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Simultaneous analysis of underivatized chiral amino acids by liquid chromatography–ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase

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Abstract

Simultaneous chiral separations of underivatized amino acids have been performed using a teicoplanin-based chiral stationary phase and ionspray tandem mass spectrometry for their ionisation and detection. Different amino acid enantiomer pairs were separated simultaneously, including those of positional isomeric amino acids (e.g., L,D-Leu/Ile, or L,D-Val/Iva). Due to the specificity of tandem mass spectrometry, co-eluting enantiomers of different amino acids could also be determined. Fifteen chiral underivatized proteinogenic and non-proteinogenic amino acids were analysed simultaneously under isocratic conditions (acetonitrile–water, 75:25) in less than 25 min. For maximum sensitivity, post-column addition of 500 m*M* aqueous HCOOH was necessary. Detection limits varied from 2.5 to 50 $\mu g\ l^{-1}$ depending on the amino acid. The signal vs. concentration relationship was linear for all D- and L-amino acids ($0.9995 \leq r^2 \leq 1$) for three orders of magnitude.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Amino acids

1. Introduction

Many amino acids contain an asymmetric centre, occurring as laevorotatory, L, or dextrorotatory, D, compounds. The analysis of both L- and D-amino acids is very important in food chemistry [1–10], geochronology [11,12] and in extraterrestrial exploration. Moreover, extraterrestrial delivery of amino acids to the primitive Earth (obtained by meteorite collisions with Earth or micrometeorite attraction [13]) could explain the homochirality of life on Earth, an important key for the origin of life [14–16]. Enantiomerically pure synthetic peptides are also of growing importance for pharmaceutical applications (recently, D-amino acids have shown beneficial effects against cancer [17] and schizophrenia [18]), making it necessary to determine the extent of racemization which may occur during synthesis [19]. Two excellent articles, reviewing the formation, chemistry, nutritional value and safety as well as the microbiology of D-amino acids, have been published by Friedman [20,21].

Due to the great complexity of the above matrices, quantitative measurements require the simultaneous resolution of a great number of optical isomers, resulting in a very difficult analytical problem. HPLC offers several ways for the enantiomeric separation of amino acids. Chiral separation by conventional reversed-phase supports can be achieved only after the formation of diastereoiso-

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meric derivatives with chiral reagents, such as 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) [22] or a combination of *o*-phthaldialdehyde (OPA) and chiral thiols [23]. Derivatives of amino acids obtained with non-chiral reagents require chiral stationary phases for their enantiomeric separation [24–26]. However, derivative instability, reagent interferences, the inability of some reagents to derivatize the secondary amino group, inadequate enantiopurity of some reagents and long preparation times are the main disadvantages for the derivatization methods [27,28].

Another approach towards the enantiomer analysis of amino acids is their direct analysis by chiral ligand-exchange chromatography (CLEC) [29–32] or by a chiral stationary phase (CSP) based on α-cyclodextrin [33], crown ether [34–38] or teicoplanin [39–44].

CLEC is based on the formation of diastereoisomeric complexes among the amino acid enantiomers, a metal ion and the chiral selector (often an appropriate L-amino acid derivative) which can be separated due to differences in complex stability. A teicoplanin-based CSP is created by covalently linking the macrocyclic antibiotic teicoplanin to spherical silica particles. Its chiral discrimination is basically due to interactions between the teicoplanin ammonium group and the carboxylate group of the amino acid [40], although additional interactions (hydrogen bonding, dipole orientation, hydrophobic and steric interactions, π–π aromatic stacking) can further increase or decrease the enantiomeric resolution [40,44]. Generally, the antibiotic prefers the D form of amino acids [40]. α-Cyclodextrin-based CSPs can only separate aromatic underivatized chiral amino acids [33] while all the other methods can separate a great number of underivatized chiral amino acids [32,35,36,38]. Besides the resolution of enantiomers, the quantification of amino acid enantiomers normally requires the separation of the enantiomers of interest from other amino acids in the sample.

