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Determination of 20 underivatized proteinic amino acids by ion-pairing chromatography and pneumatically assisted electrospray mass spectrometry

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

Institut de Chimie Organique et Analytique (ICOA), CNRS UPRES-A 6005, Université d'Orléans, B.P. 6759, Orléans Cedex 2, France

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Abstract

A qualitative determination of 20 underivatized proteinic amino acids by LC–MS is reported. The need for chromatographic separation before mass spectrometry determination is demonstrated based on the study of several amino acid pairs which have some similar characteristics. Two suitable LC–MS systems are proposed for amino acid analysis. A preliminary optimization of these systems has been investigated using evaporative light scattering detection as these two detection modes have the same chromatographic requirements. The amino acid separation was achieved on a Purospher RP-18e or a Supelcosil ABZ^{+}Plus column with tridecafluoroheptanoic acid or pentadecafluorooctanoic acid as volatile ion-pairing reagent in an acetonitrile–water mobile phase. In order to elute the most retained amino acids, an elution gradient based on simultaneously increasing the concentration of acetonitrile and decreasing the concentration of the ion-pairing reagent was used. The detection limits of the present work (without specialized optimization) varied from 0.5 to 1 mg l^{-1}. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Ion-pairing reagents; Amino acids; Carboxylic acids, Perfluorinated

1. Introduction

The great biological importance of amino acids has led to the publication of a large number of papers on their chromatographic determination. However, none of the existing protocols for the separation and analysis of amino acids provides the ideal solution, and none has been demonstrated to be superior to all the others. So far, the determination of amino acids by liquid chromatography has been dominated by pre- or post-column derivatized methods.

In the last 10 years, alternative methods for amino acid analysis without derivatization have been proposed. For the separation of underivatized amino acids, reversed-phase chromatography is ineffective because these compounds lack large hydrophobic side-chains and as a result their separation is usually achieved by ion-exchange [1–4] or ion-pair reversed-phase liquid chromatography [5–7], but the use of normal-phase liquid chromatography has also been reported [8]. The main difficulty consists in the separation of the polar amino acids [9].

Moreover, the detection of underivatized amino

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

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

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acids is a problem as most of them are very weak chromophores in the UV–vis region and possess no native fluorescence. Almost all the available detectors coupled with liquid chromatography have been used for the analysis of underivatized amino acids in direct or indirect mode: photometric and fluorimetric detection [5,10–13] as well as electrochemical [1–3], light scattering [14–17] and mass spectrometry [18].

Concerning mass spectrometry, a variety of different ionization methods have been used for the study of the free amino acids such as electron impact (EI) [19], chemical ionization (CI) [20], field desorption (FD) [21], plasma desorption (PD) [22], secondary ion mass spectrometry (SIMS), [23] fast atom bombardment (FAB) [24], laser mass spectrometry (LMS) [25] and atmospheric pressure ionization (API) [26–29]. From a variety of combined LC–MS available systems API-MS has been shown to be a preferred way to simplify the coupling of liquid inlet systems such as LC, CE and ion-pair chromatography to MS [30]. Until now, underivatized valine, leucine and their corresponding amines have been analysed by LC–MS equipped with a thermospray source [18]. Moreover histidine, tryptophan, phenylalanine and aspartic acid have been analysed by coupling capillary electrophoresis with electrospray ionization mass spectrometry [31]. However, an LC–MS coupling for the determination of the 20 underivatized proteinic amino acids has never been developed.

One of the problems in coupling LC with MS is that a volatile mobile phase is required. The use of non-volatile components in the mobile phase does not necessarily harm the API mass spectrometer but can lead to source blockages which decrease the sensitivity of the system during this period. As a result it does not affect qualitative studies but can affect seriously the accuracy of quantitative results [30,32]. Usually the analysts attempting to transpose to LC–MS an existing LC method in which the mobile phase contains inorganic modifiers such as borate, phosphate etc., are forced either to replace non-volatile by volatile modifiers such as formic acid, acetic acid or trifluoroacetic acid (TFA) [33], which does not always give identical resolution for the analyzed compounds, or to use on-line membrane suppressors to decouple non-volatile modifiers [30]. Several papers have been presented which evaluate potential additives to the mobile phase which can be volatile enough to be compatible with MS [34–36].

Semi-long perfluorinated carboxylic acids (5–9 carbons) have recently been proposed [37] as sufficiently volatile compounds to be used for the coupling of LC with API-MS.

In a recent paper [17] we have demonstrated the separation and analysis by LC–evaporative light scattering detection (ELSD) of ten of the more polar proteinic underivatized amino acids (Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala, Pro) using perfluorinated carboxylic acids as volatile ion-pairing reagents.

In this paper we develop a chromatographic method using perfluorinated carboxylic acid for the determination of the 20 proteinic amino acids without derivatization by using LC–ELSD and then LC–API-MS.

