ELSEVIER

Journal of Chromatography A, 896 (2000) 253–263

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Parameter optimization for the analysis of underivatized protein amino acids by liquid chromatography and ionspray tandem mass spectrometry

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

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

Received 10 February 2000; received in revised form 14 April 2000; accepted 2 May 2000

Abstract

The analysis of the 20 underivatized protein amino acids by liquid chromatography ionspray tandem mass spectrometry is investigated in positive ion mode. First, by direct infusion, amino acid fragmentation was investigated based on the product-ion mass spectrum of the parent compound in three different collision energies (10, 20, 30 eV). Then, the relative abundance of fragment ions was studied as a function of the collision energy in order to select the product ion with the highest abundance and to obtain the maximum sensitivity for each amino acid, by using the optimum collision energy. Depending on the amino acid, the loss of H_2O or NH_3 or CH_2O_2 was selected as the product ion from the molecular ion $[M+H]^+$ in selective reaction monitoring mode. 15 eV was chosen as a mean value of collision energy to obtain satisfactory sensitivity for the simultaneous determination of the 20 protein amino acids. In spite of the specificity of mass spectrometry, and in order to obtain maximum sensitivity, several pairs of amino acids had to be separated. The separation of these amino acids pairs was achieved in less than 20 min by using a porous graphitic carbon column and nonafluoropentanoic acid as ion-pairing reagent. Detection limits depending on the amino acid varied from 500 fmol to 40 pmol (using a 10 μl loop).

Keywords: Mass spectrometry; Amino acids

1. Introduction

The determination of amino acids is of great importance in food extracts, biological fluids and fermentation products. Among the protein amino acids only phenylalanine, tyrosine and tryptophan have sufficient UV absorbance. Conventional spectrophotometric detection methods involve UV or fluorescence detection of amino acid derivatives obtained after pre- or post-column derivatization. However, derivative instability, reagent interferences, the inability of some reagents to derivatize the secondary amino acids, and long preparation time are the main disadvantages for the derivatization methods [1,2]. Furthermore, the effective separation of all the amino acids of interest is essential for their determination.

Alternative methods proposed for the analysis of underivatized amino acids containing electrochemical detection [3–5], indirect UV–fluorimetric detection [6–9], or direct low-wavelength UV detection [10] have not gained wide acceptance due to low sensitivity, baseline drift, potential instability, incom-

*Corresponding author.

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

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00582-3

patibility with gradient elution mode, inability of analyzing complex matrices etc.

The satisfactory detection of such underivatized molecules can be achieved only by a universal and sensitive detection. Among the universal detection methods, evaporative light scattering detection (ELSD) has been recently shown to be a good alternative for amino acid analysis with detection limits about ≥ 1 mg l^{-1} [11–15] and to be well suited to the analysis of protein hydrolysate [16].

However, the detection of choice should be mass spectrometry as this mode of detection has the advantage of providing additional structural information about the eluted compounds. Because of the great importance of the protein and peptide mapping sequence, all the available ionization methods have been tested for the ionization of amino acids such as electron impact [17], chemical ionization [18], field desorption [19], plasma desorption (20), secondary ion mass spectrometry [21], laser mass spectrometry [22], thermospray [23], fast atom bombardment (FAB) [24,25] and atmospheric pressure ionization (API) [26]. Among them 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.

Recently [12], the analysis of the 20 underivatized protein amino acids has been successfully achieved by liquid chromatography–ionspray mass spectrometry (LC–ISP-MS). A positive ion mode has been used for their ionization, as for all amino acids (even for the acidic ones) under optimized experimental conditions, higher sensitivities can be obtained for the molecular ion in the positive ion mode ($[M+H]^+$) than in the negative mode ($[M-H]^-$) [27]. It has also been demonstrated [12] that most protonated amino acids generated in the gas phase undergo fragmentation, due to the collisions with nitrogen, in the atmospheric pressure ion source {termed as in-source collisionally induced dissociation (in-source CID) by Niessen [28]}. This in-source CID could not be avoided even under the most favorable conditions — minimum curtain gas flow-rate and minimum acceleration of protonated amino acids by decreasing orifice (OR) and focusing ring voltage (RNG) — and led to additional charged ions which in some cases had the same m/z ratio as the $[M+H]^+$ of a protonated amino acid. Because of the above in-source CID fragments as well as isomeric and isobaric amino acids and carbon-13 isotopes, some amino acid mixtures had to be separated by LC before identification by MS. Several suitable and complementary chromatographic systems compatible with MS detection are nowadays available for separation of underivatized protein amino acids [11–13]. They use ion-pair reversed-phase liquid chromatography on octadecyl silica or porous graphitic carbon columns with perfluorinated carboxylic acids as the ion pairing reagent. These reagents not only give enhanced selectivities for underivatized protein amino acids towards alkyl sulfonates ion pairing reagent [29–31], but are volatile enough to be compatible with mass spectrometry. However, under these LC–MS conditions, the determination of the 20 protein amino acids could be achieved in under 30 min with detection limits only around 20 ng of injected amount.

Thus in this work, the development of a liquid chromatography–ionspray tandem mass spectrometry (LC–ISP-MS–MS) methodology is investigated to bring more specificity in the detection mode, in order to reduce analysis time and to lower the detection limits.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile (ACN) and tetrahydrofuran (THF) were obtained from J.T. Baker (Noisy le Sec, France). Trifluoroacetic acid (TFA) was obtained from Interchim (Montluçon, France) and nonafluoropentanoic acid (NFPA) from Aldrich (St Quentin-Fallavier, France). All amino acids were purchased from Sigma (St Louis, MO, USA). 18 MΩ deionized water from an Elgastat UHQ II system (Elga, Antony, France) was used for the preparation of amino acid and ion-pairing reagent solutions.