CLEC and crown ether-based CSPs have the advantage over teicoplanin-based CSPs of presenting simultaneous selectivities among different underivatized chiral amino acids [31,32,35–38]. On the other hand, CLEC and the commercial crown ether column require a non-volatile mobile phase [31,35] making their use for preparative chromatography difficult. Moreover, these techniques cannot be combined with detection methods which need a volatile mobile phase, such as evaporative light scattering detection (ELSD) or mass spectrometry (MS). Teicoplanin-based CSPs are compatible with a high percentage of organic modifiers [40–44] in the mobile phase, while CLEC and crown ether CSPs show decreased sample retention and/or a dramatic loss in column selectivity [32,38], as well as column bleeding (>15% organic modifier, only for the crown ether CSP) [38]. Lastly, crown ether CSPs cannot separate the enantiomers of secondary amino acids (proline, pipecolic acid, etc.) [35].

After separation of the underivatized chiral amino acids, low-wavelength UV is used for their detection [29–45], resulting in poor sensitivity, limited choice of mobile-phase solvents and additives, as well as incompatibility toward ''real world'' samples [44]. Considerable progress has been made in the last 2 years concerning the chromatographic separation and detection of underivatized amino acids [45–50]. Recently [51,52], a comparative study of several commercial detection systems including ELSD, refractive index detection, conductivity detection, low-wavelength UV detection, simple and tandem MS has been carried out for underivatized amino acids. Due to its high sensitivity and incomparable specificity [48,50,51], tandem MS appears to be the most promising detection method.

In this study, a teicoplanin CSP was coupled to tandem MS for the simultaneous analysis of several underivatized chiral amino acids. The ability of this column to separate several pairs of amino acid enantiomers was also investigated.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from J.T. Baker (Noisy le Sec, France). Isovaline (Iva), citruline (Citr), sarcosine (Sar), α-aminobutyric acid (α-ABA), α-aminoisobutyric acid (α-AIBA), 3,4-dihydroxyphenylalanine (Dopa), α-methylleucine (α-mLeu), aspartic acid (Asp), asparagine (Asn), glutamine

(Gln), glutamic acid (Glu), serine (Ser), threonine (Thr), glycine (Gly), alanine (Ala), proline (Pro), valine (Val), methionine (Met), tyrosine (Tyr), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophan (Trp), lysine (Lys), histidine (His), arginine (Arg) and formic acid were purchased from Sigma (St. Louis, MO, USA). All amino acids were injected in their racemic form, except for Gln and Dopa, which were mixtures of about equal quantities of the L and D forms. Deionized water (18 MΩ) from an Elgastat UHQ II system (Elga, Antony, France) was used as HPLC-grade eluent and for the preparation of amino acid solutions (500 μg ml^{-1}).

2.2. Apparatus

Retention and selectivity studies on the teicoplanin CPSs were carried out with a LC–ELSD system composed of a Beckman (Fullerton, CA, USA) Model 128 System Gold binary pump, a Gilson (Villiers le bel, France) Model Bio732 autosampler fitted with a 10 μl loop, a Shimadzu (Kyoto, Japan) C-R6A integrator and a Sedere (Vitry s/Seine, France) Model Sedex 55 evaporative light scattering detector set as follows: drift tube temperature, 60°C; nebulizer gas pressure, 2.2 bar; photomultiplier, 9. ELSD has proved to be [46] an inexpensive way to develop chromatographic methods that are directly transposable to LC–MS.

LC–ionspray (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 triple quadrupole mass spectrometer with IonSpray as ion source. The mass spectrometer was operated in positive ion mode. Nitrogen was used as curtain and collision gas. The MS–MS parameters have been optimized previously [47] and 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). NEB = 7 (nebulizer gas) corresponds to a flow-rate of 0.95 l min^{-1} and 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 carried out by a Perkin-Elmer series 200 autosampler (Toronto, Canada) fitted with a 20-μl loop. A Harvard Model 22 syringe pump was used for sheath liquid studies. The optimum sheath liquid flow-rate was found to be 5 μl min^{-1}. The mixing tee used for post-column liquid addition was purchased from Jour Research (Onsala, Sweden). The optimum post-column addition liquid flow-rate was found to be 2.5 μl min^{-1}.

