ELSEVIER

Journal of Chromatography A, 870 (2000) 245–254

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Ion-pair chromatography on a porous graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids

P. Chaimbault, K. Petritis, C. Elfakir*, M. Dreux

Institut de Chimie Organique et Analytique (IC.O.A), CNRS UPRES-A 6005, Université d'Orléans, BP 6759, 45067 Orléans Cedex 2, France

Abstract

The analysis of twenty underivatized protein amino acids has been achieved on porous graphitic carbon packing material (Hypercarb). Five perfluoroalkyl carboxylic acids (trifluoroacetic, heptafluorobutyric, nonafluoropentanoic, tridecafluoroheptanoic and pentadecafluorooctanoic acid) have been studied as ion-pairing reagent. Several parameters (equilibration time, quantities adsorbed onto the chromatographic support, concentration and nature of the ion-pairing reagent, as well as temperature effect) have been studied leading to the complete separation of these compounds in gradient elution mode. Evaporative light scattering detector has allowed the detection of these non UV–visible absorbent molecules. The chromatographic methodology developed can also be easily coupled with pneumatically assisted electrospray mass spectrometry.

Keywords: Amino acids; Perfluorocarboxylic acids; Porous graphitic carbon; Stationary phases, LC

1. Introduction

Amino acids belong to one of the most important classes of natural compounds as they take part in many important well known physiological processes. One of them is peptide and protein building and concerns mainly twenty α-amino acids [1].

Numerous papers have been published already on the subject of their chromatographic determination. With the exception of tryptophan, tyrosine and phenylalanine which possess an aromatic group, the other seventeen protein amino acids are lacking in chromophore groups. Therefore, the majority of the chromatographic methods proposed for their mixture characterisation involves pre- or post column derivation to allow UV, visible or fluorometric detection.

Only a few publications concern the analysis of underivatized amino acids. Various separation techniques as well as the associated detection mode were employed.

Among the detection modes, indirect photometric or fluorometric detection [2–5], electrochemical [6–8], light scattering [9–13] and even mass spectrometry [14,15] have been proposed.

Concerning separation techniques, liquid chromatography (LC) is widely used but separation using capillary electrophoresis [15] and even supercritical fluid chromatography [9] have also been reported. In the field of LC, several methodologies have also been tested. For example, Schuster [16] has proposed normal-phase chromatography. Due to the ionic properties of amino acids, ion-exchange has also

*Corresponding author. Tel.: +33-384-17-074; fax: +33-384-17-281.

E-mail address: Claire.Elfakir@univ-orleans.fr (C. Elfakir)

0021-9673/00/$ – see front matter
PII: S0021-9673(99)00863-8

allowed their separation [6–8,17]. However, due to the weak hydrophobic character of amino acids, RPLC is not efficient enough to separate the most polar ones. In contrast, ion-pairing chromatography is more appropriate [3,12,13,18–20]. Whatever the methodology, analysis of the most polar underivatized amino acids remains a great challenge. We reported in two recent papers [12,13] the analysis of the most polar amino acids as well as the possible determination in gradient elution of 17 of the 20 protein amino acids using ion-pair reversed-phase liquid chromatography (IP-RPLC) on octadecyl silica columns with long chain perfluorocarboxylic acids as the volatile ion-pairing reagent and an evaporative light scattering detector (ELSD). The complete determination by LC–ELSD was prevented by coelutions or by induced peaks occuring during the gradient elution phase that mask the elution of some amino acids. The use of LC–MS was necessary in order to identify all the protein amino acids. From these studies, it appears that the most efficient stationary phase for the separation was the most hydrophobic octadecyl silica support. Thus, the aim of this work is to evaluate the potential of a porous graphitic carbon (PGC) packing material and perfluorocarboxylic acids for the analysis of underivatized amino acids by LC–ELSD. Indeed, it has been shown that this stationary phase exclusively composed of rigid, two dimensional, planar carbon sheets without residual silanols is far more hydrophobic than the classical reversed-phase materials [21,22]. In addition to its strong reversed-phase behavior, delocalized π-electrons from the graphite can participate in the retention mechanism and even dominate it [23,24]. As a result PGC often exhibits unexpected properties and greater selectivities than reversed-phase toward aromatic or other conjugate systems [22,25] and polar compounds [26]. Thus, it is even possible to have retention and separation for ionic (cationic or anionic) compounds [27–30]. On PGC, the separation of two underivatized aromatic amino acids (Tyr and Phe) has already been obtained [31] and some chiral separations have been successfully achieved [32] but the separation of the 20 underivatized protein amino acids has never been investigated.

Therefore, PGC should be a possible additional column material providing an alternative separation potential to conventional reversed-phase packing material for IP-RPLC analysis of underivatized amino acids.

2. Experimental

2.1. Reagents

The twenty protein amino acids were purchased from Sigma (St Louis, MO, USA). Standards (250 mg l^{-1}) and aqueous solutions were prepared with deionized water obtained from an Elgastat UHQ II system (Elga, Antony, France). Trifluoroacetic acid (TFA), tridecafluoroheptanoic acid (TDFHA) were obtained from Interchim (Montluçon, France); heptafluorobutyric acid (HFBA), nonafluoropentanoic acid (NFPA) and pentadecafluorooctanoic acid (PDFOA) from Aldrich (St Quentin-Fallavier, France). TFA is the only HPLC-grade perfluorinated carboxylic acid; the others contain from 1 to 3% of impurities. HPLC-grade acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) were purchased from J.T. Baker (Noisy-le-sec, France).

