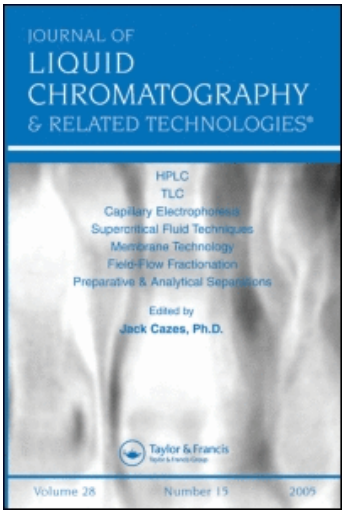

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SEPARATION OF UNDERIVATIZED SMALL PEPTIDES ON A POROUS GRAPHITIC CARBON COLUMN BY ION-PAIR CHROMATOGRAPHY AND EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

Separations of small peptides (di-, tri- and tetrapeptides) using ion-pair chromatography and evaporative light scattering detection have been investigated with a porous graphitic carbon stationary phase, Hypercarb®. Five perfluorinated carboxylic acids (trifluoroacetic (TFA), pentafluoropropanoic (PFPA), heptafluorobutanoic (HFBA), nonafluoropentanoic (NFPA) and tridecafluoroheptanoic (TDFHA) acids) have been evaluated as ion-pairing agents. Influence of the percentage of organic modifier, concentration, and chain length of the ion-pairing agent on the retention factors, k, has been examined. Equilibration times were also studied. Separations of dipeptides or tri- and tetrapeptides are presented using HFBA and NFPA at 20 mM and TDFHA at 2.5 mM.

INTRODUCTION

Considering the growing importance of small peptides as therapeutic and sweetening agents, there is an increasing interest in the development of techniques for the analysis of these compounds. Reversed-phase liquid chromatography (RP-LC) is the most widely used analytical technique for separation of oligopeptides.[1,2] A number of papers also reported enantiomeric separations of small peptides by LC with different types of chiral selectors such as cyclodextrins[3,4] or crown ether.[5] During recent years, capillary electrophoresis has also been employed for chiral separations.[6,7] Most of small peptides do not show UV absorption, and frequently peptides are derivatized to enable or improve detection by UV,[8,9] fluorimetry,[10,11] or electrochemistry.[12] To eliminate these pre- or post-column derivatization steps, separations of underivatized amino acids have been studied using evaporative light scattering detector (ELSD)[13,14] and pneumatically assisted electrospray mass spectrometry.[15] The ELSD may be considered as an universal LC detector for analytes which are less volatile than the chromatographic eluent.[16,17] It is more sensitive than a refractive index detector and perfectly compatible with gradient elution.

Our group recently published a separation of twenty underivatized amino acids using ELSD and ion-pair liquid chromatography (IP-LC) on a porous graphitic carbon column.[18] IP-LC was necessary to enhance retention of the most polar amino acids on the apolar stationary phase. Ion-pairing agents were perfluorinated carboxylic acids which have low boiling points and are, therefore, perfectly compatible with ELSD. Ion pairs are then formed between the amino groups and the anionic surfactant. Addition of trifluoroacetic acid in eluent to increase retention of peptides on alkyl silicas is well-known.[1] Perfluorinated carboxylic acids with longer n-alkyl chains are also employed[19,20] and the heptafluorobutyric acid was found to be a powerful ion-pairing agent for arginine-, histidine-, and lysine-rich peptides.[21]

The choice of a porous graphitic carbon material (PGC) for analysis of amino acids[18] was justified by its unique LC properties. This material has been developed as a very insoluble and stable LC support.[22,23] This phase shows a highly ordered crystalline surface with large bands of delocalized electrons, and has proved to be a unique material since the retention mechanism is a mixture of hydrophobic and electronic interactions.[24-26] Separations of ionic compounds are, therefore, possible.[27,28] Chiral separations have already been mentioned.[29] The objective of this work was, then, to evaluate the potential of PGC for the analysis of underivatized small peptides using ion-pair chromatography, ELSD, and perfluorinated carboxylic acids as ion-pairing agents.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile (ACN) and tetrahydrofuran (THF) were from J. T. Baker (Noisy-le-Sec, France). LC-grade water was prepared by purifying distillated water in an Elgastat UHQ II system from Elga (Villeurbanne, France).