Separation was carried out on a Chirobiotic T chiral stationary phase 250×4.6 mm I.D., 5 μm (Astec, Whippany, NJ, USA) column. The flow-rate was 0.8 ml min^{-1}. For LC–MS–MS, a split ratio of 1:20 was used to avoid too high a flow-rate in the ion source. Chromatographic separation was carried out under isocratic conditions. The hold-up time was determined by injecting an aqueous solution of sodium nitrate.

Before using the new Chirobiotic T column, 20 ml of water were percolated through the column at a flow-rate of 0.8 ml min^{-1}, then the column was conditioned for 1 h with the first mobile phase tested (water–ACN, 87.5:12.5) as recommended by the supplier [53]. After that, each time the mobile phase was changed, the column was conditioned by passing the new eluent through the column for 30 min, then waiting until reproducible retention factors were obtained.

The column temperature was regulated at 25°C by a Croco-cil (Cluzeau, France) oven.

3. Results and discussion

Without any doubt, the most specific liquid chromatographic detection method nowadays is mass spectrometry. Its high specificity decreases the necessity for powerful chromatographic systems in which all compounds of interest have to be separated before detection. It thus reduces the analysis time. In the case of underivatized protein amino acids, we recently demonstrated [46,48,49] that, before analysis by MS, only certain pairs of amino acids need to be separated due to the intrinsic problems of MS detection (isobaric and isomeric amino acids, collisionally induced dissociation fragments, carbon-13 isotope). Similarly, in spite of the high specificity of

MS, the distinction between the L and D enantiomers of amino acids is not possible as they have the same molecular mass and identical fragmentation behaviour. Among the LC methods allowing the separation of underivatized chiral amino acids, teicoplanin-based CSP seems to be the most appropriate packing material as it uses a volatile mobile phase containing a high percentage of organic modifier, ensuring both MS compatibility and spray stability [39–45,54]. However, selectivities among different amino acids have never been described with this column.

3.1. Study of retention and selectivity

The purpose of this study was the simultaneous analysis of several protein amino acids as well as other amino acids which are important for extraterrestrial exploration such as α-aminobutyric acid, α-aminoisobutyric acid, isovaline, α-methyleucine, etc. [55,56]. As the specificity of mass spectrometry allows the identification of several amino acids, even if they are co-eluted [46,48,49], special attention was given to the chromatographic separation of amino acids that cannot be distinguished by MS, such as the enantiomers of positional isomers of amino acids (e.g., L,D-Leu/Ile or L,D-Val/Iva) which have the same parent and product ions. Different ACN–water and MeOH–water ratios were tested as mobile phases for each of the above compounds as shown in Fig. 1. Increased retention and selectivities were obtained when the mobile phase contained more than 60% organic modifier. The above compounds are highly retained when there is more than 80% of ACN in the mobile phase.

For the isoleucine/leucine pair, the elution order was L-Leu<L-Ile<D-Leu<D-Ile with both ACN–water and MeOH–water in the mobile phase. For the isovaline/valine pair, the retention order was L-Val<

