_{Gly-Gln}$	$\alpha_{Gln-Cys}$	$\alpha_{Cys-Glu}$	$\alpha_{Glu-Thr}$
Purospher RP-18e	TDFHA (C$_6$)	1	1.11	1.13	1.21	1.19	0.97 (1/α=1.03)
	PDFOA (C$_7$)	1	1.08	1.11	1.06	1.09	1.15
		0.5	1.12	1.11	1.15	1.11	1.08
LiChrospher RP-Select B	PDFOA (C$_7$)	0.5	1.08	1.04	1.05	1.15	1.07
Supelcosil ABZ$^+$Plus	PDFOA (C$_7$)	0.5	1.10	1.03	1.09	1.18	1.03

$\alpha_{1-2}=k_2/k_1$.

tive amino acids reaches 1.08, their total separation is achieved due to the excellent efficiency of the chromatographic systems. Fig. 2 shows the isocratic analysis of a standard mixture of 10 amino acids with PDFOA as ion pairing agent in the mobile phase. For column-to-column comparisons, the PDFOA was adjusted in order to obtain an equivalent retention time (about 25 min) for the last eluted amino acid. To obtain this result, a PDFOA concentration equal to 0.5 mM was sufficient on Purospher RP-18e column, while 0.75 mM was required for the others. This can be explained by the fact that Purospher RP-18e column is the most hydrophobic support as characterized by the highest surface coverage and the highest retention factor value for amylbenzene (Table 1). In any case, Purospher RP-18e column proved to be the best support to achieve, with a well-defined baseline resolution, the separation of all 10 most polar amino acids. LiChrospher RP-Select B support is an octyl-bonded silica material which turned out to present hydrophobic and

silanophilic properties close to those of Supelcosil ABZ$^+$Plus, an octadecyl-bonded silica column. Although the LiChrospher RP-Select B packing material has a shorter alkyl chain bonding in the silica surface, no significant difference in the chromatographic profiles was observed (Fig. 2b and c) on these two columns. In all the surfactant concentration range studied, it was never possible to resolve the Gly–Gln pair on these two supports.

In order to decrease the retention factors of the two last eluted amino acids, a gradient mode analysis has been investigated. Fig. 3 depicts a good separation of the 10 amino acids with an acetonitrile–aqueous solution of PDFOA 0.5 mM gradient in 18 min.

4. Conclusion

Long n-alkyl chain perfluorinated carboxylic acids have proved to be excellent ion-pairing agents for the
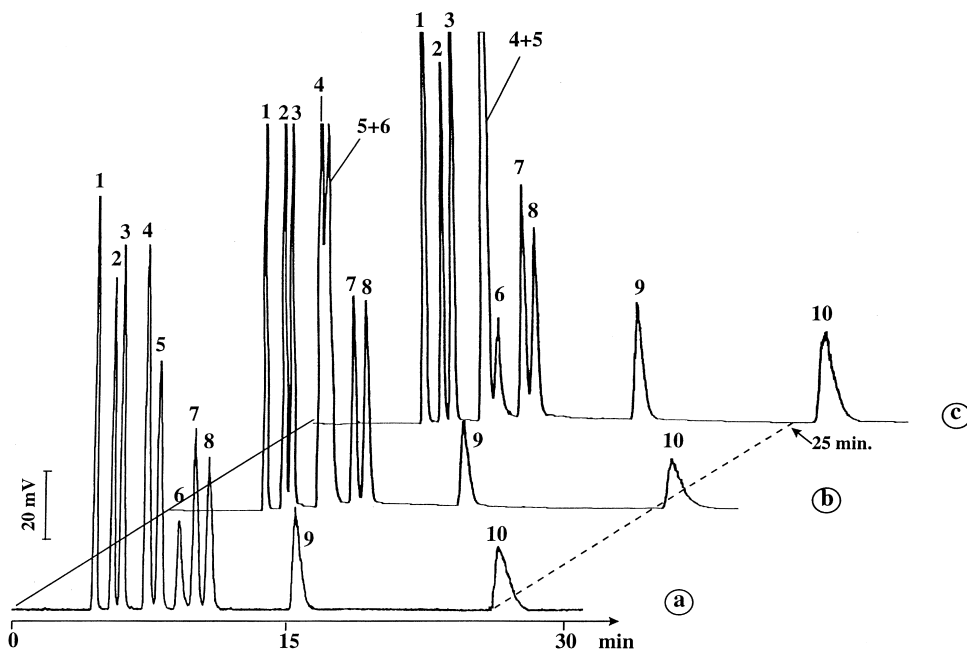