2. Experimental

2.1. Chemicals

HPLC-grade methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) were obtained from J.T. Baker (Noisy le Sec, France). Formic acid, TFA and tridecafluoroheptanoic acid (TDFHA) 99.4% were obtained from Interchim (Montluçon, France) and pentadecafluorooctanoic acid (PDFOA) 98% from Aldrich (St. Quentin Fallavier, France). Aspartic acid (Asp), asparagine (Asn), glutamine (Gln), glutamic acid (Glu), serine (Ser), threonine (Thr), cysteine (Cys), glycine (Gly), alanine (Ala), proline (Pro), valine (Val), methionine (Met), tyrosine (Tyr), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophan (Trp), lysine (Lys), histidine (His) and arginine (Arg) were purchased from Sigma (St. Louis, MO, USA). Standard mixtures of 100 mg l^{-1} for each amino acid were prepared in purified water obtained from an Elgastat UHQ II System (Elga, Antony, France).

2.2. Apparatus

The LC–ELSD chromatographic system was set

up 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.

Experiments in the LC–MS chromatographic system were performed on a Perkin-Elmer (Toronto, Canada) model LC-200 binary pump and a Perkin Elmer Sciex (Forster City, CA, USA) API 300 mass spectrometer triple quadrupole with IonSpray as ion source. The mass spectrometer was operated in positive ion mode. Ionspray voltage was set at 5 kV. The orifice and the focusing ring voltages were set at different values. Nitrogen was used as curtain gas at a flow-rate of 1.25 l min^{-1}. Injections were done by a Perkin-Elmer series 200 autosampler (Toronto, Canada) fitted with a 20-μl loop. For the study of the MS parameters a Harvard Model 22 syringe pump was used to infuse the amino acid solutions in the MS system at a flow-rate of 5 μl min^{-1}.

The equilibration of the columns was confirmed by monitoring the conductivity of the mobile phase by a Vydac conductimeter (Wescan Instrument, Santa Clara, USA) model 6000 CD.

A Beckman pH meter Model Φ 10 (Gagny, France) was used for measuring the pH of the solutions.

Separations were carried out on a Purospher RP-18e 125×4 mm I.D. (Merck, Darmstadt, Germany) column fitted with its precolumn and a Supelcosil ABZ^+Plus 150×4.6 mm I.D. (Supelco, Bellefonte, PA, USA) column. All experiments were carried out at room temperature at a flow-rate of 1 ml min^{-1}. For the LC–API-MS system, a 50 μl min^{-1} portion of column effluent was diverted to the ion source.

For gradient elution, solvent A was: 0.5 m*M* PDFOA in water for Purospher support and 1 m*M* TDFHA in water for Supelcosil ABZ^+Plus column. Solvent B was acetonitrile.

The regeneration procedure of each column after experiments and the column tests were carried out as described previously [17] i.e. methanol (50 ml), acetonitrile (50 ml), tetrahydrofuran (30 ml) then methanol (50 ml).

3. Results and discussion

Mass spectrometry (MS) is, like gas phase light scattering, a universal detection mode. The only requirement for the detection of a compound by MS is its ionization. API using electrospray or IonSpray (pneumatically assisted electrospray) is the most appropriate method for the ionization of polar as well as ionic compounds such as amino acids. Indeed, this very mild ionization mode considerably reduces the fragmentation of thermolabile solutes.

The first question to be addressed before an MS analysis is the following: for quantitative and qualitative determination of compounds is the separation necessary, since the mass spectrometer can often distinguish the compounds which differ in molecular mass or mass spectral fragmentation? Table 1 reports for proteinic amino acids, the different m/z ratio values for $[M+H]^+$ ions formed in a positive mode ionization and for the most important fragments observed in collisionally induced dissociation (CID)-MS after the $[M+H]^+$ ions have passed through the

Table 1
Observed fragmentation in CID-MS for the 20 proteinic amino acids; relative abundances are not indicated as they depend on orifice (OR) and focusing ring voltage (RNG); Im.: (Immonium) = $[H_2N{=}CH{-}R]^+$ where R is the residue of the amino acid

Amino	m/z			
acid	$[M+H]^+$	$[M+H-NH_3]^+$	$[M+H-H_2O]^+$	Im.
Gly	76			
Ala	90			44
Ser	106		88	60
Pro	116			70
Val	118			72
Thr	120		102	
Cys	122	105		76
Ile/Leu	132			86
Asn	133	116		87
Asp	134		116	88
Lys/Gln	147	130		
Glu	148		130	102
Met	150	133		104
His	156			110
Phe	166			120
Arg	175			
Tyr	182	165		136
Trp	205	188		

curtain gas of the interface. The relative abundances of these fragments are not indicated because, as we shall see below, they are strongly dependent on the orifice (OR) and the focusing ring (RNG) voltage adjustments. It can be clearly seen in Table 1 that leucine and isoleucine have the same molecular mass (M_r) and their CID-MS in the curtain gas lead to the same fragmentation. Furthermore, quadrupole resolution is not sufficient to distinguish glutamine ($M_r = 147.131$) and lysine ($M_r = 147.174$). As a result, Lys and Gln can be considered to have the same molecular mass. Moreover, identical fragmentation behavior (ammonia loss or immonium formation) is observed in CID-MS for these amino acid pairs. Hence the chromatographic separation of these two pairs is essential in order to identify and quantify each amino acid in the presence of the other one. There are two additional cases where chromatographic separation has to be implemented in order to quantify amino acids in samples:

1. The CID-MS fragment of one compound and the molecular ion of another one have the same m/z ratio (i.e. Phe and Thr).
2. The molecular mass of two amino acids differs only by one mass unit. ^{13}C has a natural abundance of 1.1%. Therefore, isotopes cannot be ignored. For example, the abundance of the $[M+H+1]^+$ ion of leucine due to one ^{13}C in the molecule is about 7.6% of that of the corresponding $[M+H]^+$ ion [27]. Thus the m/z ratio of [(^{13}C) leucine $+H]^+$ ion or [(^{13}C) isoleucine $+H]^+$ ion is equal to that of [asparagine $+H]^+$ ion. The same observations hold true for Asn and Asp or Gln and Glu.