2.2. Apparatus

The LC–ELSD system consists in a Varian 9010 (Palo Alto, CA, USA) ternary pump, a Rheodyne (Cotati, CA, USA) model 7125 injection valve fitted with a 3 μl loop, a Shimadzu (Kyoto, Japan) C-R5A integrator and a Sedere (Vitry s/Seine, France) Model Sedex 55 evaporative light scattering detector set as follows: drift tube temperature, 55°C; nebulizer gas pressure, 2.2 bar; photomultiplier, 10.

Temperature was regulated by cooling a Croco-cil (Cluzeau, France) oven then heating to the desired temperature (except for temperature effects, all experiments were done at room temperature, checked between 20 and 22°C).

Column equilibration was monitored using a Vydac conductivity meter (Wescan Instrument, Santa Clara, USA) model 6000 CD.

LC–MS was carried out using a Perkin Elmer (Toronto, Canada) model LC-200 binary pump, a Perkin Elmer Sciex (Forster City, CA, USA) API 300 mass spectrometer with IonSpray™ as ion source. Ionspray voltage was 5 kV. The orifice and

focusing ring were respectively at 20 V and 200 V. Dwell time was 200 ms. Injection were made by a Perkin Elmer (Toronto, Canada) series 200 autosampler fitted with a 3 μl loop.

Flow-rate was 200 μl min^{-1}. For LC–MS, a split of 1/10 was used to avoid too high a flow-rate in the ion source.

The porous graphitic carbon column was Hypercarb S (100×2.1 mm I.D. particle size 5 μm) from Hypersil (Runcorn, UK). Before each new equilibration, 12 ml of a Water/THF (50/50) mixture containing 0.5% TFA, then 12 ml pure THF, then 60 ml pure water were percolated through the column in order to regenerate its surface. As Na^+ is not retained on PGC [30] with water or aqueous solution of TFA as mobile phase, the column hold-up time (t_0) was estimated by injecting a NaOH solution (100 mg l^{-1} in Na) after regeneration.

3. Results and discussion

Deionized water allows the elution of 15 out of 20 underivatized amino acids. Some peaks are badly tailed and hence the separation is not efficient on a PGC support. Asp and Glu due to their negative charge at pH 7 are totally retained on the PGC surface with deionized water. This result confirms the presence of unique and strong interactions between the negatively charged centers in the analyte and the delocalized electrons on the PGC surface as previously established for common inorganic anions [30]. In other respects, the three neutral aromatic amino acids (Phe, Tyr, Trp) are not eluted with aqueous eluent and an addition of 50% ACN in the mobile phase is required to elute them near the void volume. This is in good agreement with the unique strong affinity of PGC well established for compounds with a planar structure containing aromatic ring electrons [22,25].

In order to increase the retention of amino acids on PGC support and to improve their separation, the addition of different ion-pairing reagents in the aqueous mobile phase was investigated. The choice of these reagents must be compatible with ELSD volatility requirements.

3.1. Choice of perfluorinated carboxylic acid as ion-pairing reagent

Perfluorinated carboxylic acids (TFA, HFBA, NFPA, TDFHA and PDFOA) have already been proved to be volatile ion pairing reagents suitable for LC–ELSD analysis of underivatized amino acids on octyl as well octadecyl silica support [10–13]. Hence, it was interesting to test these perfluorinated surfactants as ion-pairing reagents with PGC support and to compare the results to those obtained on classical RP packing materials.

3.1.1. Adsorbed quantities of surfactant and LC equilibration time

The adsorption of the different perfluorocarboxylic acids on the stationary phase was determined by measuring the breakthrough with a conductivity meter. Table 1 shows that the adsorbed quantities of ion pairing reagent on PGC support increases with surfactant concentration in the mobile phase as well as with the increase of the surfactant alkylchain length. Aqueous TFA mobile phases lead to an equilibration time (t_e) that corresponds to the hold up time (t_0). This means that TFA is not adsorbed on

Table 1
Influence of perfluorocarboxylic acid concentration and chain length (C_n) on the adsorbed quantities (μmol) on Hypercarb (100×2.1mm I.D.)