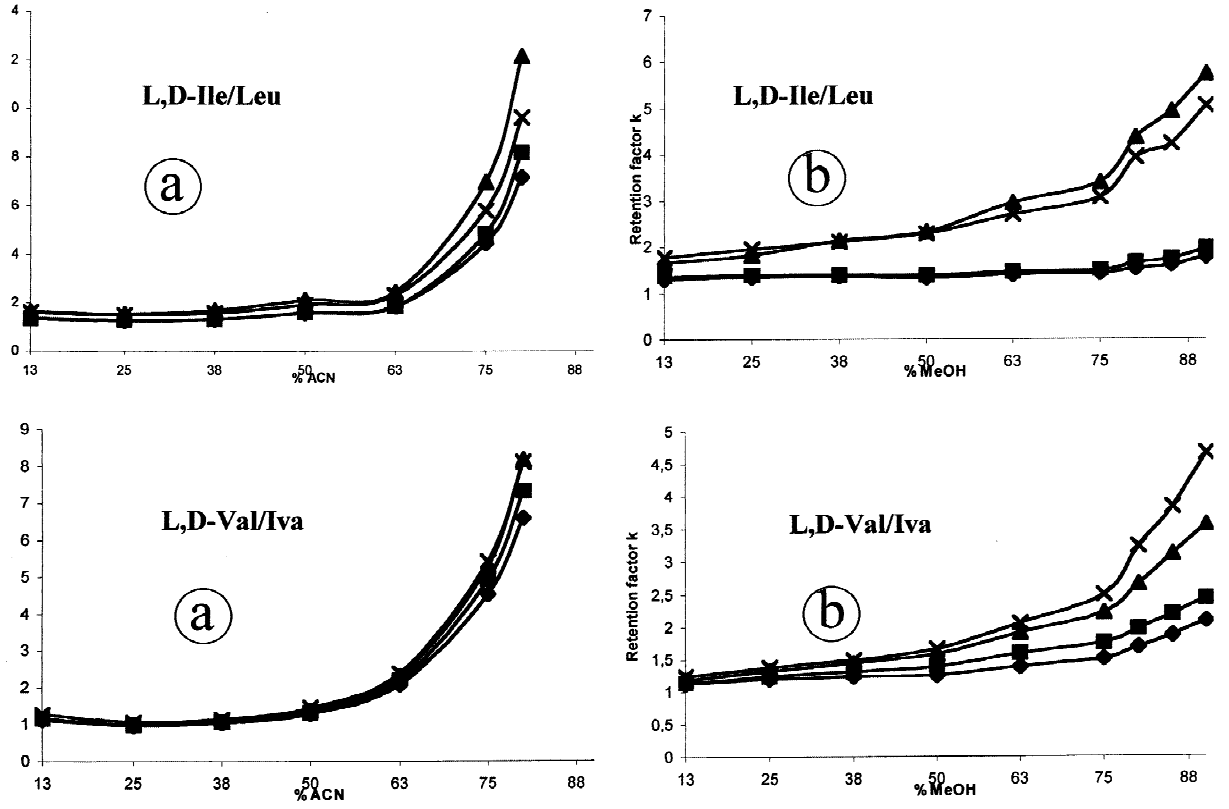

Fig. 1. Effect of the mobile-phase composition on the separation of L,D-Ile/Leu and L,D-Val/Iva, respectively. (a) ACN–water mixture as mobile phase, (b) MeOH–water mixture as mobile phase. (♦) L-Leu or L-Val, (■) L-Ile or L-Iva, (×) D-Leu or D-Val, (▲) D-Ile or D-Iva.

L-Iva<D-Iva<D-Val with both ACN–water and MeOH–water mobile phases. For L,D-α-aminobutyric acid and α-aminoisobutyric acid, the elution order was L-α-ABA<α-AIBA<D-α-ABA (data not shown). ACN–water mixtures gave better selectivities for the Leu/Ile pair, while MeOH–water mixtures offered better selectivities for the Val/Iva pair. Optimum isocratic conditions were 80% ACN for L,D-Leu/Ile and 90% MeOH for L,D-Val/Iva (see Fig. 2).