2.1.1. Apparatus

LC–ISP-MS–MS was carried out using 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. Nitrogen was used as curtain and collision gas. After optimization of MS parameters, state files were as follows; NEB=9, CUR=7, CAD=1, IS=5000, OR=20, RNG=200, Q0=−5, IQ1=−6, ST=−10, RO1=−6, IQ2=−15, RO2=−20, IQ3=−35, RO3=−25, DF=−400, CEM=2100. Quad 1: 30 (0.010), 100 (0.050), 1000 (0.400), 2000 (0.742). Quad 3: 10 (0.008), 100 (0.035), 1000 (0.285), 2000 (0.530). The NEB=9 (nebulizer gas) corresponds to a flow rate of 1.08 l min^{-1} and the CUR=7 (curtain gas) corresponds to a flow rate of 1.02 l min^{-1}. The selective reaction monitoring (SRM) mode was used to monitor the parent and product ions. The dwell time was set at 100 ms and the pause time was 5.0 ms. Injections were done by a Perkin-Elmer series 200 autosampler (Toronto, Canada) fitted with a 10-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}.

Separation was carried out on a porous graphitic carbon column, Hypercarb S 100×2.1 mm I.D., particle size 5 μm from Hypersil (Runcorn, UK). Flow-rate was 200 μl min^{-1}. For LC–MS–MS, a split 1/10 was used to avoid too high a flow-rate in the ion source. For gradient elution, solvent A was: 20 m*M* NFPA in water. Solvent B was acetonitrile.

The equilibration of the chromatographic system was confirmed by monitoring the conductivity of the mobile phase by a Vydac conductivity meter (Wescan Instrument, Santa Clara, USA) model 6000 CD. A Beckman pH meter Model Φ 10 (Gagny, France) was used to measure the pH of the solutions.

Before use of the Hypercarb column, 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 for surface regeneration.

3. Results and discussion

Current limitations of LC–ISP-MS analysis of underivatized protein amino acids have been previously listed [12] and are linked to the intrinsic problems of MS detection (isobaric and isomeric amino acids, collisionally induced dissociation fragments, carbon-13 isotope, etc.). As a result, a necessary chromatographic separation of the different amino acid pairs which have some similar characteristics has to be achieved before MS identification.

In LC–ISP-MS, it has been noted that for all the amino acids, in positive ionization mode the protonated molecule $[M+H]^+$ was the most abundant. In order to develop a tandem MS method it was necessary to establish for each amino acid, the product-ion spectra after collisionally activated dissociation (CAD) of the $[M+H]^+$ ions. Thus, the most abundant, or the most specific fragment ion should be selected as product ion in addition to the parent ion in the SRM mode. As the target compound is now monitored at two points (parent and product ion) with MS–MS, background noise is minimized, allowing an enhancement of sensitivity in comparison with simple MS mode (usually a 100-fold lower detection limit is attained).

When only one solute is analysed, things are rather easy. However, when the simultaneous analysis of several solutes is required, several MS–MS parameters must be investigated and several others must be taken into consideration.

3.1. Optimization of ionspray MS–MS conditions for underivatized protein amino acids

Previous optimization of MS parameters [12] had concluded in compromised values of OR (20 V) and RNG (200 V) for which the molecular ion of all the amino acids had the highest relative abundance. In order to investigate further fragmentation of the protonated molecules by N_2 gas within the mass analyzer (CAD) a hydro-organic solution [water–ACN (50:50) acidified with 1 m*M* TFA] of 60 mg l^{-1} of every amino acid was infused by a syringe pump at a flow rate of 5 μl min^{-1}.

The mass spectrometry was set in product-ion-scan mode by selecting the $[M+H]^+$ as parent ion and scanning in a mass area from 30 to $[M+H+3]^+$ mass units for determination of the product ions. The above procedure was repeated three times for each of the collision energies: 10, 20, 30 eV. Table 1 summarizes all the CAD fragments observed and the most abundant fragments are underlined. All peaks exceeding 2% of the total fragment ion abundance are recorded. The immonium ion $[H_2N=CH\text{-}R]^+$

Table 1
m/z values of CAD fragments of amino acids in precursor-ion-scan mode using 10 to 30 eV collision energy. For a given amino acid, the underlined m/z value corresponds to the most abundant ion fragment and the number in parenthesis corresponds to the collision energy value for which the fragment abundance is maximum. $[Im]^+=[H_2N=CH\text{-}R]^+$ where R is the residue of the amino acid

Amino acid	m/z						
	$[M+H]^+$	$[M+H-NH_3]^+$	$[M+H-H_2O]^+$	$[Im]^+$	$[M+H\text{-}63]^+$	$[M+H\text{-}64]^+$	Others
Gly	76			30 (15)			48
Ala	90			44 (15)			
Ser	106		88	60 (12)		42	
Pro	116			70 (18)			
Val	118			72 (12)	55		57
Thr	120		102	74 (13)		56	84
Cys	122	105		76 (18)	59 (32)		100, 87
Ile	132			86 (15)			69, 57, 44, 41, 30
Leu	132			86 (12)			63, 55, 44, 43, 30
Asn	133	116		87 (12)			74 (20)
Asp	134		116	88 (14)		70	74 (18), 65, 46, 43
Gln	147	130 (12)					84 (22), 56
Lys	147	130 (14)					84 (20), 56
Glu	148		130 (12)	102		84 (20)	56, 41
Met	150	133 (14)		104 (14)	87		102, 74, 61, 56
His	156			110 (19)	93		83, 56
Phe	166	149		120 (17)	103		79
Arg	175	158					130, 116 (18), 70 (30), 60
Tyr	182	165 (13)		136 (17)	119		147, 123, 95, 93, 91
Trp	205	188 (14)		159			146 (26), 132, 118