Surfactant concentration in mobile phase (m*M*)	Adsorbed quantities (μmol) of perfluoroalkyl carboxylic acid				
	PDFOA (C_7)	TDFHA (C_6)	NFPA (C_4)	HFBA (C_3)	TFA (C_1)
0.5	6.5	2.3	<0.1	<0.1	≈0
1	13	4.8	0.2	<0.1	≈0
5	nd[a]	26	1.0	0.4	≈0
15	nd[a]	nd[a]	3.0	1.0	≈0

[a] nd: not determined because of incomplete solubilisation of the perfluorinated surfactant.

the PGC surface. Fig. 1a depicts a typical equilibration profile obtained with HFBA or NFPA. Systems containing HFBA or NFPA (surfactants with respectively a C_3 or C_4 side chain) have rather short equilibration times (t_e) whatever the surfactant concentration range (0.5 to 15 m*M*) used: 90% of the mobile phase conductivity is recovered just after hold-up time. As a whole, 9 min for HFBA and 15 min for NFPA on average, at 200 μl min^{-1} flow-rate, are necessary to recover the eluent conductivity. The adsorbed quantities of HFBA and NFPA are thus negligible. It can be concluded that HFBA and NFPA have weak interactions with the PGC surface. Systems containing TDFHA or PDFOA (surfactants with respectively a C_6 or C_7 side chain) required respectively 35 minutes to 105 minutes (flow-rate 200 μl min^{-1}) to be equilibrated whatever the surfactant concentration. Fig. 1b depicts a typical equilibration profile obtained with TDFHA and PDFOA. It appears that an important quantity of TDHFA or PDFOA can be adsorbed on the porous graphitic carbon surface. The system then resembles a dynamic ion-exchange system.

3.1.2. Retention and selectivities

As on octadecyl silica bonded phases [11,12], it appears on PGC that TFA, even at high concentration, is ineffective in promoting selectivities for polar amino acids (Asp, Asn, Ala, Cys, Gln, Glu, Gly, Pro, Ser, Thr). In fact on PGC, TFA is a better electronic competitor toward organic or inorganic anions [29,30] than an ion pairing reagent toward cationic compounds. Therefore perfluorocarboxylic acids with longer side chains have been tested.

In our previous study [12] the surfactant with the longest side chain (0.5 m*M* PDFOA in water) has been proved to be the most suitable ion pairing reagent on a classical RP silica packing material. This fact is not observed on PGC support. Table 2 indicates the retention factors of the first eluted amino acids depending on the PDFOA concentration in mobile phase. On PGC support, several pairs

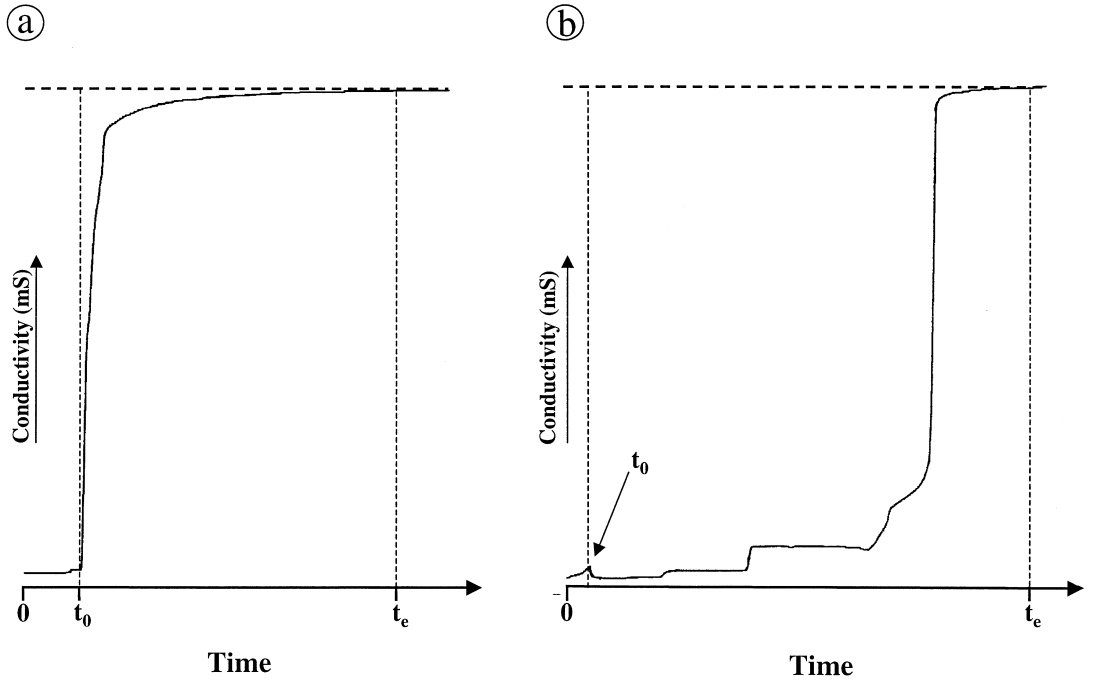

Fig. 1. Typical equilibration profiles obtained with: (a) heptafluorobutyric acid (HFBA) or nonafluoropentanoic acid (NFPA), (b) tridecafluoroheptanoic acid (TDFHA) or pentadecafluorooctanoic acid (PDFOA). t_0, hold up time; t_e, equilibration time corresponding to the eluent conductivity recovery, its value is discussed in Section 3.1.1. The eluent conductivity value varying from about 0.35 mS cm^{-1} for 1 m*M* in surfactant to 5 mS cm^{-1} for 15 m*M* in surfactant.