Trifluoroacetic acid (TFA) and tridecafluoroheptanoic acid (TDFHA) were obtained from Interchim (Montluçon, France). Pentafluoropropanoic acid (PFPA), heptafluorobutanoic acid (HFBA), nonafluoropentanoic acid (NFPA), were from Aldrich (St-Quentin-Fallavier, France). TFA is the only HPLC-grade perfluorinated carboxylic acid, the others contain from 1 to 3% of impurities.

Four peptides (L-Ala-L-Glu, L-Gly-L-Glu, L-Ala-L-Pro, L-Gly-L-Tyr) were from Rexim (Ham, France). Other small peptides were from Sigma (Steinheim, Germany). Stock solutions (1000 $mg.L^{-1}$) were prepared by weighing and dissolving peptides in a water /acetonitrile mixture (50/50,v/v) and were stored at -18°C. Final concentrations of injected solutions were comprised between 80 and 150 $mg.L^{-1}$.

HPLC Apparatus

The HPLC equipment consisted of a Merck-Hitachi LaChrom system equipped with a L7100 quaternary pump, a L7200 automatic injector, and a D7000 interface (Merck, Darmstadt, Germany). Piloting, data acquisition, and data processing were provided by HSM software.

The ELSD was a Sedere (Vitry-sur-Seine, France) model Sedex 55. The usual ELSD settings were as follows : drift tube 50°C, nebulizer gas pressure 2.2 bar.

Equilibration times were determined using a Vydac conductivity meter (Wescan Instrument, Santa Clara, USA) model 6000 CD.

The porous graphitic carbon column was Hypercarb S (100 x 2.1 mm I.D., particle size 7 µm) from Hypersil (Runcorn, UK). Flow-rate was 200 $\mu L.min^{-1}$. The analytical method developed with this LC-ELSD system could, therefore, constitute a primary step for an eventual LC-MS coupling. Between each new equilibration, 15 mL of THF then 15 mL of pure water were percolated through the column in order to regenerate its surface. The void volume of the column was determined by injecting an aqueous NaOH solution (100 $mg.L^{-1}$).

During separations of peptides, gradient elution was used. To maintain the constant con centration of the ion-pairing agent during the analysis, it was introduced in equal concentration in the aqueous and organic phases.

RESULTS AND DISCUSSION

Presentation of Small Peptides

Thirteen dipeptides which are lacking of chromophore groups, with the exception of L-Tyr-L-Glu, L-Gly-L-Tyr, and L-Lys-L-Phe, were selected (see Table 1). L-Asp-L-Asp-L-Asp, tri-, and tetramer of Lysine, and oxidized and reduced Glutathione, were also included in the list. The negative logarithms of the dissociation constant, pKa, calculated with Pallas software[30] are also presented.

A preliminary study has shown that most of these peptides are weakly retained on the PGC support with a 100% aqueous eluent. Asp-Asp, due to its three negative charges at neutral pH, is totally retained on PGC with this eluent. An identical result was observed with Asp and Glu by Chaimbault et al.,[18] confirming existence of strong interactions between the negatively charged analytes and the delocalized electron cloud on the PGC surface.[26,28] Strong affinity existing between PGC and planar aromatic compounds,[31] a total retention in pure water is also observed with dipeptides with an aromatic amino acid (Tyr-Glu, Gly-Tyr and Lys-Phe).

