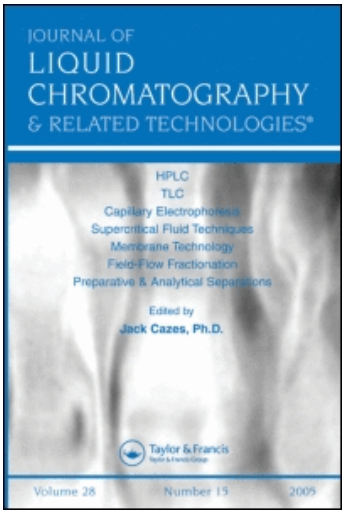

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Separation of Proteic Primary Amino Acids under Several Reversed-Phase Liquid Chromatographic Conditions

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Abstract: The reversed-phase liquid chromatographic (RPLC) analysis of proteic primary amino acids with acetonitrile-water, using pre-column derivatisation with *o*-phthalaldehyde (OPA) and *N*-acetylcysteine (NAC), was compared with RPLC modes using trifluoroacetic acid or pentadecafluorooctanoic acid and evaporative light-scattering detection, or sodium dodecyl sulphate micelles with pre- and post-column derivatisation. The importance of column lifetime, risk of potential damages in the instrumentation, retention and resolution, was considered. Among the assayed approaches, the best is still aqueous-organic RPLC with pre-column derivatisation. It not only yields the most reliable results, but allows acceptable resolution and longer column lifetime, and its implementation is simpler.

Keywords: Amino acids, Reversed-phase liquid chromatography, Acetonitrile, Trifluoroacetic acid, Pentadecafluorooctanoic acid, Sodium dodecyl sulphate

INTRODUCTION

One of the most frequent and important applications of liquid chromatography is the determination of amino acids (AAs),[1] which is usually carried out in reversed-phase mode (RPLC). Hundreds of reports can be found in the literature, where AAs are eluted under various conditions (usually with acetonitrile or methanol as modifiers at different pH). UV-visible detectors are the most common for routine analysis. However, most AAs do not absorb in the

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accessible range, and, therefore, should be derivatised to be monitored.[2] Among the recommended derivatisation reagents, the mixture of *o*-phthalaldehyde (OPA) and a thiol deserves a special mention. This mixture reacts with AAs to form isoindoles, with characteristic and suitable retention, which are highly fluorescent, allowing very low detection limits.[3,4] Also, automatic devices have been designed to carry out post-column derivatisation.[5] Alternatively, there has been an interest to apply detectors able to monitor free AAs, such as evaporative light-scattering (ELS) and mass spectrometry (MS).[5]

Besides the importance of detection, other features, such as column lifetime, risk of instrumentation damage, and especially, retention and resolution, should be taken into account to prospect the real analytical potential of a procedure. We implemented several RPLC procedures reported in the last decade for the analysis of proteic primary AAs to evaluate their performance: the aqueous-organic mode with acetonitrile-water using pre-column derivatisation with OPA and *N*-acetylcysteine (NAC), the ion pairing mode with trifluoroacetic acid (TFA) or pentadecafluorooctanoic acid (PDFOA) with direct detection of free AAs using ELS, and the micellar mode with the anionic surfactant sodium dodecyl sulphate (SDS) using pre- and post-column derivatisation. The results are presented under a comparative perspective.

EXPERIMENTAL

Reagents

Glycine (Gly) (Guinama, Barcelona, Spain) and the following *L*-AAs from several manufacturers were analysed: alanine (Ala), cysteine (Cys), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), valine (Val) (Guinama), arginine (Arg), asparagine (Asn), histidine (His), lysine (Lys) (Fluka, Buchs, Switzerland), aspartic acid (Asp), tyrosine (Tyr) (Merck, Darmstadt, Germany), glutamic acid (Glu), glutamine (Gln) (Aldrich, Milwaukee, WI), and serine (Ser) (Scharlab, Barcelona). The AAs were dissolved in 10–20 drops of 1 M hydrochloric acid (Panreac, Barcelona), then diluted with water and stored at 4°C. In all cases, the concentration of the stock solutions was 2.0×10^{-3} M. Other reagents were *o*-phthalaldehyde, *N*-acetylcysteine (Fluka), boric acid (Probus, Badalona, Spain), ethanol (Merck), acetone, methanol (Scharlab), and sodium hydroxide (AnalaR, Poole, UK), all of analytical grade.

Aqueous-organic mobile phases were prepared with acetonitrile (HPLC grade, Scharlab) and trisodium citrate dehydrate (Scharlab) as the buffer system. Hydrochloric acid was added to fix the pH in the range 3–7. Ion pair mobile phases were prepared with trifluoroacetic acid (Scharlab) or pentadecafluorooctanoic acid (Aldrich). Micellar mobile phases contained sodium dodecyl sulphate (Merck) and propanol (Scharlab), and were buffered with citrate. Nanopure water (Barnstead, Sybron, Boston, MA) was used throughout.