Table 2 sums up the problems encountered when MS detection is used for the analytical identification of the amino acids.

In order to identify the proteinic amino acids present in a given sample unambiguously, it was necessary to have several suitable and complementary chromatographic systems which can separate the different amino acid pairs discussed above.

The mass spectrometer needs a volatile mobile phase when LC–MS coupling is investigated. This is also a requirement for ELSD in order to avoid high background noise. As a consequence LC methodology previously developed with ELSD is directly compatible with MS detection. The use of ELSD is an inexpensive approach for developing the required chromatographic conditions for LC–MS and is straightforward compared with MS, as only a few parameters must be taken into consideration [38,39].

Table 2
Amino acid mixtures that have to be separated by LC before identification by MS

Amino acid mixtures	m/z ($[MH]^+$)	Justification
Leu–Ile	132	[a]
Lys–Gln	147	[a]
Pro–Asn–Asp	116–133–134	[b]
Gly–Cys	76–122	[b]
Thr–Phe	120–166	[b]
Asn–Met	133–150	[b]
Glu–Lys–Gln	148–147–147	[c]
Asp–Asn	134–133	[c]
Asn–Met–Leu–Ile	133–150–132	[b,c]
Asp–Asn–Met	134–133–150	[b,c]
Ser–Cys	106–122	[b,c]
Phe–Tyr	166–182	[b,c]

[a] Same molecular mass and identical fragmentation behavior in CID-MS.
[b] $[M+H]^+$ of one corresponds to a fragment of the other(s).
[c] $[M+1+H]^+$ of one [isotopic amino acid (^{13}C)] corresponds to the $[M+H]^+$ of the other(s).

3.1. Analysis of underivatized amino acids by LC–ELSD

In a recent paper [17] we have proposed an isocratic liquid chromatographic method using volatile ion-pairing reagent and evaporative light scattering detection for the successful separation and detection of ten underivatized polar amino acids (Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala, Pro). In this previous study, among the different perfluorinated carboxylic acids examined as ion-pairing reagents in aqueous mobile phase, the best results were obtained with TDFHA and PDFOA, yielding significantly increased selectivities for polar amino acids, over their shorter chain homologues. Moreover, among the different commercial octyl or octadecyl bonded phases tested, Purospher RP-18e support and Supelcosil ABZ$^+$Plus support provided the best performances.

Under the isocratic chromatographic conditions described in Ref. [17], it appears that basic amino acids (Lys, His and Arg) which are also polar compounds, are highly retained due to the possible

formation of a double ion pair with the ion-pairing reagent under the acidic pH conditions of the mobile phase used. In order to analyse all the proteinic amino acids, a gradient elution mode has been investigated based on the most efficient chromatographic systems proposed in Ref. [17].

Fig. 1a and b show the analysis of the 20 proteinic underivatized amino acids by LC–ELSD under gradient elution mode on Supelcosil ABZ^{+}Plus column and Purospher RP-18e column respectively. The elution gradient is based on simultaneously increasing the concentration of acetonitrile and decreasing the concentration of the ion-pairing reagent. At the end of each chromatogram a system peak is observed. System peaks (SP) are in addition to the solute peaks. SP are observed when an injection is made in a chromatographic system previously equilibrated with a mobile phase consisting of a solution with a constant concentration of an additive which is involved in the retention mechanism. They appeared after perturbation of the equilibrium, when some of the adsorbed additive molecules are desorbed and migrate along the column as an excess additive band [40,41]. In our case, it has been previously demon-