Table 2
Retention factor of the first ten eluted underivatized amino acids on PGC with PDFOA in aqueous water

Amino Acids	PDFOA concentration in the mobile phase	
	0.5 m*M*	1 m*M*
Gly	1.0	2.7
Ser	1.0	2.7
Asp	2.0	5.0
Ala	2.2	5.8
Cys	2.4	5.1
Asn	2.4	6.2
Thr	2.4	6.3
Pro	3.1	10.5
Gln	5.2	>15
Glu	9.7	>15

(Gly-Ser, Asp-Ala, Cys-Asn-Thr) cannot be separated by using PDFOA 0.5 m*M* in aqueous mobile phase. No improvement in selectivities was observed by increasing the PDFOA concentration. The Gly-Ser pair and the Asn-Thr pair remain without separation when PDFOA is used at 1 m*M*, whereas Gln and Glu cannot be eluted under these chromatographic conditions. Moreover for the Asp-Cys pair, selectivity is better with 0.5 m*M* than with 1 m*M* PDFOA. It appears that PDFOA is not suitable to separate the 10 amino acids selected. The same conclusion is obtained with TDFHA in a 0.5 to 10 m*M* concentration range. As expected in IP-RPLC, Table 3 shows that the shorter the ion-pairing reagent side chain is, the weaker the amino acid retention is for a given concentration of ion-pairing reagent in mobile phase. In section 3.1.1, the behavior of HFBA and NFPA toward the PGC stationary phase has been proved to be different from that of TDFHA and PDFOA. Thus, it can be interesting to investigate the effect on amino acid retention, of increasing HFBA or NFPA concentration in mobile phase. Higher concentrations of HFBA and NFPA lead to more acidic mobile phases (pH <2) but, due to the specific properties of PGC which allow its use in a large pH range (1 to 13) without damage, it was possible to work under such acidic conditions whereas it was not possible on classical RP packing materials. Fig. 2 shows the influence on amino acid retention factor of the NFPA concentration in aqueous mobile phase. As expected in IP-RPLC, the analysis time is significantly increased when the concentration of the ion pairing reagent is increased in mobile phase. Fig. 2 suggests better selectivities at high surfactant concentration. The best selectivities were obtained with 20 m*M* to 25 m*M* of NFPA. For higher concentrations (>25 m*M*) the retentions tend to decrease and an efficiency loss is observed, probably due to solubility problems. Moreover whatever the concentration range (1 to 40 m*M*) used, HFBA is not efficient enough to separate Cys, Asn and Asp.

3.1.3. Temperature effect

The temperature effect on the amino acid separation was investigated from the best chromatographic conditions obtained at room temperature. A few publications have studied the influence of temperature on retention behavior on the PGC surface. Okamoto et al. [33] have reported that the optimum temperature for the separation of some inorganic anions is 40°C while Karlson and Charron [34] have reported some better enantiomer resolutions at low temperature (below 0°C). In the case of polyethoxylated alcohols [35], no effect of the temperature on the retention behavior was noted. Therefore, it is not easy to predict what the temperature effect will be.

Fig. 3 shows that retention time is increased by

Table 3
Influence of the chain length (C_n) of the perfluorinated carboxylic acid on the retention factor (k) of six polar underivatized amino acids. Surfactant concentration is 1 m*M*

Perfluorinated carboxylic acid	Retention factor					
	Gly	Ser	Ala	Thr	Asn	Asp
HFBA (C_3)	0.3	0.3	0.3	0.4	0.6	0.9
NFPA (C_4)	0.4	0.4	0.5	0.6	0.8	0.9
TDFHA (C_6)	1.4	1.6	2.9	3.8	3.6	4.0
PDFOA (C_7)	2.7	2.7	5.8	6.3	6.2	5.0