Determination of Equilibration Times

Equilibration times were determined by plotting the curves representing the variation of the conductance of the effluent versus the percolation time of eluent through the chromatographic system (capillary tubes and analytical column) with a conductivity meter. Equilibration times are then specific to the HPLC system. Different concentrations (from 10 to 40 mM) were used for TFA, PFPA, HFBA, and NFPA. These concentrations lead to pH inferior to 2 but no deterioration of the carbon phase is observed since PGC is totally resistant to extreme acidic or basic conditions. For TDFHA, concentrations were inferior to 5 mM due to the weak solubility of this acid in water at 25°C. The HPLC system is equilibrated when the effluent conductance is constant and equal to the eluent conductance.

For TFA, PFPA, HFBA, and NFPA, the same conductance variations and identical equilibration times of the chromatographic system are observed. These times are equal to 25 minutes (flow-rate 200 $\mu L.min^{-1}$), whatever the concentration of the ion-pairing agent used (see Figure 1a). For TDFHA, equilibration times increase with the concentration of the acid (45 minutes for

Table 1

List of Investigated Small Peptides and Negative Logarithm of the Dissociation Constant, pKa.

Peptides	pKa* Acidic Functions	pKa* Basic Functions
L-Gly-L-Gln (G-Q)	3.25	8.04
L-Ala-L-Gln (A-Q)	3.49	8.04
L-Asp-L-Asp (D-D)	2.91/3.25/4.51	8.12
L-Lys-L-Asp (K-D)	2.91/4.51	8.04/10.15
L-Tyr-L-Glu (Y-E)	3.38/4.70/9.90	7.43
L-Lys-L-Lys (K-K)	3.61	8.04/9.85/10.45
L-Val-L-Gly (V-G)	3.52	8.29
L-Lys-L-Pro (K-P)	3.57	7.71/10.15
L-Ala-L-Pro (A-P)	3.57	7.71
L-Lys-L-Met (K-M)	3.45	8.04/10.15
L-Gly-L-Tyr (G-Y)	3.06/10.04	8.04
L-Lys-L-Leu (K-L)	3.66	8.04/10.15
L-Lys-L-Phe (K-F)	3.44	8.04/10.15
L-Asp-L-Asp-L-Asp (D-D-D)	2.91/3.25/3.80/4.51	8.12
L-Lys-L-Lys-L-Lys (K-K-K)	3.61	8.04/9.67/10.15/10.63
L-Lys-L-Lys-L-Lys-L-Lys (K-K-K-K)	3.61	8.04/9.55/9.97/10.33/10.75
Glu-Cys-Gly (G-SH)	3.58/4.18/8.37	8.01
L-Glu-L-Cys-L-Gly | L-Glu-L-Cys-L-Gly (G-SS-G)	3.30/3.88/3.91/4.48	7.71/8.32

* Calculated with Pallas software.[30]

1 mM of TDFHA, 50 minutes for 2 mM and 55 minutes for 3 mM) (see Figure 1b).

Identical equilibration times observed for the four acids (TFA, PFPA, HFBA, and NFPA) clearly show that these compounds are not adsorbed on the PGC surface. On the other hand, TDFHA has strong interactions with the carbon material. The stationary phase can then be assimilated to a dynamic cation-exchanger. These results confirm those observed by Chaimbault et al.[18]