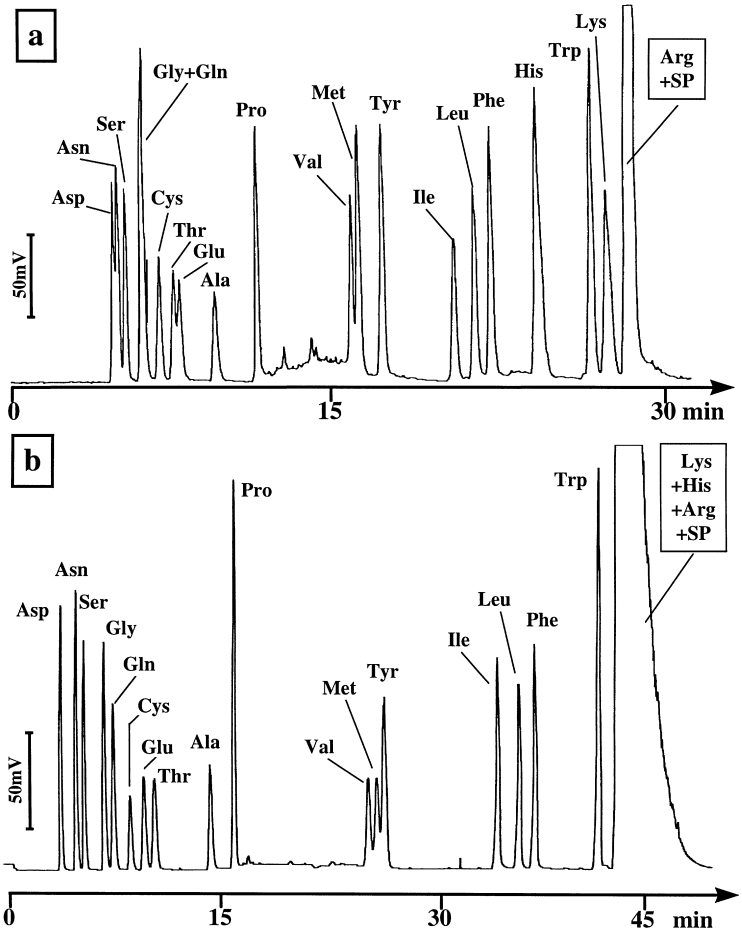

Fig. 1. LC–ELSD analysis of a 100 mg l^{-1} standard solution of the 20 proteinic underivatized amino acids in gradient elution. (a): Column: Supelcosil ABZ^{+}Plus (150×4.6 mm I.D.). Gradient elution: solvent A: 1 m*M* TDFHA in water, solvent B: acetonitrile; gradient starts at 0% B for 6 min, from 0 to 20% in 2 min, then 20% is maintained for 6 min, from 20 to 25% in 2 min, then 25% is maintained to the end of the analysis. (b) Column: Purospher RP-18e (125×4 mm I.D.). Gradient elution: solvent A: 0.5 m*M* PDFOA in water, solvent B: acetonitrile; gradient starts at 0% B for 8 min, from 0 to 19% in 5 min, then 19% is maintained for 10 min, from 19 to 28% in 10 min, then 28% is maintained to the end of the analysis. Flow rate: 1 ml min^{-1}; ELSD (see Section 2).

strated that the ion-pairing agent added to mobile phase induces a dynamic modification of the surface of the reversed-phase packing material [17]. So, by increasing the ACN percentage in the mobile phase, considerable amounts of PDFOA or TDFHA, hydrophobic agents which are more soluble in ACN than in water, were desorbed from the stationary phase and migrated towards the detector. This phenomenon can be observed with ELSD due to differences in mobile phase volatility when PDFOA and TDFHA concentration increases suddenly. Unfortunately the presence of SP masks the elution of one or more amino acids: Arg on Supelcosil column and Lys, His and Arg on Purospher support. This was an assumption as no additional solute peak was observed after the SP, and the SP area decreased when water rather than the amino acid sample was injected into the chromatographic system.

Fig. 1a shows that the Supelcosil column allows the separation of all the amino acids with TDFHA as ion-pairing reagent in about 27 min but, that under these conditions, a separation without baseline resolution for the first eluted compounds and a coelution of Gly and Gln were observed. The selectivity towards Asp, Asn and Ser can be increased using PDFOA as ion-pairing reagent as reported in Ref. [17]. However, under these new conditions the analysis time is increased and the three most retained amino acids are masked by the system peak (data not shown). In either case Gly and Gln cannot be separated on Supelcosil support.

Fig. 1b shows that the Purospher material allows the separation of the first 17 eluted amino acids with PDFOA as ion-pairing reagent with a better baseline resolution than Supelcosil column in about 45 min. The Gly, Gln pair is now resolved under these chromatographic conditions; however, the elution of Lys, His and Arg is masked by the SP. Using 1 m*M* TDFHA in water as solvent A in gradient elution it was possible to decrease the analysis time to around 27 min, but the separation performances for the first eluted amino acids were not so satisfactory and the elution of basic amino acids is still masked by the system peak (data not shown).

For a given ion-pairing reagent in mobile phase, the elution order of amino acids is the same on the two selected supports. When TDFHA is used rather than PDFOA in mobile phase the elution order is modified only for two pairs: Glu–Thr and Trp–His. The order of elution for the basic and more hydrophobic amino acids is easily explained. The amino acids Val, Met, Tyr, Ile, Leu, Phe and Trp are eluted in direct relation to their lipophilicity [42]; moreover His, Lys and Arg (when they separated) are eluted in accordance with their basicity. For the first ten amino acids the elution order is more difficult to explain as it depends on charge and polarity.

Until now, the separation of Leu and Ile has always been a problem, due to the structural similarity and the close physicochemical properties (p*K*, polarity, molecular volume, etc.) of these compounds but now, under the chromatographic conditions proposed in this paper, a satisfactory separation of two amino acids is observed.

This preliminary study proposes two complementary LC–ELSD systems where 17 out of 20 amino acids can be identified and where all the co-elutions that could prevent a further LC–MS analysis have been resolved.

3.2. Analysis of the underivatized amino acids by LC–MS

MS detection has the advantage of providing structural information about the eluted compounds. Moreover, coelutions of compounds can be resolved in the event of different m/z ratios.