Chromatographic Procedures

Aqueous Organic RPLC with Pre-column Derivatisation and UV Detection

The derivatisation mixture was prepared by mixing 5 mL of 1.25×10^{-2} M OPA solution in ethanol with 200 mL of 0.1 M boric/borate buffer at pH 9.5, then adding aqueous 2.0×10^{-2} M NAC to get the following concentrations: 2.5×10^{-4} M OPA and 4.0×10^{-4} M NAC. The reagent was renewed weekly and stored at 4°C, protected from light with aluminium foil. An aliquot of each AA solution was mixed with 3 mL of the OPA-NAC reagent to form the isoindoles, which were diluted with water to 10 mL.

The separation was carried out with an Inertsil ODS-3 column (5 μm particle size, 250 mm × 4.6 mm I.D., pH range: 2.0–7.5) (Análisis Vínicos, Tomelloso, Spain), connected to a Kromasil C18 guard column (5 μm particle size, 30 mm × 4.0 mm I.D.) (Scharlab). The derivatives were eluted in the isocratic mode with acetonitrile–water mixtures containing 5–27.5% (v/v) of organic solvent, and buffered with 5.0×10^{-3} M citric/citrate at pH 6.5, to get a training set of retention data that was applied to predict the optimal separation conditions in both isocratic and gradient modes. The volume percentage range of acetonitrile used to get the training set was adapted to the polarity of each AA: Asn, Ser, and Gln (5.0–15.0); His (7.5–15.0); Gly, Arg, and Thr (7.5–17.5); Ala (10.0–22.5); Cys (15.0–22.5); Tyr and Val (15.0–25.0); Met, Ile, and Trp (17.5–27.5); and Phe, Leu, and Lys (20.0–27.5). The mobile phases and the injected solutions in this procedure and the procedures described below were filtered through 0.45 μm membranes (Micron Separations, Westboro, MA, and GE Osmonics, Minnetonka, MN).

Elution of Free AAs with Trifluoroacetic Acid and ELS Detection

A reported procedure,[6] which employs an Alltima C18 column (5 μm particle size, 250 mm × 4.6 mm I.D., pH range: 1.5–10.5) and pre-column (7.5 mm × 4.6 mm) (Alltech, Deerfield, IL), and 0.1% (v/v) trifluoroacetic acid (TFA)/acetonitrile mobile phases, was implemented. The signal was monitored with an ELS detector, which was operated at 95°C, using nitrogen as the nebulizer gas at a flow rate of 2.0 L/min.

Elution of Free AAs with Pentadecafluorooctanoic Acid and ELS Detection

The concentration of PDFOA in the assayed mobile phases was in the range 2.0×10^{-4}–1.0×10^{-3} M (pH = 3–4). The regeneration of the column (elimination of the surfactant) was made by flushing acetonitrile, methanol, and again acetonitrile, during 30 min in each case. Three different columns were tested: the Alltima C18 used in the previous procedure, a Kromasil

C18 (5 μm particle size, 125 mm × 4.6 mm I.D., pH range: 3.0–7.0) (Análisis Vínicos) with a guard column containing the same packing (30 mm × 4 mm I.D.), and a Zorbax Eclipse XDB–C8 (5 μm particle size, 150 mm × 4.6 mm, pH range: 3.0–8.0) (Agilent), together with the Kromasil C18 guard column. The ELS detector was operated in the range 50–95°C, depending on the PDFOA concentration. Nitrogen flow rate was 2.0–3.0 L/min.

Micellar RPLC with Pre-column Derivatisation and UV Detection

The OPA-NAC derivatisation mixture for aqueous-organic RPLC was again used to form the AAs isoindoles. A Spherisorb ODS-2 column (5 μm particle size, 125 mm × 4.6 mm I.D., pH range: 3.0–7.0) (Scharlab) was placed after a guard column with the same packing (30 mm × 4.6 mm I.D.). A training set of mobile phases containing micellar sodium dodecyl sulphate and propanol, in the ranges 0.05–0.15 M and 0–3.0% (v/v), respectively, was used to predict the optimal separation. The pH was buffered, with 0.01 M citric/citrate, in the range 3–6.

Micellar RPLC with Post-Column Derivatisation and UV Detection

The conditions were the same as in the pre-column mode, except for the concentration of propanol that was in the range 0–8.0% (v/v).

Apparatus

The chromatograph (Agilent, Waldbronn, Germany, Model HP 1100) adopted diverse configurations with the following modules: isocratic and quaternary pumps, thermostated column compartment, automatic sampler, UV-visible variable multiwavelength, diode array (DAD), and Alltech evaporative light-scattering (Deerfield, IL, Model ELSD 2000) detectors. UV detection at 335 nm was applied in all pre- or post-column derivatisation procedures. Post–column derivatisation was implemented with a Pickering system (Mountain View, CA, Model PCX5100), consisting of two pumps, a mixer and a reactor. Pre-column derivatisation was not automated, but the derivatives were always injected 10 to 20 min after mixing the reagents.