Thus, the teicoplanin column not only has the ability to separate the L and D forms of amino acids, but also the enantiomers of the positional isomers of amino acids (methyl group on the α, β or γ carbon atoms). All L-amino acids having the methyl group on the α carbon were eluted before the L-amino acids having the methyl group on the β carbon, while all L-amino acids having the methyl group on the β carbon were eluted before the L-amino acids having the methyl group on the γ carbon. This was not always the case for D-amino acids, as their retention is also governed by the chiral recognition of the teicoplanin column. Separation of isomer amino acids is not the only selectivity observed with this column. Selectivities between isobaric amino acids were also obtained. With ACN–water (75:25) as mobile phase, L-hydroxyproline (the D form was not available) was separated from its isobars L,D-Leu/Ile, and sarcosine was separated from its isobars L,D-alanine (data not shown). For the rest of this study, ACN–water (75:25) was used as the mobile phase as it resolved a larger number of critical separations (among amino acids that have to be separated [48,50]) than MeOH–water mixtures.

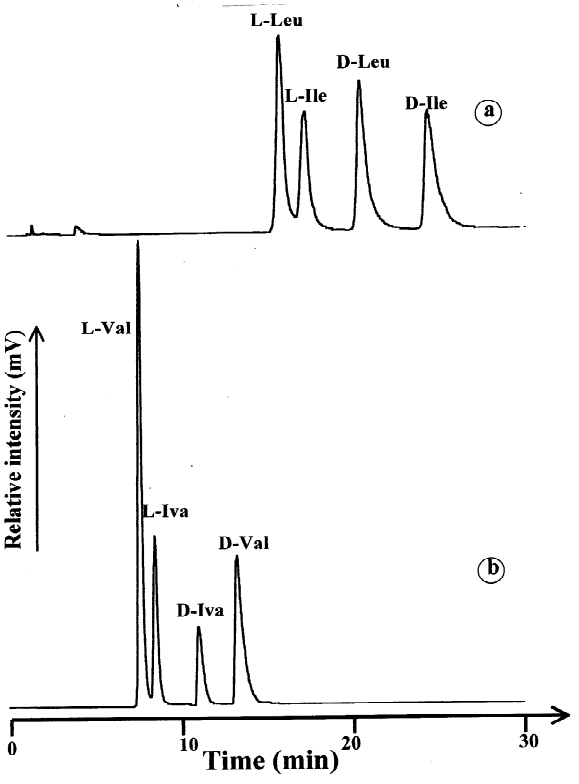

Fig. 2. LC–ELSD optimum isocratic chromatographic conditions for the separation of (a) L,D-Leu/Ile, ACN–water (80:20) as mobile phase, (b) L,D-Val/Iva, MeOH–water (90:10) as mobile phase; flow-rate, 0.8 ml min^{-1}, ELSD (for settings, see Section 2).

3.2. Study of sheath liquid parameters

The MS parameters (orifice and ring voltage, optimum collision energy as well as optimum parent and product ions) were previously optimised for amino acid analysis [46,48]. All non-protein amino acids analysed in this study were optimised in the same way.

The MS response of the amino acids was then studied by analysing four amino acids (Phe, Ile, Gln, and Pro) under optimum chromatographic conditions (ACN–water, 75:25) With this mobile phase, the MS response was rather disappointing as the overall detection limits were about 1 mg l^{-1}. These detection limits were inferior to those obtained previously with water–perfluorocarboxylic acid mobile phases [48]. Moreover, it has been demonstrated that the higher the concentration of perfluorocarbocylic acid in the mobile phase, the greater the MS response of the amino acids [46].

In order to increase the MS response of the amino acids, an aqueous sheath liquid was added that contained different concentrations of formic acid. Formic acid was preferred to trifluoroacetic acid or its higher homologs because it eliminated the possibility of signal suppression [57]. To investigate the influence of sheath liquid on the MS response of amino acids, six different concentrations of HCOOH (5, 10, 50, 100, 500, 1000 m*M*) were applied. As shown in Fig. 3, the increase in amino acid response was spectacular. Amino acid responses were about