Due to their zwitterion nature amino acids can be positively or negatively charged which allows either a positive or a negative mode of MS detection. In our study low concentrations of strong acids are used as ion-pairing reagents. The pH value of the mobile phase is 3 or 3.3 (depending on the surfactant concentration). As a result all the amino acids are protonated under these conditions. Moreover, Hiraoka et al. [43] has shown that the amino acids can be detected with higher sensitivities in the positive mode than in the negative mode, as we confirmed by observing the intensity of the amino acids in the two detection modes after infusion. Furthermore, the ion-pairing reagent is negatively charged, so in a positive detection mode it cannot enter into the mass spectrometer and pollute it. From the above the use of the positive detection mode was judged to be more appropriate.

Fig. 2a and b show the selected ion monitoring

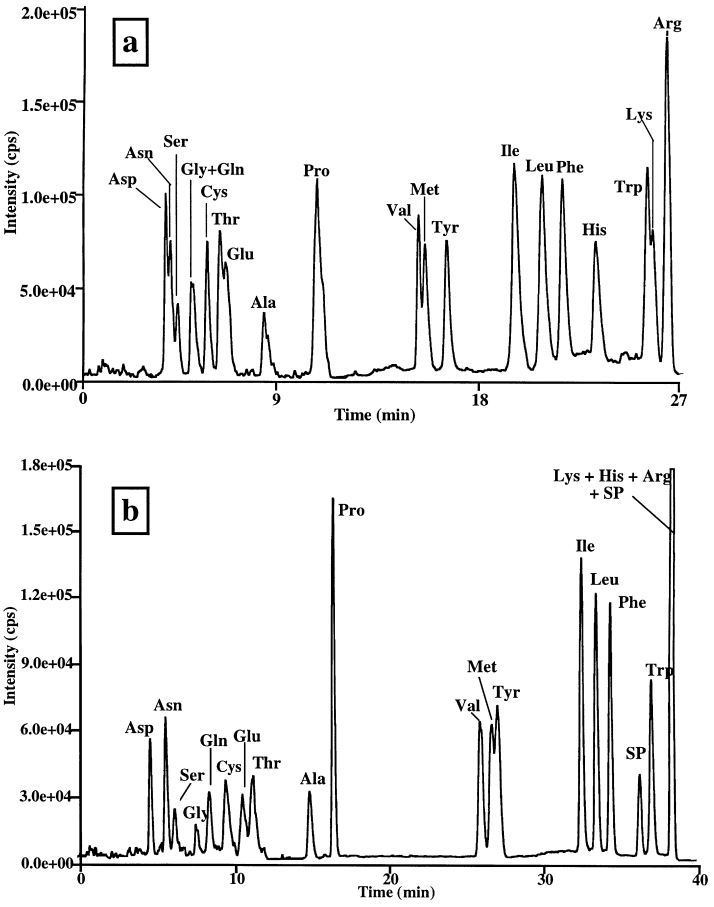

Fig. 2. LC–MS analysis of a 100 mg l^{-1} standard solution of the 20 proteinic underivatized amino acids in gradient elution. (a): Column: Supelcosil ABZ^{+}Plus (150×4.6 mm I.D.); gradient elution: solvent A: 1 m*M* TDFHA in water, solvent B: acetonitrile; gradient starts at 0% B for 6 min, from 0 to 20% in 2 min, then 20% is maintained for 8 min, from 20 to 25% in 2 min, then 25% is maintained to the end of the analysis. (b): Column: Purospher RP-18e (125×4 mm I.D.). Gradient elution: solvent A: 0.5 m*M* PDFOA in water, solvent B: acetonitrile; gradient starts at 0% B for 8 min, from 0 to 17% in 5 min, then 17% is maintained for 8 min, from 17 to 28% in 10 min, then 28% is maintained to the end of the analysis. Flow-rate: 1 ml min^{-1}. Split: 1/20. Mass spectrometry (IS: 5 kV, OR: 20 V, RNG 200 V).

(SIM) of the 20 amino acids (18 ions) obtained by LC–MS under similar LC–ELSD conditions to those used in Fig. 1. The chromatographic profiles obtained by LC–ELSD or LC–MS are comparable in terms of retention time. The signal stability is improved after some minutes by the introduction of ACN as has already been shown [44]. Resolutions are sometimes lower in LC–MS than in LC–ELSD, probably due to differences in the apparatus. The hypothesis that some amino acids (Arg on the Supelcosil column and Lys, His and Arg on the Purospher column) are masked by the system peak when ELSD is used, is now confirmed by MS. However, the absolute and relative signal intensities observed in LC–MS and LC–ELSD are different (see Figs. 1 and 2). Several reasons could explain this fact. It has been shown that ELSD response is directly linked to the sample mass [39] in a homologue series, whereas MS response is linked to ionization of the solutes. Generally, for a given solute in MS, the abundance of $[M+H]^{+}$ ions formed depends on its proton affinity, on the nature of the solvent and the presence of acidic or basic modifiers in mobile phase [29,45,46]. For amino acids, it has already been established that each amino acid has a different proton affinity [29] and recent

studies have demonstrated that the intensity of the $[M+H]^+$ signal does not drop significantly on the 'wrong' side of the pK_a [28,44]. Moreover, a suppression of the analyte ion signal has been observed in the presence of a significant concentration of surfactants in the mobile phase [47,48]. However, in our experimental conditions, as shown in Fig. 3, the increase in surfactant concentration in mobile phase increases the intensity of the $[M+H]^+$ signal.