The pH was measured with a Crison potentiometer (Barcelona, Model GLP21), provided with a combined glass-Ag/AgCl electrode. The column temperature was fixed throughout the runs at 25°C. The flow rate was kept constant at 1 mL/min, and the injection volume was 20 μL. The dead time was measured as the first deviation of the baseline, whereas the dwell time in the aqueous-organic mode was obtained by monitoring a blank gradient at 280 nm, increasing acetone concentration from 0 to 1% (v/v) in 20 min.

DATA ACQUISITION AND TREATMENT

The chromatographs were controlled with an Agilent Chemstation. Home built in routines, written in MATLAB 6.5 (The Mathworks, Natick, MA) were developed for the pre-column aqueous organic and the micellar procedures to find the optimal separation conditions. Optimisations were based on the prediction of the separation quality in a predefined number of experimental conditions, using the product of elementary peak purities as objective function.[7,8]

RESULTS AND DISCUSSION

Aqueous Organic RPLC with Pre-Column Derivatisation and UV Detection

One of the typical reagents for pre-column derivatisation of primary AAs is OPA, which needs the presence of a thiol to form isoindoles.[3,9] The thiol most widely used has been mercaptoethanol. However, the instability of its isoindoles limits a proper quantitation, unless an automatic device is coupled to the chromatograph to control the time between the addition of the derivatisation reagent and injection into the chromatograph. NAC has been reported as a good alternative to mercaptoethanol, due to the increased stability.[3,10] With NAC, there is no significant reduction in the absorbance (or fluorescence) of isoindoles at least up to 60 min, for most AAs.[3] Isoindoles derived from Cys, Gly, and His still present some instability problems that can be minimised by controlling the concentration of the OPA-NAC reagent.

The chromatographic elution of isoindoles is usually carried out with mobile phases containing acetonitrile or methanol. A literature survey reveals that both solvents are selected indistinctly. Acetonitrile was used here, since the resolution was good and solvent concentration in the mobile phase was significantly smaller. The retention of isoindoles shows a sigmoidal dependence with the pH of the mobile phase, with the largest retention at acidic pH. A $pH = 6.5$ was selected to avoid the long retention of isoindoles at low pH and restrict the consumption of acetonitrile (below 30%).

The isocratic mode allows reaching maximal separation, but with the inconvenience of yielding prohibitive analysis times for mixtures involving compounds in a wide range of polarities. Indeed, the proteic primary AAs can be baseline resolved in isocratic conditions, but under absolutely unpractical analysis times. Figure 1a shows the optimal isocratic chromatogram of the mixture, obtained by restricting the analysis time to ca. 80 min. The most hydrophobic AAs can be easily separated, but the reduction of the analysis time to acceptable values produces a strong overlap of the less retained AAs, especially for the pairs Glu/Asp, Ser/Gln, and Thr/Gly. In spite of the low practical usefulness of isocratic elution of AAs with