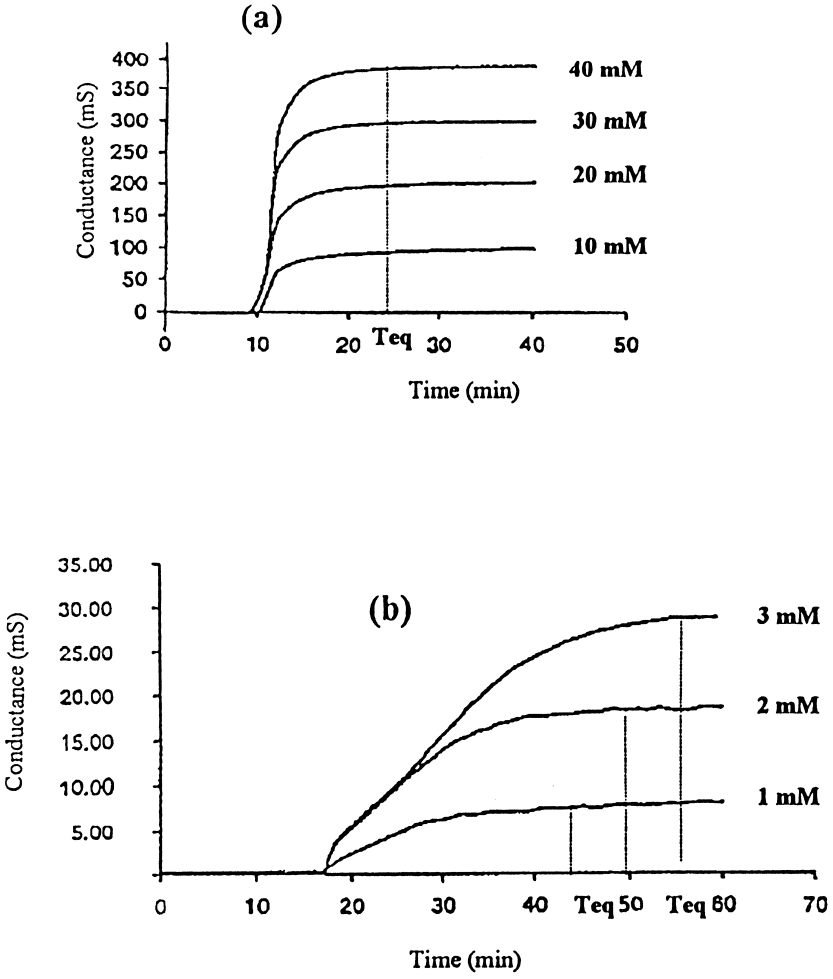

Figure 1. Variations of the conductance (mS) of the effluent with the percolation time (min) of eluent through the chromatographic system with (a) TFA, PFPA, HFBA, or NFPA, and (b) TDFHA. t_{eq} is the equilibrium time.

Retention and Selectivities

The variations of the retention factors, k, of dipeptides with the nature and the concentration of the ion-pairing agents were first investigated. The concentration range for TFA, PFPA, HFBA, and NFPA was from 0 to 30 mM, and

only from 0 to 5 mM for TDFHA. Using TFA, several dipeptides are eluted near the void volume, even with a 40 mM TFA solution as eluent. Therefore, TFA is not a convenient ion-pairing agent to separate various structural small peptides on a carbon support.

Important differences of retention between dipeptides with a Lysine unit and the others (Val-Gly, Ala-Pro, Gly-Gln, Asp-Asp, and Ala-Gln) were observed whatever the ion-pairing agent used. In consequence, the percentage of acetonitrile in the eluent was set at 15% for the first group (Lysine dimers) and at only 5% for the second group. The higher retention factors of Lysine dimers may be explained by the presence of two (cationic) amino groups in the peptide structure, instead of only one for other peptides. Figure 2a and 2b, respectively, show the curves representing the variation of k versus the concentration of the ion-pairing agent NFPA for the five dipeptides Val-Gly, Ala-Pro, Gly-Gln, Asp-Asp, and Ala-Gln, and for the lysine dimers. As expected in ion-pair chromatography, retention factors increase with the concentration of the ion-pairing agent. Retention factors observed using PFPA and HFBA are weaker, whereas they are higher using TDFHA (curves not presented), confirming an increase of retention with the chain length of the surfactant.