Fig. 2. LC analysis of a standard solution of 10 underivatized amino acids under isocratic conditions on: (a) Purospher RP-18e 125×4 mm I.D. (eluent, PDFOA 0.5 mM in water); (b) LiChrospher RP-Select B 125×4 mm I.D. (eluent, PDFOA 0.75 mM in water); and (c) Supelcosil ABZ$^+$Plus 150×4.6 mm I.D. (eluent, PDFOA 0.75 mM in water). Injected amount, 2 ng of each amino acid; flow-rate, 1 ml min^{-1}; evaporative light scattering detection (for settings, see Section 2); elution order, (1) Asp; (2) Asn; (3) Ser; (4) Gly; (5) Gln; (6) Cys; (7) Glu; (8) Thr; (9) Ala; (10) Pro.
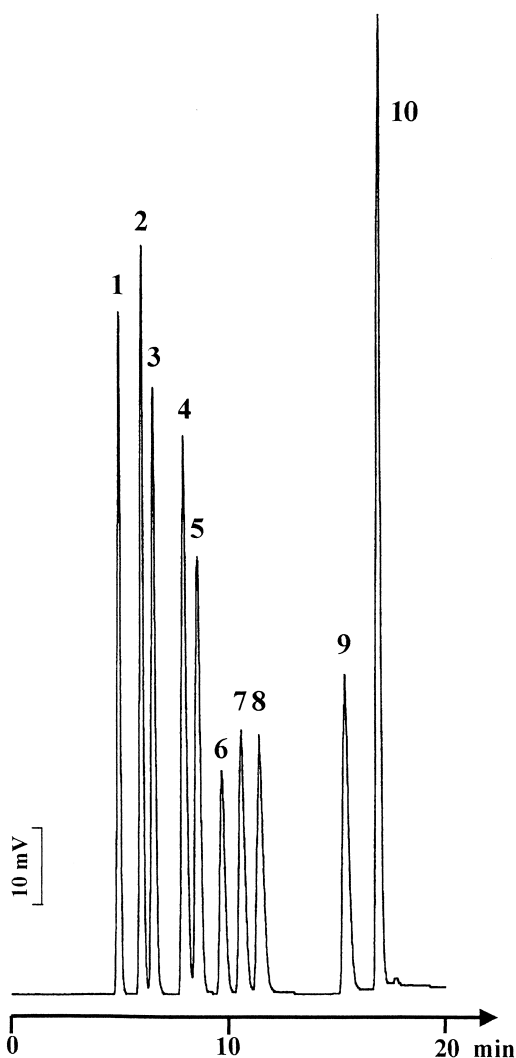
Fig. 3. LC analysis of 10 underivatized amino acids with a gradient elution. Mobile phase, aqueous solution of PDFOA 0.5 m*M* (A); acetonitrile (B). Gradient profile, 0% B during 8 min, from 0 to 17% B in 5 min then 17% B is maintained to the end of the analysis; flow-rate, 1 ml min^{-1}; evaporative light scattering detector (for settings, see Section 2); column, Purospher RP-18e (125×4 mm I.D.). Same elution order and injected amount as Fig. 2.

separation of polar amino acids. TDFHA and PDFOA gave the best results and should be the ion pairing agents of choice when separation of polar amino acids is required. The column Purospher RP-18e proved to be the most efficient stationary phase under these conditions. Light scattering detection has

been shown to be a direct and simple mode of detection for underivatized amino acids.

The separation of 10 polar amino acids observed in 30 min in an isocratic elution mode can be obtained in only 20 min in a gradient elution mode without loss of resolution. Work is therefore in progress using this basic study to analyse the 20 peptidic amino acids [27] by LC-ELSD, which constitutes the ideal primary development step to LC–MS analysis [28]. Quantitative analysis (response factor and detection limits) as well as complex matrix studies remain to be pursued in the near future.

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