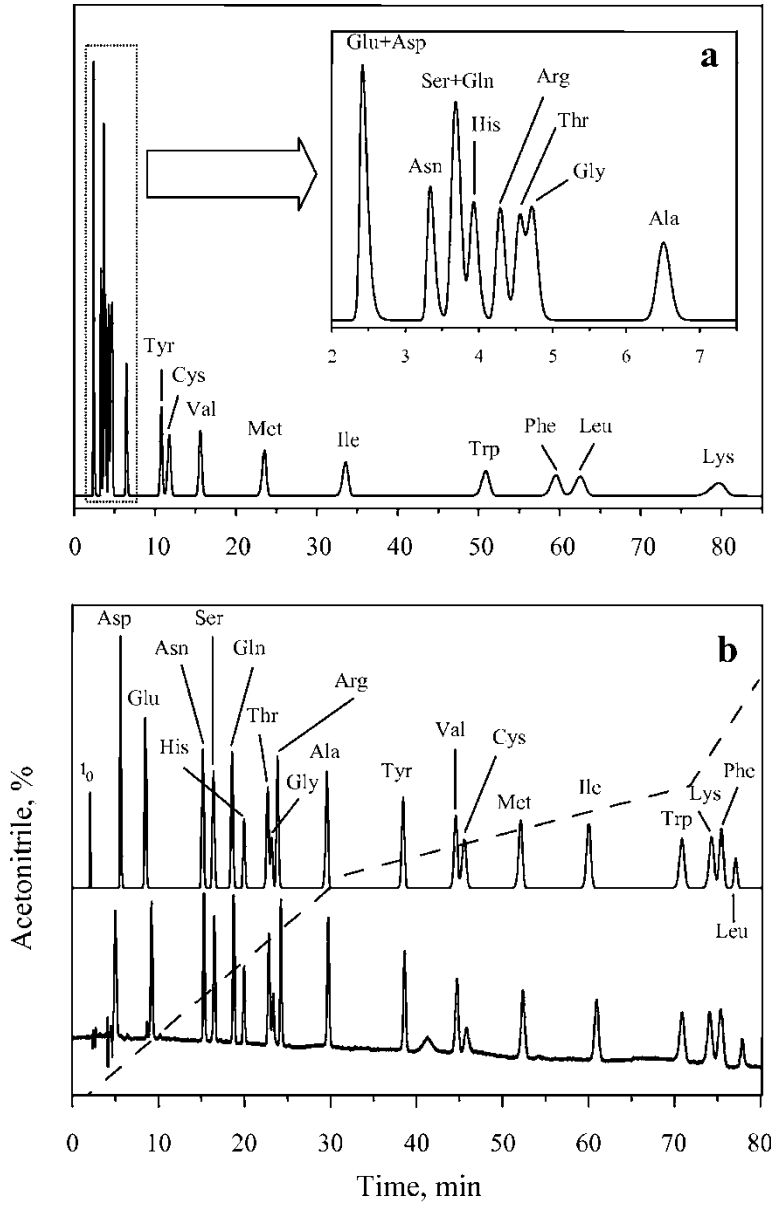

Figure 1. Aqueous-organic RPLC with pre-column derivatisation at pH 6.5 with UV detection: (a) isocratic elution (17.7% acetonitrile), and (b) trilinear gradient (mixing eluents A: 5% acetonitrile and B: 27.5% acetonitrile; gradient run: linear increase from 5 to 16.5% in 30 min, then reaching 20.75% at 70 min, and finally, 27.5% at 80 min. The concentration of injected AAs was 6×10^{-6} M, except for cysteine (2×10^{-5} M). Flow rate was 1 mL/min. The dwell time (1.79 min) in gradient elution is considered in the chromatogram.

aqueous-organic eluents, this chromatogram is shown with comparative purposes regarding the procedures described below.

Gradient elution with acetonitrile–water allows a good and easy separation of proteic primary AAs, previous formation of the isoindoles. Although most derivatives were baseline separated using a simple linear gradient, a severe overlap still remained for Cys/Val and Lys/Phe/Leu. The application of a trilinear gradient yielded acceptable resolution, with a small overlap for Thr/Gly (Fig. 1b). The analysis time can be reduced by investing more time in the optimisation of the gradient by adding more steps to adapt the elution profile to the local resolution needs of each critical peak cluster, or by shortening the column length or increasing the flow rate, with still good results. Thus, a chromatographic procedure that allowed the separation of 29 AAs isoindoles in 25 min has been reported, which used a 150 mm long Hypersil ODS column, a flow rate of 2.1 mL/min and a more complex gradient.[4] Alternatively, MS detection can be another valid solution to reduce the analysis time.[11]

Limits of detection (LODs) with DAD were in the range $1-2 \times 10^{-7}$ M. With a fluorescence detector ($\lambda_{ex} = 337$ nm and $\lambda_{em} = 454$ nm), the LODs decrease at least one order of magnitude.[4,12]

Elution of Free AAs with Trifluoroacetic Acid and ELS Detection

Direct injection of AAs avoids problems associated to the formation of derivatives (i.e. quantitative formation and stability). Also, the injected solution contains no reagent that could interfere with the analytes. However, the instrumental cost related to detection increases, and highly polar compounds interact inefficiently with RPLC bonded stationary phases. Thus, the separation of hydrophobic free AAs is easily achieved with conventional RPLC, whereas polar AAs coelute within the void volume region.

Ion-pair RPLC has been proposed to give an answer to this problem.[13,14] In this RPLC mode, ion pairs are formed between the acidic or amino forms of AAs and a cationic or anionic agent, respectively. Stationary phases have often greater viability with anionic additives. TFA has been proposed with ELS as a detection mode for the analysis of underivatised AAs.[15] This is the most common volatile ion-pair agent, due to its high purity and water solubility.[16] Another advantage, is that it is not adsorbed on the bonded phase and there is no need of flushing mobile phase to equilibrate the column. The sensitivity of ELS is, however, poorer when compared to other usual suitable detectors when a chromophore is available. Also, the ELS unit must be vented to a fume hood, since waste products may be a health risk for laboratory users.

The sigmoidal acid-base curves for free AAs are shifted to lower pH with respect to the derivatives, due to the smaller pK of the carboxylic group. Appropriate elution needs, therefore, rather low pH. TFA grants this acidic medium (the pH of an aqueous 0.1% solution of TFA was 1.8). This requires

also a special column with an extended working pH. An Alltima C18, which is a double capped polymeric bonded-phase column recommended for the separation of free AAs,[6] was selected for the analysis. According to the manufacturer, the capacity and selectivity of an Alltima C18 column remains unaltered after at least 500 hours of continuously running a TFA/acetonitrile mobile phase.[17]

We first reproduced the recommended conditions,[6] which involved gradient elution by mixing 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B), and ELS detection. Eluent A was run during the first 5 min, the concentration of eluent B being then raised to 40% (v/v) in the next 20 min. Our results coincided with those reported, with minor changes in retention times. However, the reported application[6] included only 11 proteic primary AAs (the less polar). A similar procedure was reported that used the same gradient and a Hypersil ODS column for 10 AAs.[18] In order to get a complete perspective, we eluted the 19 proteic primary AAs isocratically in a number of separation conditions, and found the following:

The 12 most polar AAs (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Lys, Ser, and Thr) were significantly retained only with mobile phases of TFA containing less than 10% acetonitrile. However, band broadening was always excessive with regard to peak separation (e.g. retention times for 0.1% TFA in 5% acetonitrile were in the range 4.9–5.8 min). The retention of Ile, Leu, Met, Phe, Trp, Tyr, and Val was, instead, too long, often $>$60 min for mobile phases of TFA with acetonitrile below 5%.