For the same ion-pairing agent, selectivities are better at high concentrations, and the best ones are obtained with TDFHA. However, NFPA may be considered as a more suitable ion-pairing agent than TDFHA to separate dipeptides, since correct selectivities are observed with smaller k; moreover, the equilibration step is faster (25 minutes instead of 55 minutes with 3 mM of TDFHA). Considering the two other acids, PFPA and HFBA, mediocre selectivities are observed for several pairs of dipeptides (i.e. Asp-Asp/Ala-Pro, Ala-Gln/Gly-Gln, Lys-Leu/Lys-Met, Lys-Pro/Lys-Asp).

The curves obtained with the three dipeptides with an aromatic group (Gly-Tyr, Tyr-Glu, and Lys-Phe) have been plotted for HFBA and NFPA but are not reported in this paper. Due to the presence of an aromatic group in their structures, these peptides are highly retained on the carbon material and a 25% ACN eluent is required to observe retention factors inferior to 20 with NFPA at 20 mM.

The influence of the percentage of organic modifier (ACN) in eluent on retention of five dipeptides (Val-Gly, Gly-Gln, Ala-Gln, Ala-Pro, Asp-Asp) was also studied. NFPA was used as ion-pairing agent at a 10 mM concentration. Retention factors rapidly decrease with the increase of ACN content in eluent, the eluotropic strength of mobile phase being more important.

Separation of Small Peptides

The first tests of separations of dipeptides were achieved using NFPA as an ion-pairing agent at 20 mM. Considering the large differences between

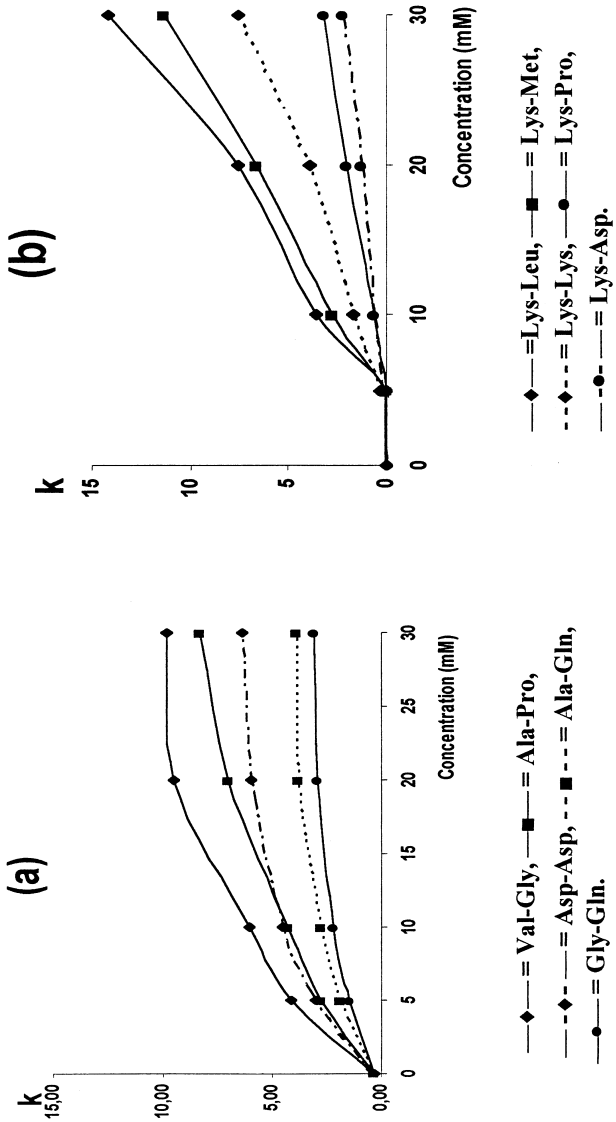

Figure 2. Variations of the retention factors, k with the concentration of the ion-pairing agent NFPA on a PGC column using a mobile phase containing (a) 5% or (b) 15% of acetonitrile.

retention factors of peptides, a gradient elution using ACN as organic modifier was applied. A first chromatogram was obtained with a linear gradient from 0 to 100% of ACN in 60 minutes. Three groups of peaks were observed: the first group composed of peptides with no lysine unit and no aromatic group, the second one constituted the peptides with a lysine unit, and the last one contained peptides with an aromatic group (Gly-Tyr, Tyr-Glu, and Lys-Phe).