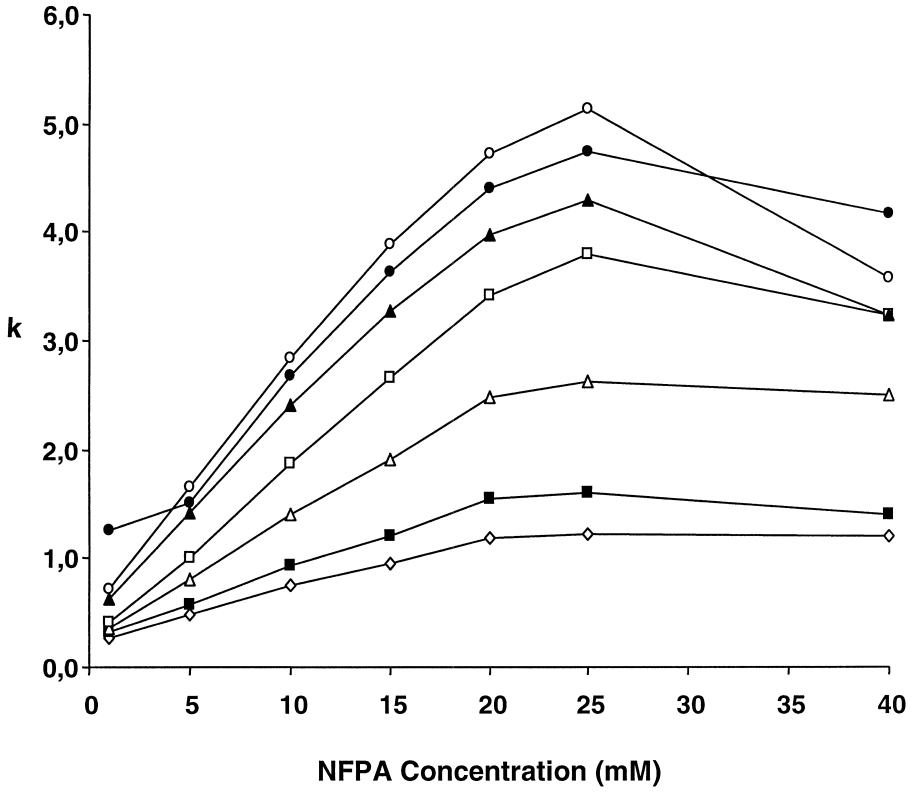

Fig. 2. Influence of the nonafluoropentanoic acid (NFPA) concentration in an aqueous mobile phase on the retention factor, *k*, of seven underivatized amino acids. Gly (◊), Ser (■), Ala (Δ), Thr (□), Asn (▲), Cys (●), Asp (○).

decreasing temperature but the amino acids do not all exhibit the same behavior toward temperature. The elution order of Asn, Asp and Cys is strongly dependent on the temperature: Cys is eluted before, between or after Asn and Asp respectively at 10°C, 20°C or 30°C. The polar amino acids are partially separated at 21°C. The resolution observed at this temperature is 1.02 for the Asn-Cys pair and 0.8 for the Cys-Asp pair. In contrast, Fig. 4 shows the excellent separation with satisfactory baseline resolution obtained at 10°C with 20 m*M* NFPA in aqueous mobile phase. Under these conditions all pairs are separated with a resolution better than 1.32. For amino acid analysis on PGC support, decreasing temperature plays an important role in improving separation. The elution order is as follows: first Gly is eluted then Ser, Ala, Thr, Cys, Asn, Asp, Pro, Gln and finally Glu. It was interesting to note that for the same amino acids, the elution order was different on octadecylsilica bonded material [12]. Moreover, the use of surfactant with a shorter side chain in mobile phase on PGC support than on C_{18}-bonded silica column involves that the optimized chromatographic system on PGC support has a faster equilibration time (about 15 min) than the previous system proposed on octadecyl silica bonded material (about 200 min).

Using a combination of these two isocratic chromatographic systems which have similar analysis time (about 30 min), will allow to identify the polar amino acids present in complex matrices without a derivation step.

3.2. Separation of the 20 protein amino acids using gradient elution

At 10°C the elution strength of the aqueous mobile phase containing 20 m*M* NFPA is too weak to elute

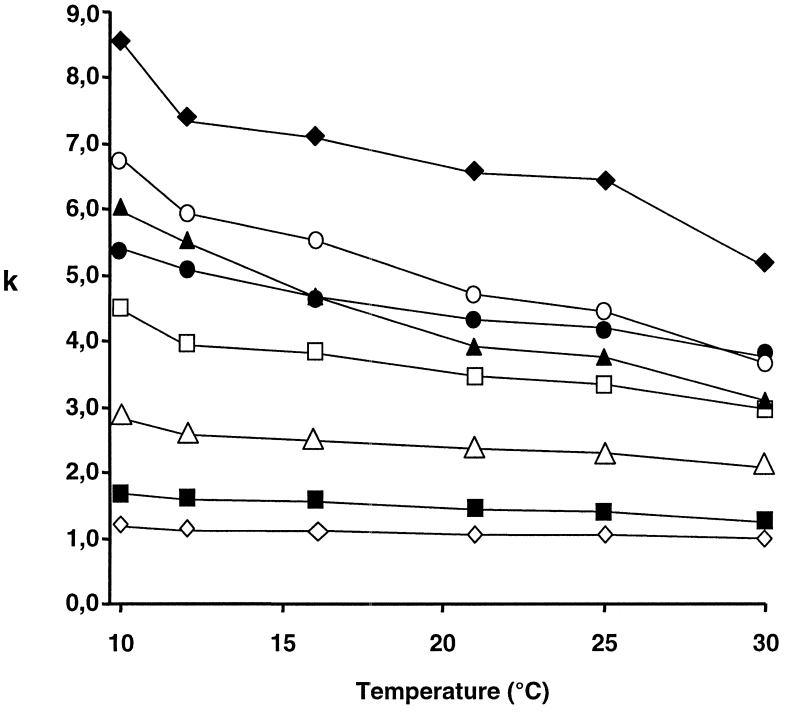

Fig. 3. Influence of the temperature on the retention factor as well as the selectivity of the first eight eluted underivatized amino acids. Eluent: Aqueous mobile phase containing 20 m*M* of nonafluroropentanoic acid (NFPA). Flow rate: 200 μl min^{-1}. Detection: Evaporative Light Scattering Detector (ELSD) set as in section 2.2. Gly (◊), Ser (■), Ala (Δ), Thr (□), Asn (▲), Cys (●), Asp (○), Pro (♦).

the other ten protein amino acids (Val, Ile, Leu, Met, Lys, His, Arg, Phe,Tyr, Trp). Thus it was necessary to investigate gradient elution conditions to analyse simultaneously the 20 protein amino acids. Two approaches to gradient separation were evaluated.