Figure 2a shows a chromatogram of a mixture of the 16 most polar AAs, eluted with 0.1% TFA in 5% acetonitrile. Leu, Phe, and Trp were strongly retained in these conditions, and required a significantly larger concentration of organic solvent to be eluted in convenient retention times. Figure 2b shows the separation of the nine less polar AAs with 0.1% TFA in 15% acetonitrile (polar AAs eluted within the void volume region). Ion-pair RPLC with TFA is, thus, not useful to analyse the whole mixture of proteic primary AAs.

Elution of Free AAs with Pentadecafluorooctanoic Acid and ELS Detection

Several perfluorinated carboxylic acids with longer *n*-alkyl chains have been proposed as ion-pair agents to solve the drawback of TFA of being unable to resolve the most polar AAs.[13] PDFOA and tridecafluoroheptanoic acid (TDFHA) are currently the most suitable reagents compatible with ELS and MS detection. PDFOA has been recommended as the best for ELS detection of underivatised AAs,[13] owing to the low background noise. TDFHA contains fewer impurities and is preferred for MS detection.[19] Both reagents modify dynamically the surface of the stationary phase. The adsorbed amount and the analysis time increases significantly with the concentration of the reagent in the mobile phase.[13]

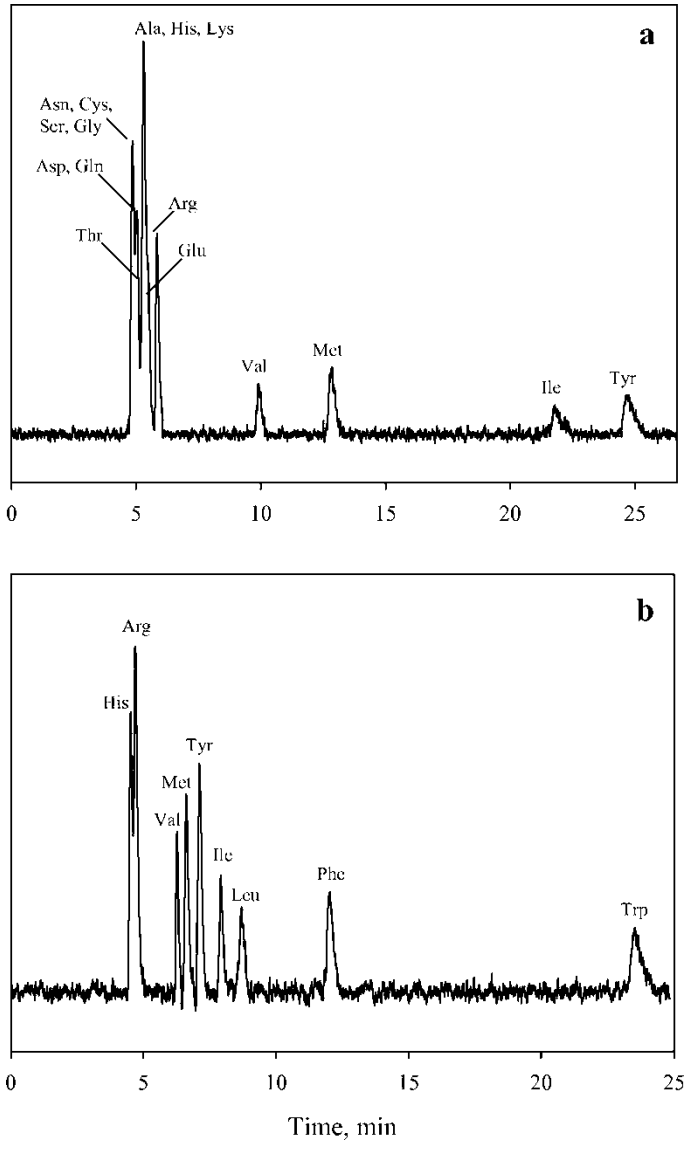

Figure 2. Ion-pair RPLC of free AAs with TFA at pH 1.8 using ELS: (a) 0.1% TFA in 5% acetonitrile, and (b) 0.1% TFA in 15% acetonitrile. The concentration of injected AAs was 2×10^{-3} M.

Accordingly, we tried PDFOA with ELS, but the results were discouraging. In comparison with TFA, PDFOA retains excessively the less polar AAs. This means, that the compounds should be swept off the column after each run with pure acetonitrile or a mobile phase containing a high content

of this solvent. However, this ion-pair agent seemed promising, due to its complementary behaviour with respect to TFA.