Resolutions being too weak, the slope of the elution gradient was modified (from 0 to 50% of ACN in 60 minutes). The corresponding chromatogram is shown Figure 3. A well-defined baseline resolution is observed for all dipeptides, with the exception of Tyr-Glu and Gly-Tyr which are coeluted. Different modifications of the elution gradient have been tested to separate these two dipeptides but no significant improvement in resolution was observed. The

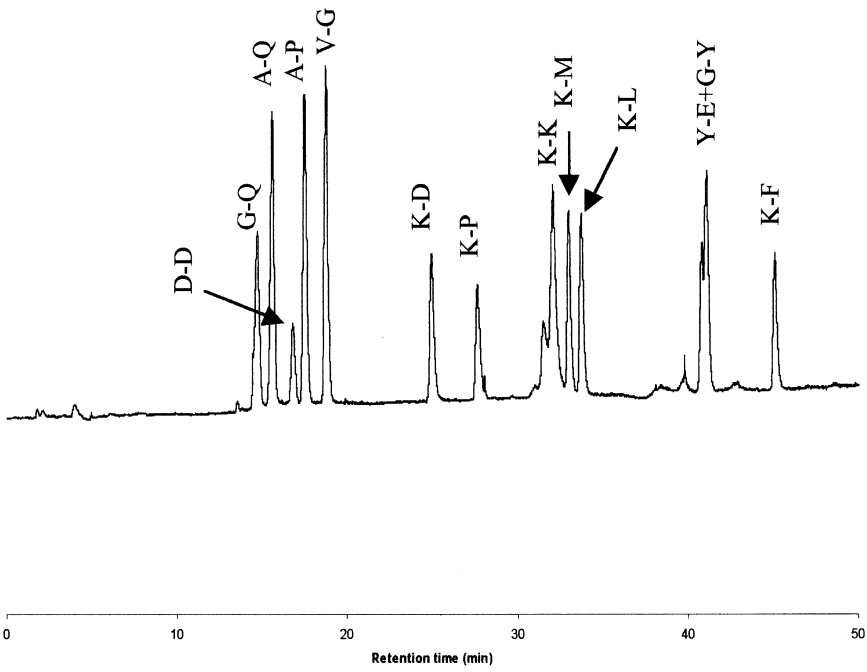

Figure 3. LC analysis of 13 small peptides on PGC support by ion-pair chromatography (see Table 1 for abbreviations of peptides). Mobile phase: water/acetonitrile (each containing NFPA at 20 mM), gradient profile: 0% of acetonitrile to 50% in 60 minutes. Flow-rate: 200 $\mu L.min^{-1}$, evaporative light scattering detection: drift tube 50°C, nebulizer gas pressure 2.2 bar.

separation of these two dipeptides is achieved using HFBA at 20 mM (see chromatogram Figure 4). However, a total loss of resolution between the high polar dipeptides (Ala-Gln, Gly-Gln, and Val-Gly) is observed, as expected, considering bad selectivities observed in the previous section.