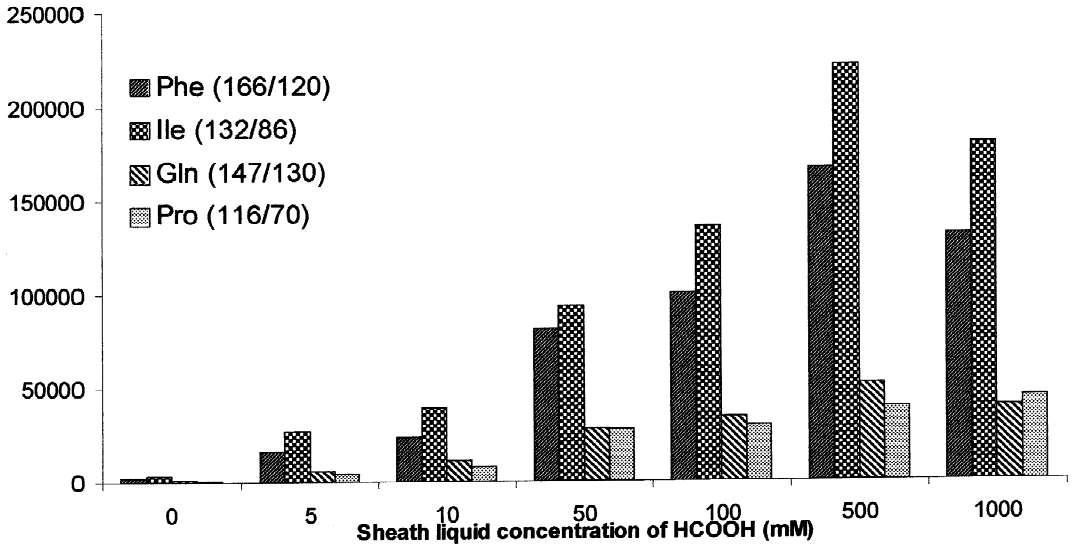

Fig. 3. Effect of different concentrations of formic acid as sheath liquid on the response of amino acids. The number in parentheses represents the parent and product ions. Sheath liquid flow-rate, 5 μl min^{-1}.

90-fold higher with 500 m*M* HCOOH as sheath liquid than without a sheath liquid. The amino acid response increased for all amino acids with a maximum at 500 m*M* HCOOH, and decreased for higher concentrations. Only the response of Pro was increased further in 1000 m*M* HCOOH.

Although the amino acid signal intensities were increased with the sheath liquid, signal stability was reduced. Instead of an increased regular noise, there were negative spikes, implying spray instability. The greater the concentration of HCOOH in the sheath liquid, the more the signal instability increased. This problem was overcome by adding the HCOOH solution as a post-column additional liquid, as shown in Fig. 4. Although the signal intensities were 5% lower, signal stability was excellent. The optimum post-column liquid addition flow-rate was found to be 2.5 μl min^{-1}. Another advantage of the post-column additional liquid is that it can be used with turboionspray as ion source, which does not support the application of a sheath liquid.

These results confirm our previous observations [46] that an acid mobile phase improves MS response for amino acids. These results contradict previous studies which claim that the pH of the mobile phase does not affect the MS response for amino acids [58,59].

3.3. Simultaneous analysis of underivatized chiral amino acids by LC–ISP-MS–MS

Several critical chiral amino acid pairs that have to be separated before MS analysis as previously described [46,48] can be analysed under the opti-

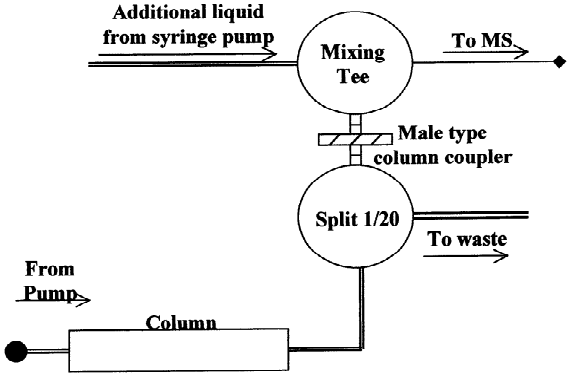

Fig. 4. Scheme of post-column liquid addition.

mized isocratic conditions used (ACN–water, 75:25) Fig. 5 shows the extracted ion currents for the simultaneous analysis of 15 amino acids in less than 25 min. Although several amino acids are co-eluted (see Fig. 6a), the specificity of mass spectrometry allows the specific analysis of each pair of enantiomers (see Fig. 5).