(where R is the residue of the amino acid) was observed for 17 out of 20 amino acids (except for Gln, Lys and Arg). The acid (Asp, Glu) and hydroxyl (Ser, Thr) protein amino acids gave additional fragments corresponding to a loss of water from the protonated molecule (loss of 18 units). The amino acids Cys, Asn, Gln, Lys, Met, Phe, Arg, Tyr and Trp gave ions which correspond to a loss of NH_3 from the $[M+H]^+$ (loss of 17 units). The loss of 63 mass units from the protonated molecules can correspond either to the loss of an NH_3 from the immonium fragment $[Im-NH_3]$ and/or to the formation of the immonium ion from the fragment $[M+H-NH_3]^+$. The $[M+H\text{-}64]^+$ corresponds either to an elimination of a water molecule from the immonium fragment $[Im-H_2O]$ and/or to the formation of the immonium ion from the fragment $[M+H-H_2O]^+$. One CAD fragment at m/z 74 corresponding to the $[NH_2=CH-COOH]^+$ immonium ion is common for several amino acids. For some amino acids several other fragmentations are observed. These CAD fragments have already been observed by other ionization methods and the mechanism of their formation has been explained [17–26]. The amino acids Leu and Ile gave low abundance specific fragments allowing their differentiation, confirming recent observations [25,32] and in contradiction with earlier results [33]. Unfortunately, for the investigated collision energies CAD did not give specific fragments for the isobaric amino acids Gln and Lys.

Understanding of the fragmentation of a molecule has to be completed by that of the relative abundance of the formed ions for the selection of the product ion in the SRM mode. The relative abundance of a product ion can vary depending on the collision energy. Furthermore, the choice of the collision energy will depend on the overall optimum collision energy of the 20 underivatized protein amino acids in the case of simultaneous analysis of these compounds.

Fig. 1 depicts the relative fragment abundances versus the collision energy (in the range 10–40 eV) for Gly, Arg, Ile, Tyr, Cys, Asp, Asn and Gln. It appears that for each amino acid, an increase in the collision energy (increase of internal energy of $[M+H]^+$) involves a decrease in the corresponding