The intensity of the $[M+H]^+$ signal for amino acids also depends on CID-MS fragmentation. Fig. 4 shows that low OR and RNG voltage values were more favourable than high values to a low fragmentation of $[M+H]^+$ ions but as a whole, the MS signal is weaker at low OR and RNG values than at high values. The molecular peak appears in all cases and the CID-MS fragmentation can be reduced but not totally avoided, because of the need to use a curtain gas in LC–MS coupling. Hence, since LC allows the separation between compounds which present MS problems linked to fragmentation (Table 2), intermediary OR and RNG voltages values are used to obtain a better sensitivity for the amino acid analysis.

In the chromatographic system proposed with Supelcosil column as stationary phase, the lack of separation between Gly ($M_r=75$) and Gln ($M_r=146$) observed by LC–ELSD (Fig. 1a), which prevents their quantification by LC–ELSD, is not a problem in LC–MS (Fig. 2a). Fig. 5a depicts the mass spectrum obtained at 4.8 min which is the specific retention time of Gly and Gln under these chromatographic conditions. In this spectrum two main ions were observed. The ion seen at m/z 76 corresponds to the $[M+H]^+$ ion of Gly and this seen at m/z 147 corresponds to $[M+H]^+$ ion of Gln. So by extracting ion current (XIC) corresponding to these two specific m/z ratios (Fig. 5(b)) it is now possible to

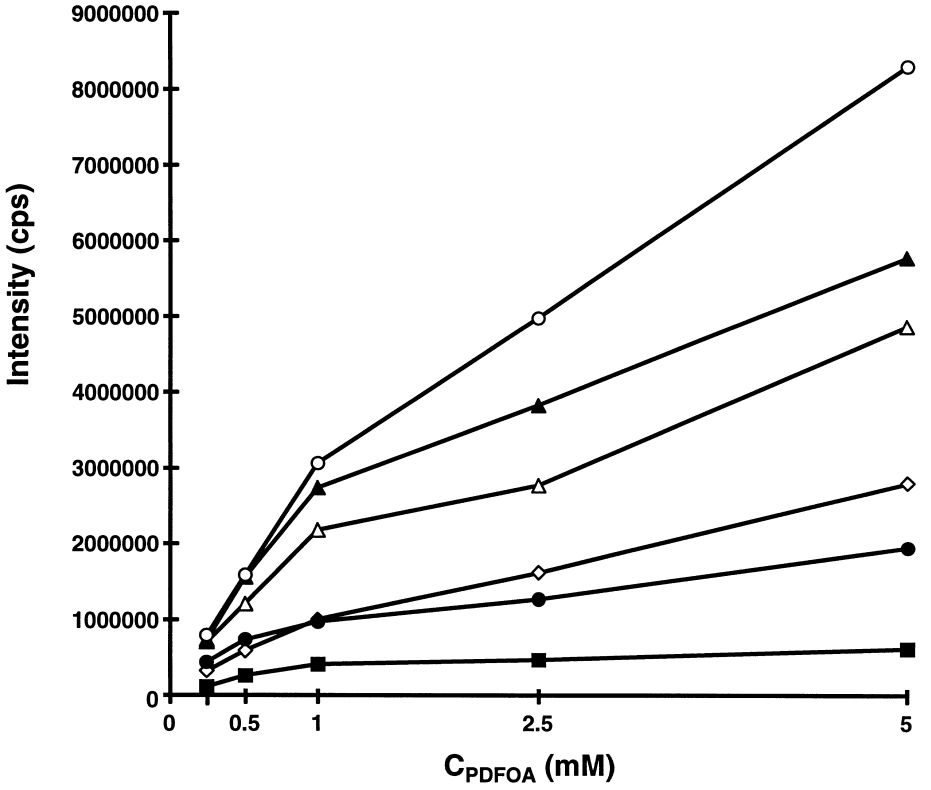

Fig. 3. Influence of the concentration of PDFOA (mM) on the MS response for some amino acids. Glu (■), Met ((◇), Phe (△), Trp (●), Val (▲) and Pro (○); infusion of the solutes by the syringe pump.