Eleven of the 19 chiral underivatized protein amino acids can be analysed simultaneously under these chromatographic conditions. These amino acids are: Thr, Trp, Phe, Leu, Ile, Gln, Pro, Ala, Val, Met and Tyr. The other eight amino acids cannot be analysed under these chromatographic conditions for different reasons. The acidic amino acids Asp and Glu are eluted at the void volume, while the basic amino acids Lys, His and Arg are not eluted. L,D-Ser

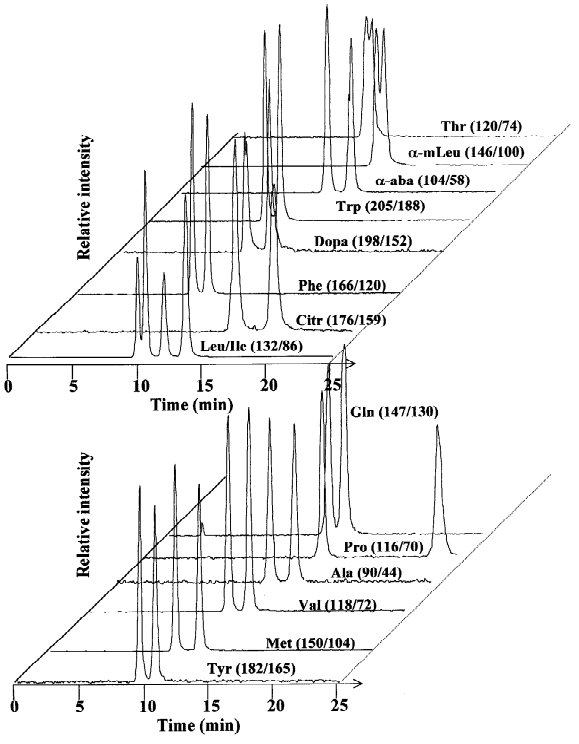

Fig. 5. Simultaneous isocratic analysis of 15 underivatized chiral amino acids (5 mg l^{-1} each) by LC–ISP-MS–MS. Extracted ion currents for 15 underivatized L,D-amino acids. Eluent, ACN–water (75:25); flow-rate, 0.8 ml min^{-1}; split, 1:20; post-column liquid addition, 500 m*M* HCOOH as described in Fig. 4; MS parameters, see Section 2.