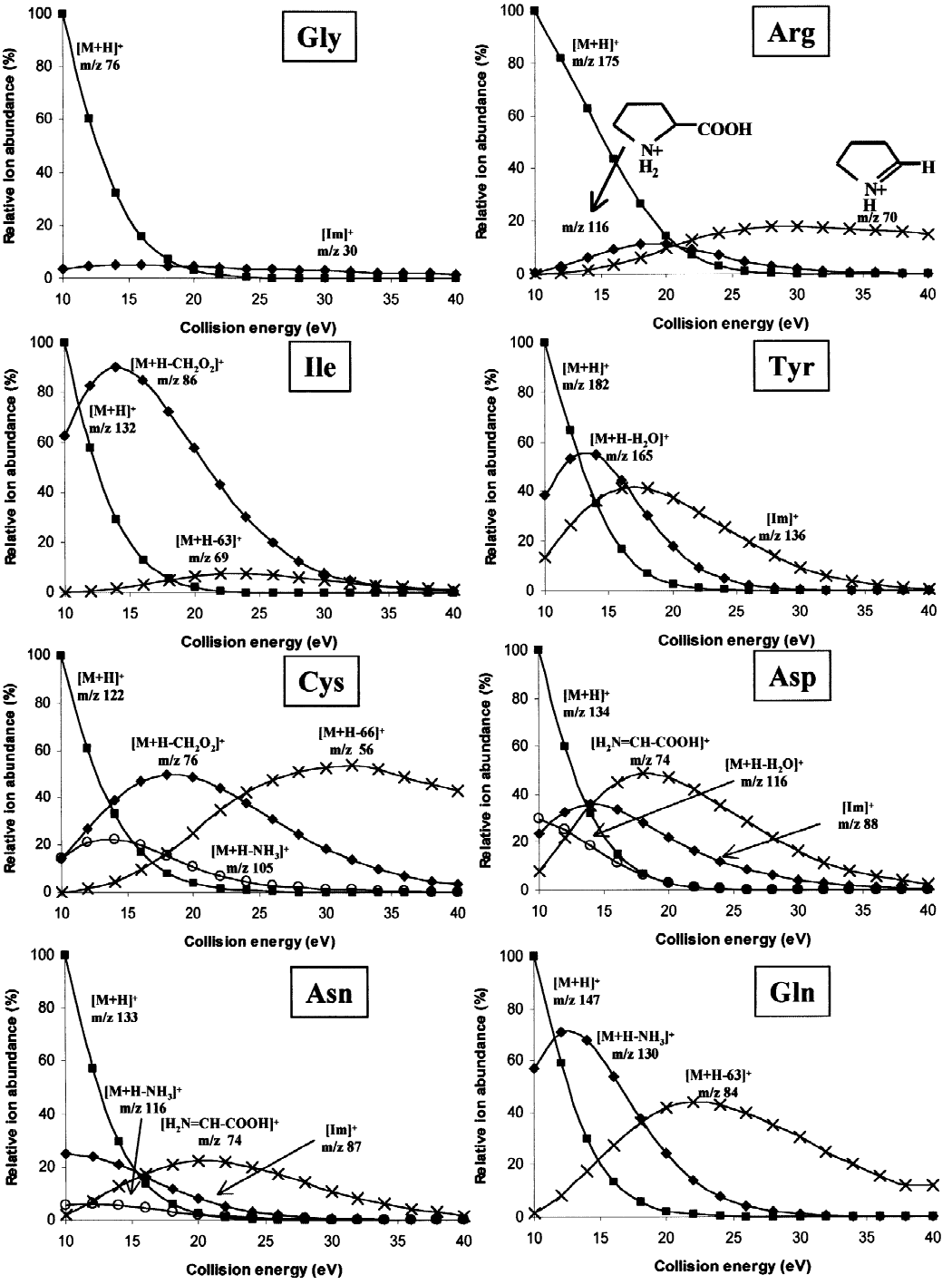

Fig. 1. Breakdown graphs of the underivatized amino acids Gly, Arg, Ile, Tyr, Cys, Asp, Asn and Gln. $[Im]^+ = [H_2N{=}CH\text{-}R]^+$ where R is the residue of the amino acid.

$[M+H]^+$ ion with the resulting formation of the CAD fragments. By increasing the collision energy, the CAD ion abundance passes through a maximum and for the majority of amino acids, the optimum of the breakdown graph was corresponded to a collision energy value between 12 and 20 eV. Moreover, Table 1 specifies in parenthesis the collision energy value for which the abundance of the main fragment ion of each amino acid is maximum. Therefore, an intermediary collision energy value equal to 15 eV was fixed as a satisfactory compromise for further studies in relation to the simultaneous analysis of the 20 protein amino acids.

With a 15 eV collision energy in the case of Gly (Fig. 1), the parent ion appeared at m/z 76 and a product ion (with a weak abundance) appeared at m/z 30 from Gly moiety by MS–MS. Thus, Gly can be detected with a combination of m/z 76→30 in SRM. In the case of Tyr (Fig. 1) the choice of the SRM conditions is not as limited. The parent ion appeared at m/z 182 and two product ions appeared at m/z 165 and 136 with a similar relative abundance. Tyr can be specifically detected either with a combination of m/z 182→165 or 182→136 in SRM in accordance with the fragmentation of the other amino acids simultaneously analysed. Due to its highly thermochemically stable guanidinium ion [R-NH-C(=NH_2)NH_2]$^+$, Arg did not give intensive fragments in the range of 15 eV. For the amino acid Asp, the CAD fragments of m/z 88 (immonium) and m/z 74 (corresponding to the ion [NH_2=CH–COOH]$^+$ [20,25]) are the ones with the highest abundance at 15 eV. For Asn the immonium fragment should be selected as product ion as it is the most abundant at 15 eV. The CAD fragment corresponding to $[M+H-NH_3]^+$ must obviously be selected as product ion for Gln as shown from Fig. 1.

The use of two mass filters in a tandem mass detection substantially increases the detection selectivity. For example in the case of the Gly–Cys pair, an in-source CID fragment of Cys was observed at m/z 76 that is the m/z ratio for the $[M+H]^+$ ion of Gly, however, this in-source CID immonium cannot further fragment as the parent ion of Gly to give an immonium ion at m/z 30. As a result, it now became possible to differentiate Gly and Cys by tandem MS detection whereas it was not possible by simple MS detection. The same holds for the pair Phe–Thr.

However, as can be seen from Table 1, for maximum sensitivity (high abundant CAD fragments selected as product ions) several amino acid pairs must be separated. First of all, the two isobar amino acids Gln and Lys and two isomers Leu and Ile have to be separated due to their identical fragmentation at low collision energy. The large in-source CID fragmentation for Met also leads to some difficulties. Indeed, an important fragment ion at m/z 133 [corresponding to the NH_3 loss from the parent ion (m/z 150)] was formed when its $[M+H]^+$ ion passed through the curtain gas of the interface. This fragment ion has the same mass as the parent ion of Asn. Thus unfortunately the CAD fragment of the in-source Met fragment gives a m/z ratio equal to 87, which is the m/z ratio of the product ion of Asn. Consequently the chromatographic separation of Asn and Met remains a necessity. The same problem is valid for the pair Asp–Pro.

The chromatographic separation of Asn and Asp has to be implemented as the molecular mass of these two amino acids differs only by one mass unit and as the ^{13}C isotopes cannot be ignored. The m/z ratio of $[(^{13}C)Asn+H]^+$ ion is equal to that of the parent ion $[M+H]^+$ of Asp and then the same m/z ratio is observed for their two immonium fragments selected as product ions. The problem is similar for the two isobar amino acids Lys and Gln in relation to Glu or for the two isomers Leu and Ile in relation to Asn.

Moreover, in the case of the pair Tyr–Phe, the ^{13}C isotopic in-source CID fragmentation of Tyr with m/z 166 ($[^{13}CTyr+H-NH_3]^+$) with further decomposition in the Q2 area gives the immonium ion with m/z 120. These two fragments correspond to the parent and product ion of Phe. The same problem occurs for the Cys–Ser pair. Lastly, the Pro–Arg pair which did not need to be separated by LC before MS detection can constitute a problem with tandem MS detection. The parent ion of Arg is very stable, but its fragmentation led to the protonated form of Pro (m/z 116) and then to the immonium form of Pro at m/z 70, which is also the product ion selected for the Pro detection.