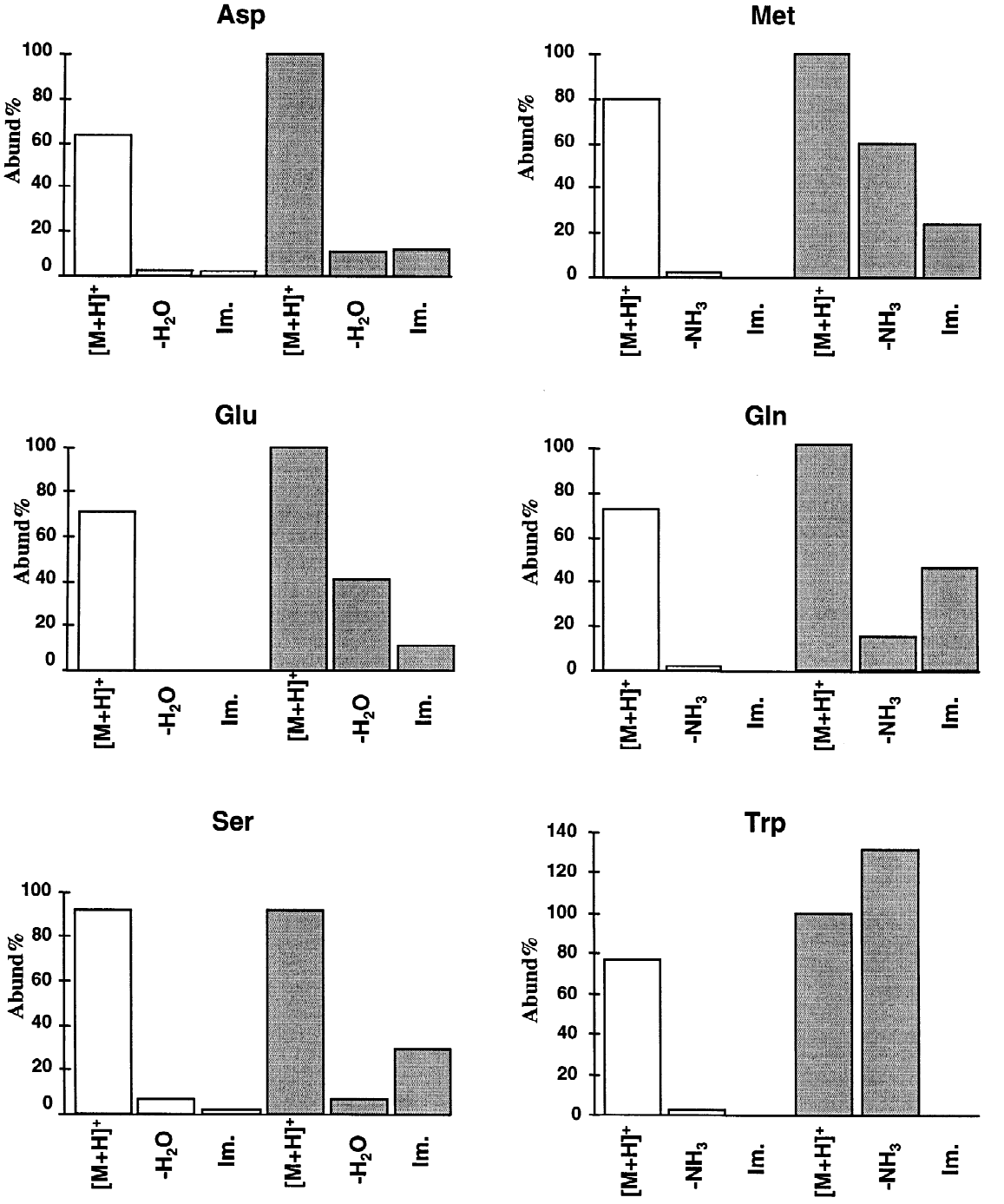

Fig. 4. Influence of the orifice (OR) and focusing ring (RNG) voltage on the CID-MS. RNG voltage is ten times OR voltage. In white: OR = 10 V, in grey OR = 30 V. Abundance scales were normalized using $[M+H]^+$ at OR = 30 V as 100% (abundances below 1% cannot appear on the histogram); infusion of the solutes by the syringe pump.

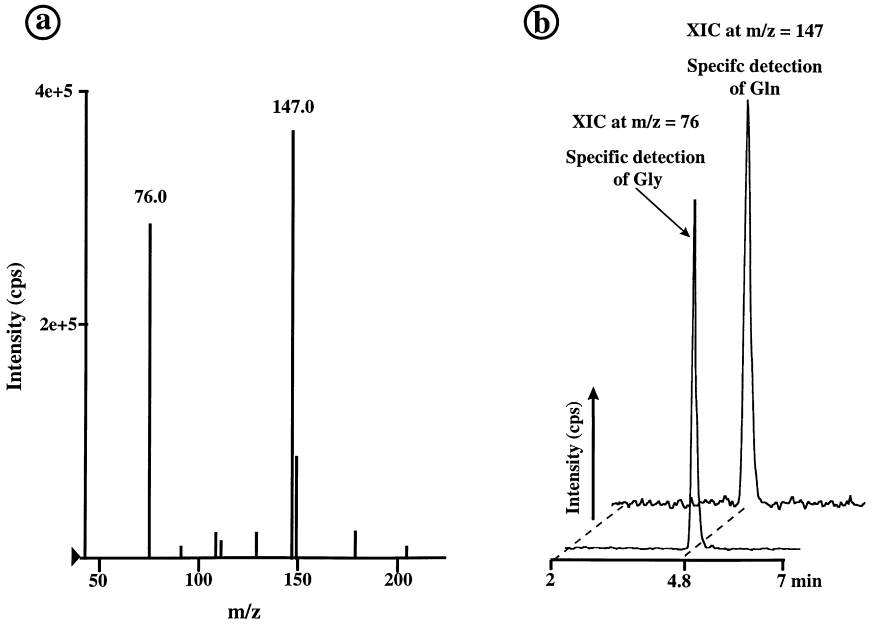

Fig. 5. Specific detection of Gln and Gly coeluted at 4.8 min under the chromatographic conditions reported in Fig. 2a. (a) Mass spectrum (at 4.8 min) by ion spray at +5 kV, orifice and ring voltages are respectively 20 V and 200 V. (b): XIC at $m/z=76$ and $m/z=147$ from the SIM of Fig. 2b.

identify by MS the presence of each amino acid in a sample and to quantify each in the presence of the other. All coelutions that can not be evidenced by XIC have been resolved by the chromatographic separation. For example, isotopic Leu and/or Ile and/or the $[M+H-NH_3]^+$ fragment of Met could induce an error in the determination of Asn if they are not separated as shown from the XIC at m/z 133 (see Fig. 6). The same goes for all the problems of Table 2.