Another alternative to separate Tyr-Glu and Gly-Tyr consists of employing TDFHA at 2.5 mM (see chromatogram Figure 5). However, an increase in the analysis time is observed (100 minutes instead of 50 minutes with NFPA). Moreover, a system peak is observed between 40 and 50 minutes. This observation has already been mentioned in ion-pair chromatography for analysis of amino acids using TDFHA as surfactant, and can be explained by the desorption of the ion-pairing agent with the increasing ACN percentage in the mobile phase.[15] Dipeptides Lys-Met and Lys-Leu are not separated on this chro-

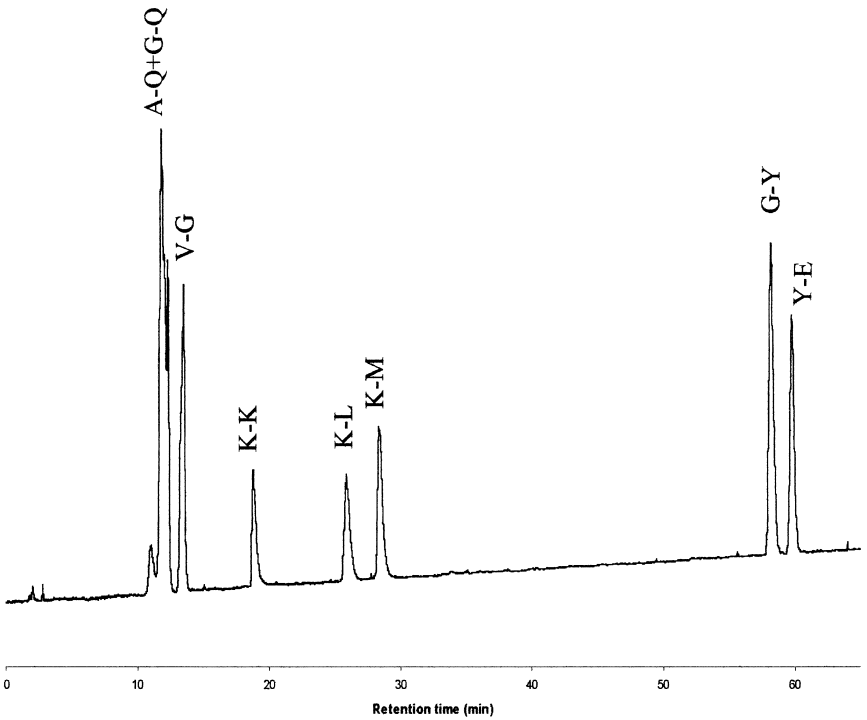

Figure 4. LC analysis of 8 small peptides on PGC support. Mobile phase: water/acetonitrile (each containing HFBA at 20 mM), gradient profile: 0% of acetonitrile to 30% in 80 min. Same other experimental conditions as Figure 3.