However, some of the above problems can be resolved, although to the detriment of sensitivity. The problem among Asn, Met, Ile and Leu can be resolved by a 4-fold loss of sensitivity for Asn if the

$[M+H-H_2O]^+$ ion is selected as product ion. A roughly 6-fold loss of sensitivity (for Ser) is necessary to resolve the undetermination between Ser and Cys by selecting the loss of water as product ion for Ser. The problem between Phe and Tyr can be resolved if the loss of NH_3 is selected as product ion for Phe, leading however to a dramatic decrease in sensitivity. A similar dramatic decrease in sensitivity takes place if the problem between Ile and Leu has to be resolved by selecting the specific CAD fragments of Leu and Ile as product ions (i.e. m/z 41 for Ile and m/z 43 for Leu). Finally, for Asp and Asn the undetermination can be resolved by choosing the specific CAD fragment of Asp (i.e. 46).

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

We reported in a recent paper [13] the determination in gradient elution of all the 20 protein amino acids using ion-pair reversed-phase chromatography on porous graphitic carbon column and evaporative light scattering detection. The analysis time was about 40 min. Furthermore, as discussed earlier, when mass spectrometry is used as detection method the need for a total separation of the amino acids is not necessary for their determination and so the analysis time can be decreased by using a more eluting mobile phase. Moreover, it has been shown [34] that increased concentration of an organic solvent ($\geq$10%) improves MS signal stability.

Table 2 sums up each parent and product ion selected for each amino acid in the SRM mode in view of their simultaneous analysis. The choice of the product ion was made according to two criteria: improvement over selectivity and improvement over detection sensitivity (signal/noise ratio). Thus for Arg, the product ion selected is also the $[M+H]^+$ ion. Moreover, by taking a look at the breakdown graph of Cys for example (Fig. 1) the ion with m/z 76 should be chosen as product ion. However, by selecting the ion with m/z 105 as product ion, higher signal/noise ratios were obtained for the present chromatographic conditions.

Fig. 2a shows the SRM of the 20 amino acids (18 ions) obtained by LC–ISP-MS–MS. The specificity of the SRM mode allowed the chromatographic single run analysis of the 20 underivatized protein

Table 2
Choice of the parent and product ions for the LC–ISP-MS–MS analysis of the 20 underivatized protein amino acids. Detection limits obtained for the 20 underivatized protein amino acids in MS and MS–MS mode for the same chromatographic conditions

Amino acid	Parent ion (m/z)	Product ion (m/z)	Detection limit MS (pmol)	Detection limit MS–MS (pmol)
Gly	76	30	60	30
Ala	90	44	100	10
Ser	106	60	40	9
Pro	116	70	20	4
Val	118	72	40	4
Thr	120	74	30	3
Cys	122	105	50	20
Ile	132	86	10	4
Leu	132	86	20	4
Asn	133	87	80	40
Asp	134	88	30	8
Lys	147	130	70	10
Gln	147	130	40	5
Glu	148	130	20	4
Met	150	104	50	20
His	156	110	40	20
Phe	166	120	10	0.3
Arg	175	175	20	8
Tyr	182	165	10	0.5
Trp	205	188	30	0.5