The first approach was to use a concentration gradient in which the concentration of the ion pairing reagent in aqueous mobile phase was decreased over the run. This method proved to be of limited utility. The mobile phase gradient was obtained as follows: NFPA 20 m*M* in aqueous solution to pure water in 10 min, this composition was then maintained for a further 30 min. It was then possible to elute at a flow-rate of 200 μl min^{-1} in less than 25 min Val (t_r=18 min), Lys (t_r=21 min), Met and Leu which are coeluted (t_r=22.5 min), then Ile (t_r=23.2 min) but aromatic amino acids cannot be eluted under these conditions. In order to keep a constant pH value during the run, the following gradient elution conditions were also tested: NFPA 20 m*M* in aqueous solution to TFA 20 m*M* in 10 min then this composition was maintained for a further 30 min. The peaks observed are tailing and retention times increase slightly. For example, Lys is eluted in 24.5 min and Ile in 31.6 min.

The second approach involved decreasing the concentration of ion pairing reagent and increasing the amount of the organic modifier simultaneously, thereby decreasing the retention time of the most hydrophobic and aromatic amino acids. Satisfactory gradients were obtained by this method.

Acetonitrile and methanol were tested as organic modifiers. Under isocratic conditions, the elution strength of mobile phase containing 40% (v/v) MeOH is similar to that of mobile phase containing 15% (v/v) ACN. When MeOH is used rather than ACN no differences in selectivities were observed thus, ACN is selected for further investigation. The elution of aromatic amino acids requires at the very least 20% (v/v) ACN in mobile phase for Phe and Tyr and 30% (v/v) ACN for the most retained amino acid, Trp. Fig. 5 depicts the analysis of 20 un-

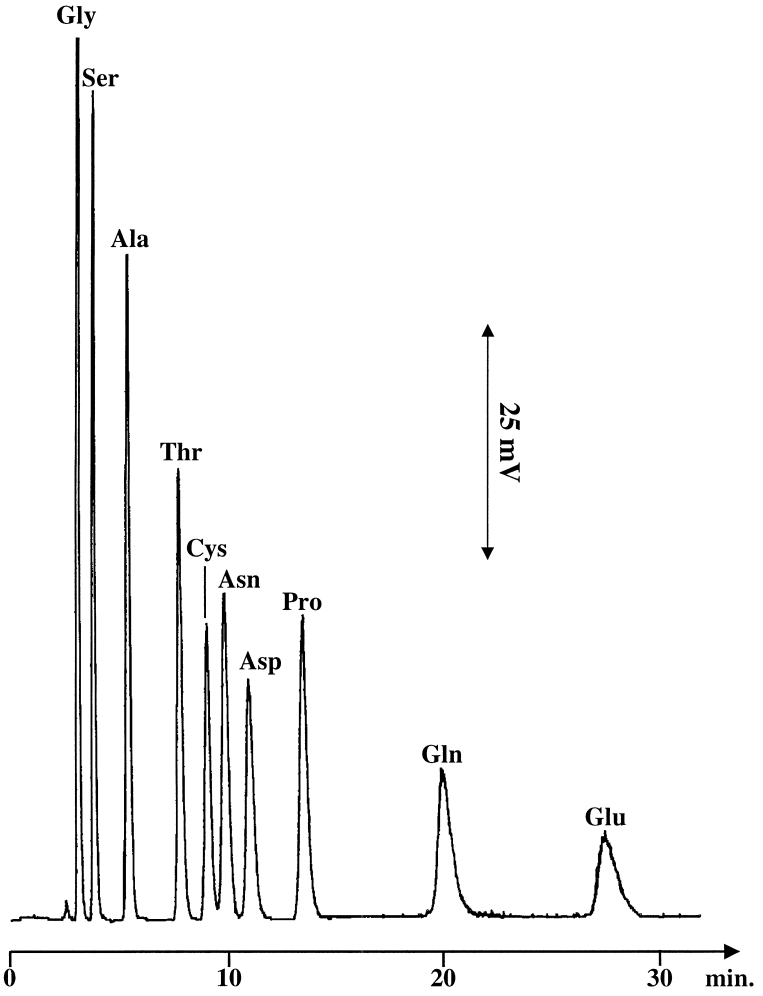

Fig. 4. LC analysis of a standard solution of 10 underivatized amino acids under isocratic conditions on PGC support. Column temperature, 10°C; eluent, NFPA 20 m*M* in water; flow-rate, 200 μl min^{-1}; evaporative light scattering detection (for settings, see section 2.2).

derivatized amino acids achieved in 40 min with an ACN gradient. The cycle time was 60 min including the reequilibration time. The mobile phase gradient was obtained from eluent A (20 m*M* NFPA in aqueous mobile phase) and eluent B (Acetonitrile) as follows: from 0 to 15% (v/v) ACN in 10 min, then 26% (v/v) ACN in 10 min, then 50% (v/v) ACN in 10 min and finally 50% (v/v) ACN maintained until the end of the analysis. The elution order is different on PGC and on RP packing material. On PGC first Gly is eluted then Ser, Ala, Thr, Cys, Asn, Asp, Pro, Gln, Glu, Val, Lys, Leu, Met, Ile, His, Arg, Phe, Tyr and finally Trp whereas on classical RP column first Asp is eluted then Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala, Pro, Val, Met, Tyr, Ile, Leu, Phe, Trp, His, Lys and finally Arg.