In order to evaluate the possibilities of PDFOA, the separation of the nine most polar proteic primary AAs (Ala, Asn, Asp, Cys, Gln, Glu, Gly, Ser, and Thr) was examined, using three columns: the Alltima C18 used with TFA, a Kromasil C18, and a Zorbax Eclipse XDB-C8. The system was operated according to the reported recommendations.[13] The mobile phase contained PDFOA in the 2.0×10^{-4}–1.0×10^{-3} M concentration range. The ion pair was swept off the column after several runs, by passing through in this order, acetonitrile, methanol, and acetonitrile. The background noise was decreased by adding PDFOA to the injected mixture in the same concentration as in the mobile phase. Several problems were, however, found in the three columns.

At the beginning of the PDFOA absorption process, the signal showed baseline fluctuations, which were more or less periodic. The fluctuations disappeared after 2 hours of running the ion-pair agent. However, in all assayed conditions, saturation was not reached. This conclusion was inferred from the progressive increase in retention times along consecutive injections, especially for Ala, the less polar AA in the analysed mixture (Fig. 3). Also, after the fourth or fifth injection, the background noise increased significantly, with numerous spikes in some chromatograms, which were accompanied by baseline drift. For the C8 column, the retention time shifts were smaller, but the noise and drift were identically observed. Peak features were acceptable for the first injections carried out with each column, but gradually degraded. Apparently, the column could be regenerated through cleaning cycles with acetonitrile and methanol, since the retention times obtained in the first injection (in the range 3–11 min for the set of AAs) were recovered. However, the Alltima C18 column, which was used in most runs, was finally irreversibly damaged.

Another procedure was tried, where the column was equilibrated as before by flushing PDFOA during 2 hours, five injections were then made, and finally, 20% acetonitrile was passed through to eliminate the excess of ion-pair agent. This was evidenced by a sudden increase in the signal. When the baseline was recovered (in one minute), PDFOA was flushed during 15 min before performing the next set of five injections. The repeatability (%) in the peak areas along one of these series was: Asp (8.8), Asn (3.4), Ser (5.2), Gly (7.5), Gln (7.1), Cys (10.7), Glu (26), Thr (14.5), and Ala (14.5). LODs were in the range $1–5 \times 10^{-6}$ M, which improved previously reported LODs with PDFOA in one order of magnitude ($1–2 \times 10^{-5}$ M).[20] Operating in this way, the retention times of AAs in the first run were recovered. However, since the column again showed signs of deterioration, the analyses were stopped to avoid further damage.

Above and beyond these difficulties, the resolution of free AAs was only satisfactory after running PDFOA during at least 2 hours before the first injection, especially for the pair Cys/Glu, and in a lesser extent, Asn/Ser. The former pair was often unresolved in the three assayed columns. It should be considered, that if the whole mixture of proteic primary AAs