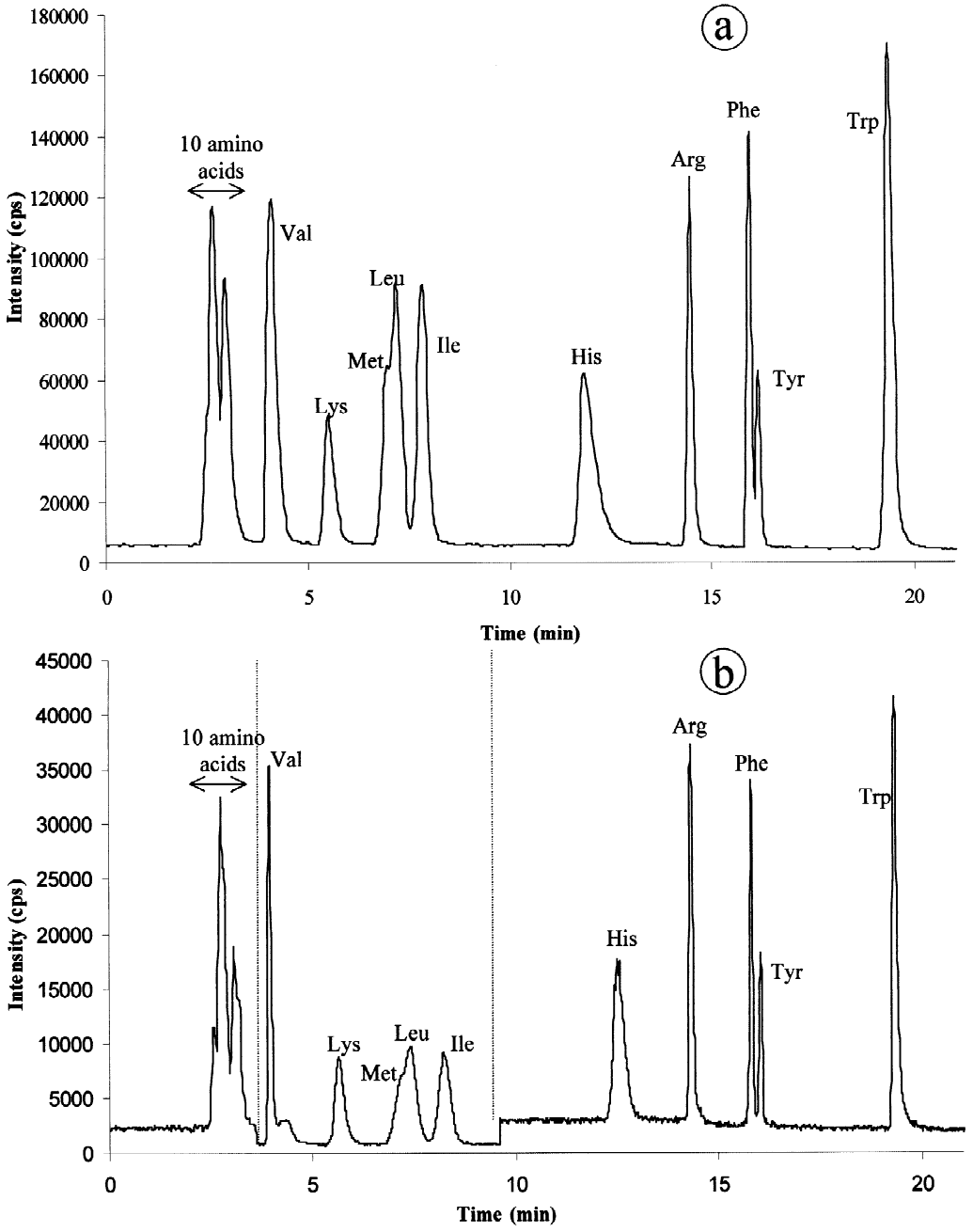

Fig. 2. LC–ISP-MS–MS analysis of the 20 underivatized protein amino acids in gradient elution. Gradient elution: solvent A: 20 m*M* NFPA in water, solvent B: acetonitrile; gradient starts at 10% B for 1 min, from 10 to 72% in 7 min, then from 72% to 90% in 1 min, then 90% is maintained to the end of the analysis. Flow rate: 200 μl min^{-1}, Split 1/10; MS parameters: (see Section 2) (a): SRM of of a 50 mg l^{-1} standard solution of the 20 underivatized protein amino acids (18 ions) (dwell time: 100 ms). (b): SRM of a 10 mg l^{-1} standard solution of the 20 underivatized protein amino acids ''cut'' in three sections: from 0 to 3 min (10 ions, dwell time: 200 ms), from 3.2 to 9 min (4 ions, dwell time: 400 ms), from 9 min until the end of analysis (5 ions 300 ms).

amino acids in less than 20 min. Recent advantages of Perkin-Elmer triple quadrupole instruments have given rise to the linear accelerating high-pressure collision cell (LINAC) [35] which allows shorter dwell times in the selective ion monitoring (SIM) mode, thereby allowing to monitor more SIM transition during one chromatographic run without sensitivity loss. However, if higher dwell times are needed, it is possible as shown in Fig. 2b to ''cut'' the chromatogram in three sections; from 0 to 3 min where 10 ions are monitored, from 3 to 9.5 min (monitor of 4 ions) and lastly from 9.5 min until the end of the analysis (monitor of 5 ions). This operation allows the 1–4-fold increase of the dwell time.

Fig. 3 shows the extracted ion currents (XIC) of several amino acids. Fig. 3a–c shows the solution of two problems: Ala and Thr are co-eluted at 2.67 min (Fig. 3a and b) but the specificity of mass spectrometry allows their detection due to the difference of their parent and product ions, whereas for the isomers Leu and Ile (Fig. 3c), their determination is achieved due to their chromatographic separation (t_{Leu}=7.2 min, t_{Ile}=7.84 min). As shown in Fig. 3d traces of Gln and Lys can be observed in the XIC of Glu (148→130) for the isotopic reasons. Fig. 3e and f show the interference of the Arg in the XIC of Pro (116→70). For the same concentration for Pro and Arg (50 mg l^{-1}) the interference induced from Arg is 50-fold smaller than the peak of Pro.

Table 2 sums up the detection limits of all the amino acids in simple MS and tandem MS mode for the same chromatographic conditions. Analysis by tandem MS gave 2–60-fold lower detection limits than simple MS. Detection limits for tandem MS