The main advantage of the chromatographic system optimized on PGC for the separation of 20 amino acids, in comparison with the analysis on Purospher RP 18e support [13], is the absence of a system peak occuring during the gradient elution. This is probably due to the better volatility of NFPA with respect to PDFOA and also perhaps because of the very low quantities of this surfactant adsorbed on the PGC surface in contrast with that of PDFOA.

Under these LC–ELSD chromatographic condi-

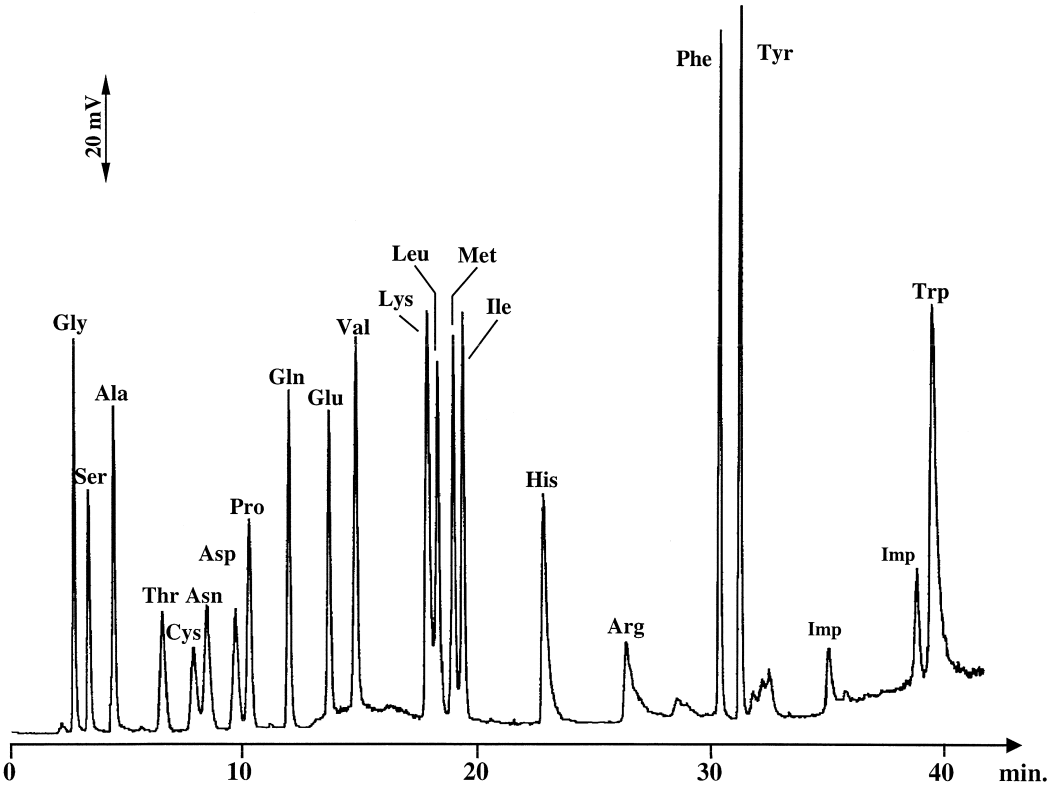

Fig. 5. LC–ELSD analysis of the 20 underivatized amino acids in gradient elution on Hypercarb (100×2.1 mm I.D.). Eluent A: nonafluoropentanoic acid (NFPA) 20 m*M* in an aqueous mobile phase. Eluent B: Acetonitrile. Gradient profile is: from 0 to 15% ACN in 10 min, then 26% ACN in 10 min and finally 50% ACN in a further 10 min. 50% ACN is maintained until the end. Column temperature is 10°C. Flow rate: 200 μl min^{-1}. Detection: Evaporative Light Scattering Detector (ELSD) set as in section 2.2. Imp: Impurity.

tions the determination of the 20 protein underivatized amino acids is achieved with detection limits varying from about 1.25 mg l^{-1} (injected amount around 30 pmol) for most of the amino acids to about 2.5 mg l^{-1} (injected amount around 60 pmol) for Asp and Cys.

This separating methodology can also be coupled with MS detection via a pneumatically assisted electrospray ion source, as NPFA is very volatile and no system peaks have been observed. The LC–MS chromatographic profile looks quite similar to the one obtained with LC–ELSD [36]. The detection limit for LC–MS methodology without further optimization is varying from 3 mg l^{-1} (e.g. Asp and Ala) to 0.2 mg l^{-1} (e.g. Leu, Ile, Phe) but for Asn the detection limit is only 7 mg l^{-1} (because the eluent is particularly noisy at the mass corresponding to Asn, probably due to a surfactant impurity). Currently, it is really easy to obtain a detection limit about 50 pmol of amino acid by LC–MS.