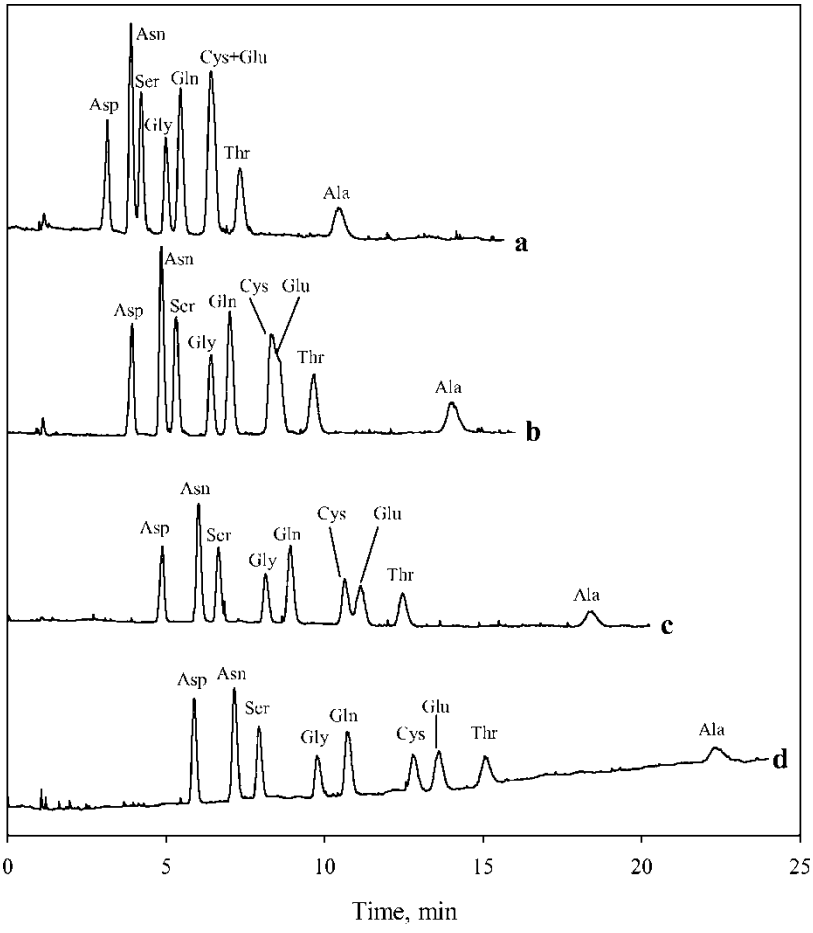

Figure 3. Ion-pair RPLC of free AAs with 5×10^{-4} M PDFOA at pH 3.4 using ELS. The first chromatogram (a) was run 2 hours after beginning the PDFOA flush. The time between the end of each chromatogram and the following injection was 30 min (b–d). The concentration of injected AAs was 5×10^{-4} M, except for cysteine (1×10^{-3} M).

were analysed, the less polar compounds should be swept off the column with acetonitrile after each run, but this will also remove the ion-pair agent off the column, forcing the reequilibration and introducing an undesirable delay between consecutive analyses.

In recent work,[19] good repeatability was observed for AAs with a short (50 mm length) C_{18} column and a gradient at a flow rate of 0.2 mL/min that mixed 5×10^{-4} M TDFHA (solvent A) and pure acetonitrile (solvent B), increasing the concentration of organic solvent from 0 to 25% in 15 min. TDFHA was then flushed during 15 min to equilibrate the column, allowing

a throughput of two samples per hour. According to the authors, at least the first gradient should be discarded. With other perfluorinated carboxylic acids, the equilibration time amounted up to 2 hours, the gradient time up to 30 min, and the reequilibration time 30 more min. The authors attributed the gradual drifts in retention times to column fouling with the analysed biological matrix, which was overcome by flushing pure acetonitrile for an hour every 14–16 chromatographic runs.[19] Other authors have interpreted the drifts as a consequence of the accumulation of the ion-pair agent on the stationary phase, and solved it by flushing pure acetonitrile every six consecutive analysis.[21] In both reports, tandem mass spectrometry (MS/MS) detection was applied to complete the partial selectivity yielded by the chromatographic separation. This allowed a shorter chromatographic column, and consequently, shorter analysis time. This is not possible for ELS detection.

Micellar RPLC with Pre-Column Derivatisation and UV Detection

RPLC columns can be also used with aqueous solutions of surfactants above the CMC, being the anionic SDS the most convenient.[22] In micellar RPLC, the mobile phases contain micelles and monomers of surfactant. Also, the long hydrophobic chain of SDS is inserted in the bonded organic layer of the column, with the sulphate group protruding outside, which gives a negative charge to the stationary phase. Silanols and C_{18} bonded chains are thus covered with the surfactant monomers, which change radically the behaviour of the stationary phase. The possibility of using a surfactant instead of an organic solvent is certainly attractive. However, peak efficiency is rather poor with pure micellar eluents, and the elution strength often insufficient. Both problems are usually solved by the addition of a small amount of an organic solvent.

The retention of AAs with micellar eluents is small compared to aqueous-organic eluents (compare Figs. 1a and 4a), the lower the polarity of the solute, the larger the reduction in retention. This effect is similar to that achieved using a gradient in conventional RPLC (Fig. 4a), and explains that gradients are usually unnecessary with micellar eluents.

OPA-NAC derivatives were formed following the same procedure described for aqueous-organic RPLC. The dependence of retention with pH was also similar: the neutral species dominant at low pH were retained, whereas the anionic species were virtually unretained due to repulsion with the anionic surfactant covering the stationary phase. Micellar eluents shift; however, retention drops to higher pH values, owing to the stabilisation of the neutral species produced by micelles. Maximal separation among peaks was obtained at pH 3 (minimal pH assayed). Propanol was added to improve peak properties and resolution. However, the efficiencies were, in all cases, still low ($N < 1000$).[23] The separation space was short, and due to the high number of compounds eluting within the same time window, the resolution was poor (Fig. 4a).