The SP observed by LC–ELSD, when TDFHA ($M_r=364$) is used as ion-pairing reagent does not disturb the determination of the 20 amino acids by LC–MS. Indeed the molecules eluted in this system peak do not give a signal at m/z values selected for the SIM of the 20 amino acids. On the contrary, the PDFOA ($M_r=414$) induces two system peaks. The first SP, before the elution of Trp, gives a signal at the m/z ratio corresponding to the $[M+H]^+$ ion of Tyr and the second SP, at the end of the chromatogram, gives signals at the m/z ratios corresponding to the $[M+H]^+$ ions of Gly, Cys, Asn, Arg, His, Lys, Asp, Tyr and Trp. As the PDFOA does not enter into the mass spectrometer, these ions are probably due to impurities in the PDFOA (98% purity) which accumulate during column equilibration and are desorbed at the same time as the surfactant. From the above, only the masses corresponding with Lys, His, and Arg prevent their quantification.

Under these LC–MS conditions, the determination of the 20 proteinic underivatized amino acids is achieved with detection limits varying from 0.5 mg l^{-1} (corresponding to an injection amount of 10 ng) for Pro, His, Phe, to 1 mg l^{-1} (injected amount 20 ng) for Gly, Ala, Ser.

4. Conclusion

In this study a new method for the analysis of the 20 underivatized proteinic amino acids by LC–API-MS is described. Before LC–MS coupling, a number of amino acid pairs must be separated by an adequate LC system. In order to perform these separations, semi-long perfluorinated carboxylic acids were used as volatile ion-pairing reagents. ELSD proved to be an inexpensive way to develop a chromatographic

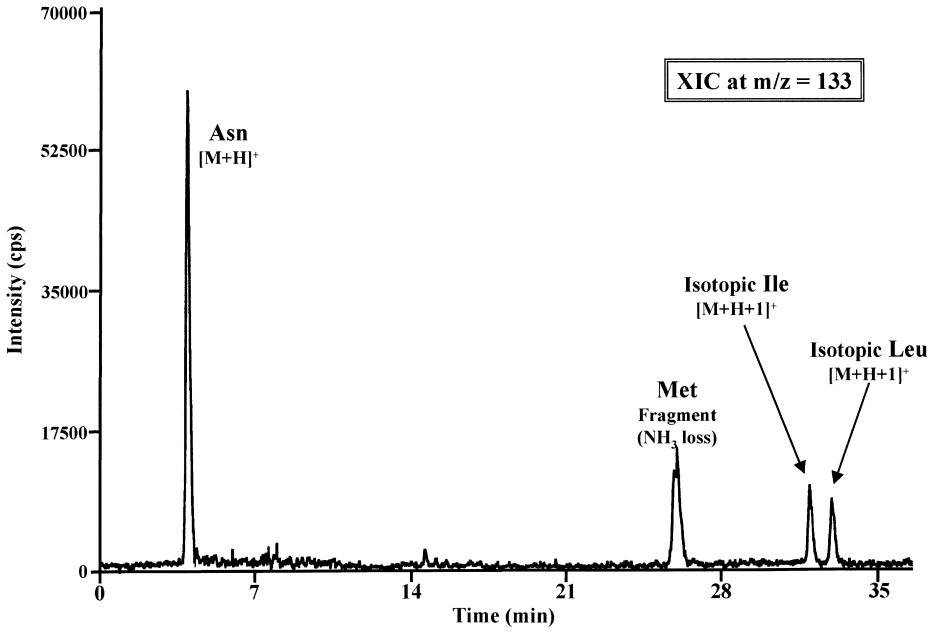

Fig. 6. XIC at $m/z = 133$ obtained on Purospher RP-18e (experimental conditions as described in Fig. 2b) shows the need of a separation for Asn, Met, Leu and Ile.

method, directly transposable in LC–API-MS. The identification of 17 of the 20 amino acids can be achieved by LC–ELSD, while the use of LC–API-MS allowed the identification of all the proteinic amino acids. The higher purity of TDFHA allows the determination of the basic amino acids (Lys, His, Arg) on either of the columns.

The proposed LC–MS system can be limited if an analysis of complex matrices is required, as amino acids have small molecular mass. Indeed, the presence in the sample of compounds with the same or higher molecular mass may interfere with the detection of these targeted analytes. Furthermore, the detection limits of this work can be improved (about an order of magnitude) through use of smaller internal diameter columns and/or TurboIonSpray as source which increases the efficiency of ion evaporation and allows the utilization of higher flow-rates for the mobile phase without splitting requirement. The future of this work is the development of a tandem-MS methodology to bring more specificity in the detection mode in order to reduce analysis time, to lower the detection limits and to quantify amino acids in complex matrices.

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