4. Conclusion

PGC column has a great potential for the analysis of amino acids. *n*-Alkyl chain perfluorinated carboxylic acids have proved to be excellent ion-pairing reagents for the amino acid separation and compatible with an evaporative light scattering detection.

The optimized chromatographic system has a faster equilibration time than the previous system proposed on octadecylsilica bonded material. The elution order of the amino acids on PGC support is quite different from that on ODS packings. These two LC–ELSD systems therefore provide complementary informations and can be used favorably

as complementary techniques for identification of trace amounts of minor amino acids present in mixture with a major amino acid for example. The optimized LC–ELSD system was directly suitable to LC–MS. ELSD proved to be an inexpensive way to develop a LC–MS method. Quantitative analysis (precision, reproducibility and validation) as well as complex matrix studies is currently in progress.

References

[1] L. Stryer, Biochemistry, 3rd ed., W.H. Freeman and Company, New York, 1988, Chapter 2.
[2] L.E. Vera-Arila, M. Caude, R. Rosset, Analusis 10 (1982) 43.
[3] J. Crommen, P. Herné, J. Pharm. & Biomed. Anal. 2 (1984) 241.
[4] J. Crommen, G. Schill, D. Westerland, J. Chromatogr. 461 (1989) 429.
[5] M. Eslami, P. Hoshemi, M.N. Sarbolaki, J. Chromatogr. Sci. 31 (1993) 480.
[6] L.E. Welch, W. R Lacourse, D.A. Mead Jr, D.C. Johnson, Anal. Chem. 61 (1989) 555.
[7] P. Luo, F. Zhang, R. P Baldwin, Anal. Chem. 63 (1991) 1702.
[8] J.M. Marioli, L.E. Sereno, J. Liq. Chromatogr. & Rel. Technol. 19 (1996) 2505.
[9] V. Camel, D. Thiebault, M. Caude, M. Dreux, J. Chromatogr. 605 (1992) 95.
[10] H.J. Chaves das Neves, Z. Braya-Morais, Anal. De Quim. 93 (1997) 98.
[11] H.J. Chaves das Neves, Z. Braya-Morais, J. High Resol. Chromatogr. 20 (1997) 115.
[12] K. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, J. Chromatogr. A 833 (1999) 147.
[13] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, J. Chromatogr. A 855 (1999) 191.
[14] R.G.J. van Leuken, G.T. C Kwakkenbos, A.L.L. Duchateau, J. Chromatogr. 647 (1993) 131.
[15] W. Lu, G. Yang, R.B. Cole, Electrophoresis 16 (1995) 487.
[16] R. Schuster, Anal. Chem. 52 (1981) 617.
[17] C. Murren, D. Stelling, G. Felstead, J. Chromatogr. 115 (1975) 236.
[18] J. Saurina, S. Hernandez-Cassou, J. Chromatogr. A 676 (1994) 311.
[19] Y. Yokoyama, O. Ozaki, H. Sato, J. Chromatogr. A 739 (1996) 333.
[20] Y. Yokoyama, T. Amaki, S. Horidoshi, H. Sato, Anal. Sciences 13 (1997) 963.
[21] N. Tanaka, T. Tanigawa, K. Kimata, K. Hosota, T. Araki, J. Chromatogr. 549 (1991) 29.
[22] J. Kriz, E. Adamkova, J.H. Knox, J. Hora, J. Chromatogr. A 663 (1994) 151.
[23] B.J. Bassler, R. Kaliszan, R.A. Hartwick, J. Chromatogr. 461 (1989) 139.
[24] E. Forgacs, T. Cserhati, Chromatographia 33 (1992) 356.
[25] B.J. Fish, J. Pharm. & Biomed. Anal. 11 (1993) 517.
[26] M.C. Hennion, V. Coquart, S. Guenu, C. Sella, J. Chromatogr. A 712 (1995) 287.
[27] C.K. Lim, Biomed. Chromatogr. 3 (1989) 92.
[28] H.C. Ehrsson, I.B. Wallin, A.S. Andersson, P.O. Edlund, Anal. Chem. 67 (1995) 3608.
[29] G. Gu, C.K. Lim, J. Chromatogr. 515 (1990) 183.
[30] C. Elfakir, P. Chaimbault, M. Dreux, J. Chromatogr. A 829 (1998) 193.
[31] J.E. Mama, A.F. Fell, B.J. Clark, Analytical Proceedings 26 (1989) 71.
[32] Q.-H. Wan, P.N. Shaw, M.C. Davies, D.A. Barrett, J. Chromatogr. A 765 (1997) 187.
[33] T. Okamoto, A. Isozaki, H. Nagashima, J. Chromatogr. A 800 (1998) 239.
[34] A. Karlsson, C. Charron, J. Chromatogr. A 732 (1996) 253.
[35] P. Chaimbault, C. Elfakir, M. Lafosse, J. Chromatogr. A 797 (1998) 83.
[36] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, 23rd International Symposium on High Performance Liquid Phase Separations and Related Techniques, HPLC'99, 30 May–4 June 1999, Grenada, Spain, Poster.