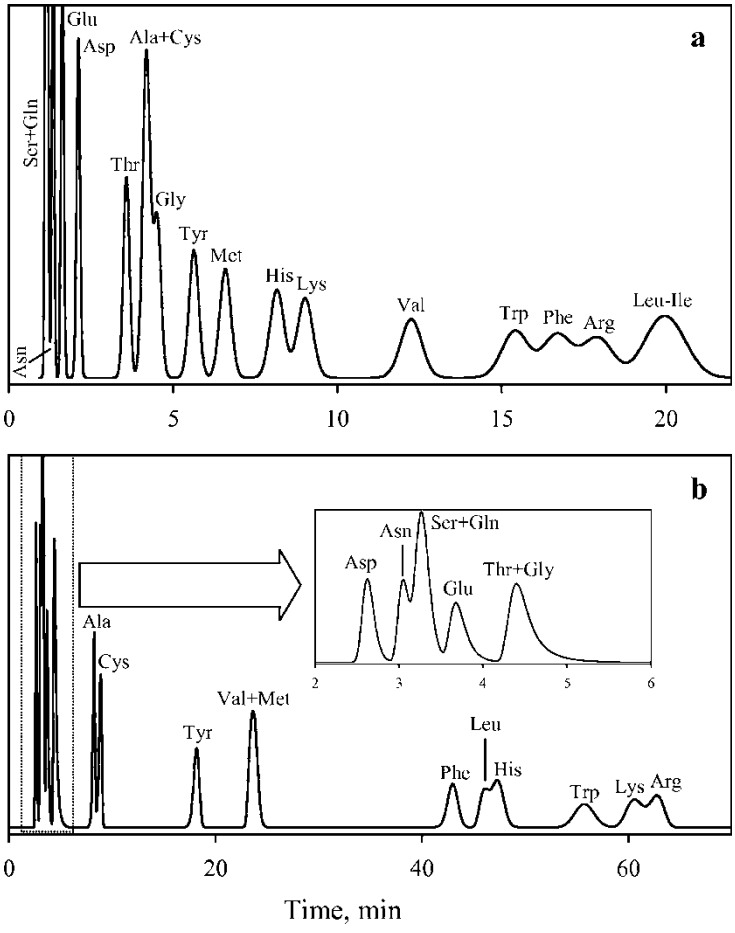

Figure 4. Micellar RPLC at pH 3.0 with UV detection: (a) pre-column derivatisation (0.051 M SDS/1.7% propanol), and (b) post–column derivatisation (0.058 M SDS/0.3% propanol). The concentration of injected AAs was 1×10^{-4} M.

Micellar RPLC with Post-Column Derivatisation and UV Detection

Detection with ELS of free AAs eluted with micellar mobile phases is not possible due to the nonvolatile character of SDS. UV detection with post-column derivatisation was, therefore, implemented. This approach implies, however, some loss of resolution performance and reproducibility due to mixing of the mobile phase with reagents, which is minimised if the derivatisation step is automated. For this purpose, the Pickering system was used. Unfortunately, this system was not designed for micellar mobile phases: the pipes suffered frequent leaks and the system pumps required daily careful cleaning to avoid damage. In addition to the increase in instrumental cost,

the derivatisation reagents should be pumped continuously into the reactor, which increased the analysis cost even more.

Free AAs were eluted again at the column's lowest feasible working pH (3.0), where maximal retention was observed. This pH was, however, located within the sudden drop in retention at the increasing pH. This implied that the adjustment of pH was more critical for free AAs than for isoindoles eluted with the micellar eluents. In contrast, the efficiencies for free AAs were rather large, mostly in the range 4000–7000, which are similar to those obtained for isoindoles eluted with acetonitrile–water ($N = 5000–9000$). Figure 4b shows a chromatogram run in the optimal conditions. Although the achieved resolution is still unsatisfactory, it improves the separation of free AAs eluted with aqueous organic eluents (in the absence and presence of ion-pair agents).

CONCLUSIONS

The analysis of AAs is still a challenging topic. Hence, new procedures are frequently reported. Nowadays, RPLC with aqueous-organic mobile phases is the usual technique for AAs, but it presents the drawback of requiring previous derivatisation to allow detection. This RPLC mode was compared with the other modes, which make use of ion-pair agents (TFA or PDFOA), or anionic micelles (SDS above its CMC). All these procedures were implemented in our laboratory and studied to evaluate their features.

Wherever MS detection is not available, the best methodology is still the conventional aqueous-organic RPLC using pre-column derivatisation and gradient elution. Many reports have been published with good results, using different columns, mobile phase compositions, pH values, gradient programs, and flow rates.[4,10] This RPLC mode yields the most reliable results and allows acceptable resolution, longer column lifetime, and simpler method implementation. In addition, OPA-NAC and other reported AAs derivatisation reagents produce fluorescent compounds, which allow very low LODs.

Ion-pair RPLC was particularly problematic. Free AAs show a wide range of polarities and cannot be analysed within the same run, even with the application of a gradient of an organic solvent that decreases the retention of the less polar AAs. The cooperation of two different ion-pair agents (TFA and PDFOA) in independent runs was considered to get adequate retention and resolution for all AAs. TFA resolves the less polar AAs, leaving the most polar ones cluttering within the void volume region. Meanwhile, PDFOA allows the retention of the most polar AAs, but other AAs are excessively retained. However, column reequilibration with PDFOA is very slow and there is a significant risk of irreversible damage. Also, perfluorinated carboxylic acids are corrosive, and proper safety cautions should be taken to avoid skin and eye contact, besides ELS and MS venting requirements.

Micellar RPLC gave interesting results, improving those obtained with TFA and PDFOA. SDS is adsorbed on the bonded stationary phase, reaching saturation at the CMC, and can be easily removed off the column. Also, retention times are highly reproducible for replicated injections. However, detection of free AAs cannot be carried out with ELS or MS, due to the presence of the nonvolatile surfactant in the mobile phase. Free AAs can only be detected through post-column derivatisation, which should be automated. Unfortunately, the surfactant makes the operation of the automatic device problematic.

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