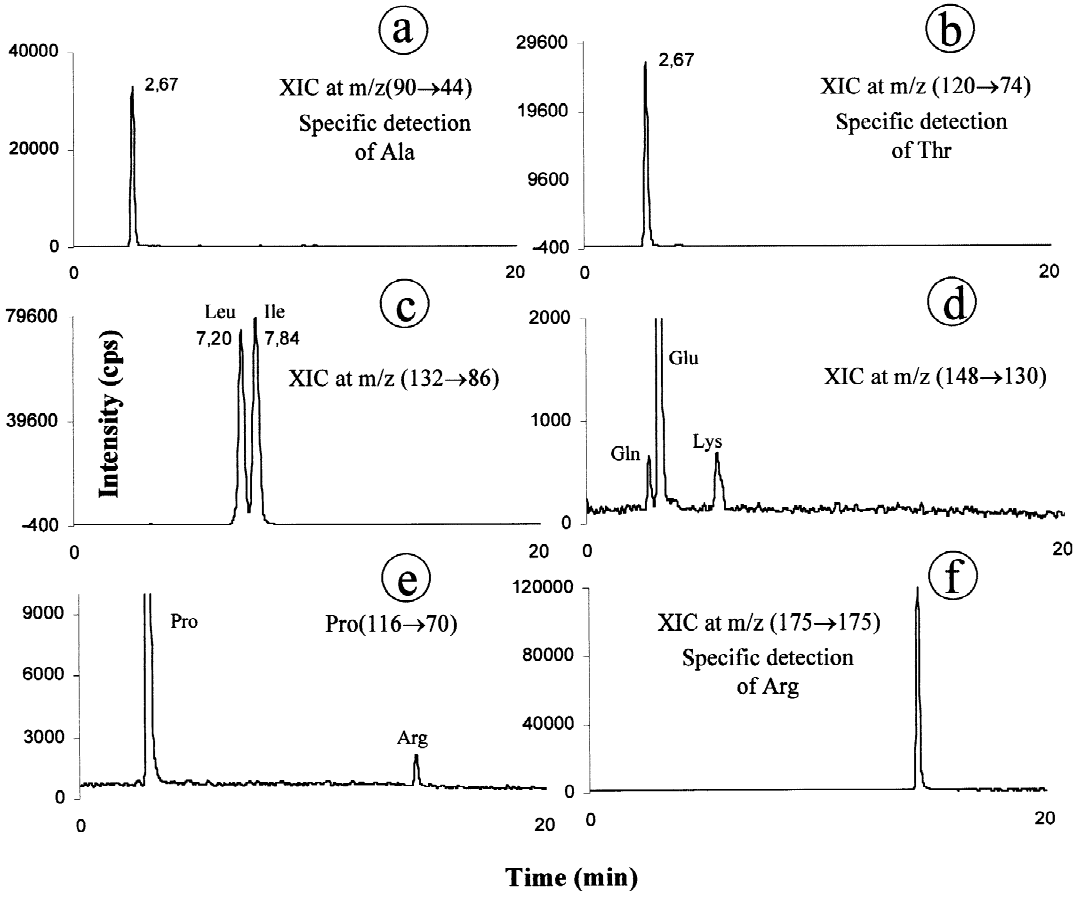

Fig. 3. XIC of the amino acids Ala, Thr, Leu, Ile, Glu, Pro and Arg.

varied depending on the amino acid from 500 fmol to 40 pmol (using a 10 μl loop). In spite of the low abundance CAD fragment of Gly, detection limits were equivalent to other amino acids, as the m/z 76→30 was the mass with the lowest noise. However, the overall sensitivity of the underivatized protein amino acids was of the same order of magnitude as that of post-column derivatization UV spectrophotometric methods [2] and is far from being the one generally announced for MS methods (i.e. in the order of fmol).

This low sensitivity can be explained by the following reasons: the M_r of underivatized protein amino acids is between 75 (Gly) and 204 (Trp) and as shown by Fig. 4, the area from m/z 50–250 is highly ''noisy'' due to solvent background (an additional reason for operating in tandem MS mode rather than simple MS). Noise is caused not only by the existence of small molecules but also from the fragmentation of molecules with higher molecular mass which may give ions in the above area. However, blank injection analysis has excluded the possibility of having increased amounts of background ions in the mobile phase which have the same precursor and product ion with the amino acids analyzed. Moreover, it has been shown [36,37] that ion sampling and ion transmission decrease for low mass ion, albeit below m/z 150.

The problem of sensitivity will be resolved with instrumentation improvements in the mass analyzer. Moreover, software improvements allow the simultaneous monitoring of two or more product ions which can be interesting for amino acids such as Tyr, Cys, Asp, Lys and Met that give two fragments with about equal intensities.

4. Conclusion

For the first time, the 20 underivatized protein amino acids have been analyzed by LC–ISP-MS–MS. The selection of the appropriate fragments as well as the selection of the appropriate collision energy has been shown to be of ultimate importance for optimum sensitivity. In spite of the specificity of mass spectrometry the chromatographic separation of some amino acid pairs remains necessary. The limits of detection of this method were of the same order of magnitude as that of post-column derivatization UV spectrophotometric methods. This MS method is shown to be more specific than conventional fluorimetric methods, because it allows the co-elution of some amino acids.

Furthermore, when analyzing amino acids in complex matrices by using conventional detectors there is always some doubt as to whether the peaks measured are actually those of target compounds or other co-eluted compounds, whereas LC–ISP-MS–MS can give more structural information and therefore a positive identification of the analyzed amino

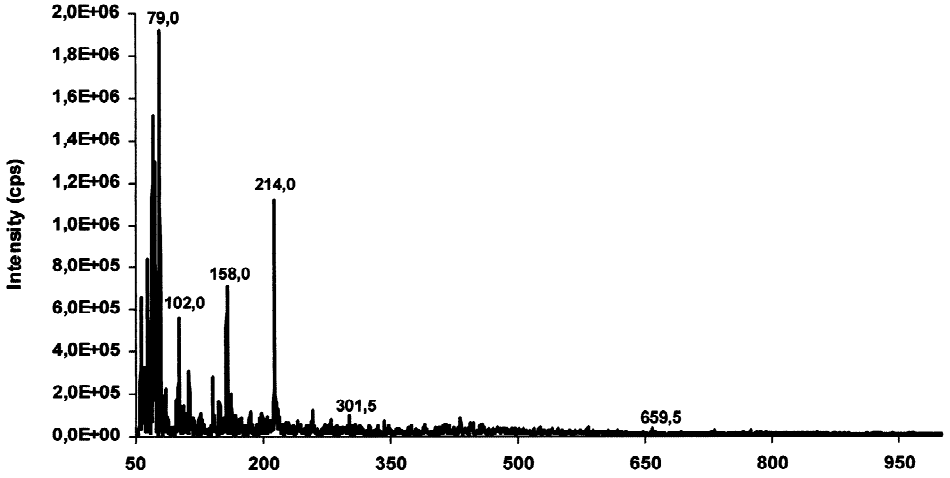

Fig. 4. Mass spectra of the mobile phase showing the high background noise in low mass range.