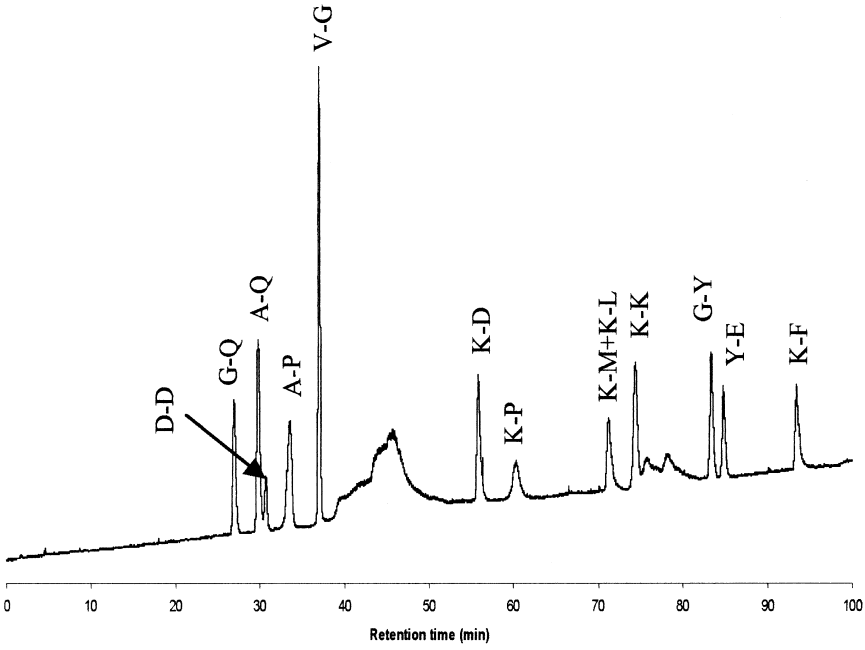

Figure 5. LC analysis of 13 small peptides on PGC support. Mobile phase: water/acetonitrile (each containing TDFHA at 2.5 mM), gradient profile: 0% of acetonitrile to 30% in 100 min. Same other experimental conditions as Figure 3.

matogram, but the mobile phase was not optimized to improve resolution between these peptides.

The separation of oxidized and reduced glutathione and tri- and tetrapeptides was also investigated using NFPA at 20 mM. These compounds were not included in the previous list since they are coeluted with dipeptides having a lysine unit. The chromatogram is presented Figure 6. Excellent resolution is observed.

CONCLUSION

PGC has proven to be a convenient stationary phase to separate small peptides by ion-pair chromatography. Its high stability over the full range of pH allows separations with strongly acidic pH mobile phases. ELSD permits a

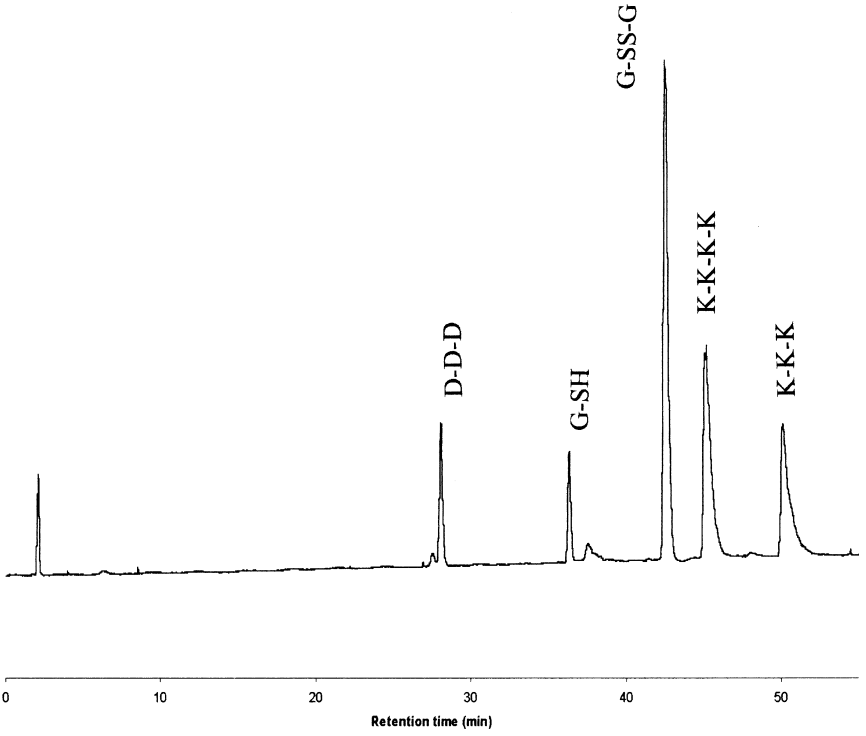

Figure 6. LC analysis of tri- and tetrapeptides and oxidized and reduced glutathione on PGC support. Mobile phase: water/acetonitrile (each containing NFPA at 20 mM), gradient profile: 0% of acetonitrile to 40% in 70 minutes. Same other experimental conditions as Figure 3.

direct analysis of peptides without pre- or post-column derivatization. The perfluorinated carboxylic acids used as ion-pairing agents being volatile, separations obtained with an elution gradient mode are totally compatible with ELSD. Moreover, this LC-ELSD system constitutes an ideal primary step to develop analysis by LC-MS, as demonstrated with amino acids.[15]

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