acid. Moreover, by overcoming the derivatization step, the easier automatization of the LC method is obtained as only a pre-purification step may be necessary in the case of physiological samples in order to protect the chromatographic support. Lastly, rapid developments in the instrumentation of ion sources and mass spectrometers as well as in the corresponding software will lead to higher sensitivities. The future of this work will be the determination of amino acids in complex matrices.

References

[1] H. Godel, P. Seitz, M. Verhoef, LC–GC Int. 5 (1992) 44.
[2] D. Fekkes, J. Chromatogr. B 682 (1996) 3.
[3] P. Luo, F. Zhang, R.P. Buldwin, Anal. Chem. 63 (1991) 1702.
[4] J.M. Marioli, L.E. Sereno, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 2505.
[5] L.E. Welch, W.R. Lacourse, D.A. Mead Jr., D.C. Johnson, Anal. Chem. 61 (1989) 555.
[6] M. Eslami, P. Hoshemi, M.N. Sarbolaki, J. Chromatogr. Sci. 31 (1993) 480.
[7] P.G. Simonson, D.J. Pietrzyk, J. Liq. Chromatogr. 16 (1993) 597.
[8] J. Crommen, G. Shill, D. Westerland, J. Chromatogr. 461 (1989) 429.
[9] J. Crommen, P. Herné, J. Pharm. Biomed. Anal. 2 (1984) 241.
[10] L.E. Vera-Arila, M. Caude, R. Rosset, Analusis 10 (1982) 43.
[11] K. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, J. Chromatogr. A 833 (1999) 147.
[12] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, J. Chromatogr. A 855 (1999) 191.
[13] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, J. Chromatogr. A 870 (2000) 245.
[14] H.J. Chaves-des-Neves, Z. Braya-Morais, Anal. Quim. 93 (1997) 98.
[15] H.J. Chaves-des-Neves, Z. Braya-Morais, J. High Resolut. Chromatogr. 20 (1997) 115.
[16] J.A. Peterson, L.J. Lorenz, D.S. Risley, B.J. Sandmann, J. Liq. Chromatogr. Rel. Technol. 22 (1999) 1009.
[17] K. Biemann, J.A. McCloskey, J. Am. Chem. Soc. 84 (1962) 3192.
[18] M. Eckersley, J.H. Bowie, R.N. Hayes, Int. J. Mass Spectrom. Ion Proc. 93 (1989) 199.
[19] H.U. Winkler, H.D. Beckey, Org. Mass Spectrom. 6 (1972) 655.
[20] S. Bouchonnet, J.P. Denhez, Y. Hoppilliard, C. Mauriac, Anal. Chem. 64 (1992) 743.
[21] L.K. Liu, K.L. Busch, R.G. Cooks, Anal. Chem. 53 (1981) 109.
[22] W. Kulik, W. Heerma, Biomed. Mass Spectrom. 15 (1988) 419.
[23] R.G.J. Van Leuken, C.T.C. Kwakkenbos, A.L.L. Duchateau, J. Chromatogr. 647 (1993) 131.
[24] C.D. Parker, D.M. Hercules, Anal. Chem. 58 (1986) 25.
[25] N. N Dookeran, T. Yalcin, A.G. Harrison, J. Mass Spectrom. 31 (1996) 500.
[26] H. Kambara, Anal. Chem. 54 (1982) 143.
[27] K. Hiraoka, K. Murata, I. Kudaka, J. Mass Spectrom. Soc. Jpn. 43 (1995) 127.
[28] W.M.A. Niessen, J. Chromatogr. A 794 (1998) 407.
[29] J. Saurina, S. Hernandez-Cassou, J. Chromatogr. A 676 (1994) 311.
[30] Y. Yokoyama, O. Ozaki, H. Sato, J. Chromatogr. A 739 (1996) 333.
[31] Y. Yokoyama, T. Amaki, S. Horidoshi, H. Sato, Anal. Sci. 13 (1997) 963.
[32] A.G. Hulst, C.E. Kientz, J. Mass Spectrom. 31 (1996) 1188.
[33] M.F. Bean, S.A. Carr, G.C. Thorne, M.H. Reilly, S.J. Gaskell, Anal. Chem. 63 (1991) 1473.
[34] K.W.M. Siu, R. Guevremont, J.C.Y. LeBlanc, G.J. Gardner, S.S. Berman, J. Chromatogr. 554 (1991) 27.
[35] W.M.A. Niessen, J. Chromatogr. A 856 (1999) 179.
[36] D.R. Zook, E.P. Grimsrud, J. Am. Soc. Mass Spectrom. 2 (1991) 232.
[37] D.R. Zook, A.P. Bruins, Int. J. Mass Spectrom. Ion Processes 162 (1997) 129.