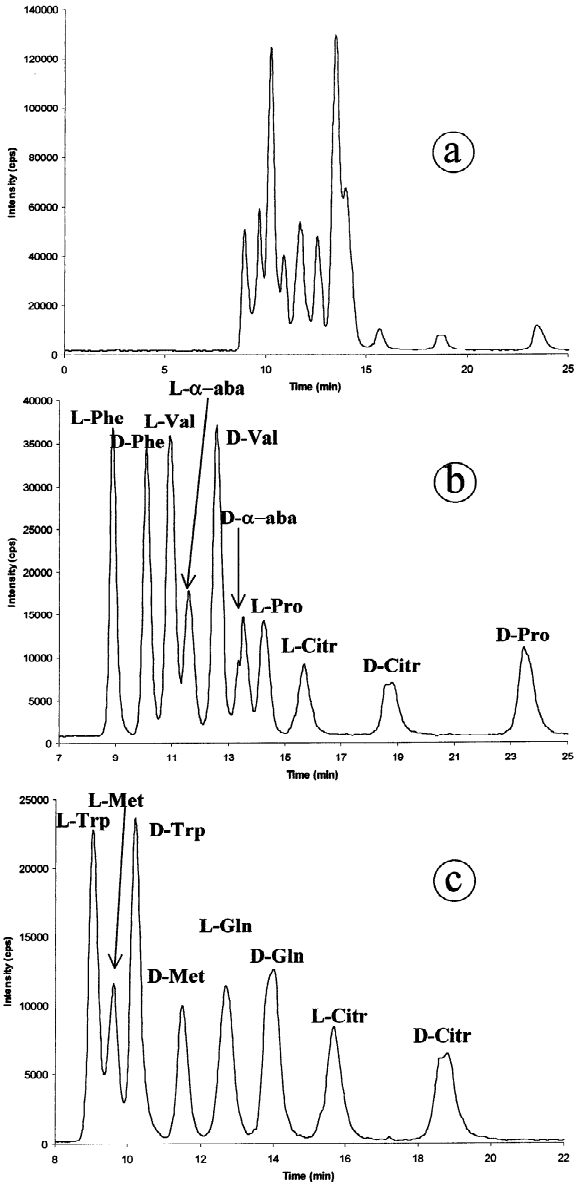

Fig. 6. Simultaneous isocratic analysis of 15 underivatized chiral amino acids (5 mg l^{-1} each) by LC–ISP-MS–MS. (a) Selective reaction monitoring of all analysed amino acids and (b,c) selective reaction monitoring of underivatized chiral amino acids that can be separated simultaneously. Same experimental conditions as in Fig. 5.

are not separated, while L,D-Cys are very strongly retained ($k > 15$) and are excluded from this study. Lastly, although L,D-Asn are separated, L-Asn is co-eluted with D-Ile, which precludes determination of Asn due to the carbon-13 isotope problem described previously [46,48]. However, the D form of Asn can be specifically analysed. Except for Asn, all the other pairs of amino acids that have to be separated before MS analysis (isomers, isobars, etc.) [46,48] are separated under these chromatographic conditions.

Furthermore, several other chiral amino acids can be analysed simultaneously with the 11 proteinogenic chiral amino acids, including Citr, Dopa, α-mLeu and α-ABA. The best enantiomeric selectivities were obtained for Pro, while the worst were for α-mLeu and Thr. As shown in Fig. 6b and c, under these chromatographic conditions up to five underivatized chiral amino acids can be separated simultaneously. Such selectivities cannot be obtained with water–methanol mixtures as mobile phase, due to the overall lower retention factors.

In order to test the linearity of the calibration curve, as well as the limits of detection and the possible application of this method to amino acid analysis of meteorite extracts, several chiral and achiral amino acids that have already been detected in the Murchison meteorite were analysed [14–16,55,56]. Specifically, a mixture of the chiral amino acids Ala, α-mLeu, α-ABA, Pro, Val, Thr, Leu, Ile, Met, Phe and Tyr and the achiral Gly, Sar and α-AIBA were analysed simultaneously at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 mg l^{-1}. L-Gln (5 mg l^{-1}), which has not been found in meteorites, was used as internal standard.

As shown in Table 1, the calibration curves were linear over three orders of magnitude while the limits of detection for this method are from 2.5 to 50 μg l^{-1}. These detection limits are better than those previously described for achiral amino acid analysis

Table 1
Selective reaction monitoring (SRM) of parent (Q1) and product ion (Q3), linearity and limits of detection of the analysed amino acids. Same chromatographic conditions as in Fig. 5

Amino acid	SRM Q1→Q3	Equation	r^2	LOD (μg l^{-1})
Gly	76→30	$y = 0.0100x + 0.0006$	0.9999	50
L-Ala	90→44	$y = 0.1120x + 0.001$	0.9999	35
D-Ala	90→44	$y = 0.1188x + 0.029$	1	40
Sar	90→44	$y = 0.0982x + 0.0028$	1	50
L-α-ABA	104→58	$y = 0.2416x + 0.0116$	0.9997	8
D-α-ABA	104→58	$y = 0.2464x + 0.0018$	1	10
α-AIBA	104→58	$y = 0.2247x + 0.0011$	0.9999	12.5
L-Pro	116→70	$y = 0.2199x + 0.0018$	1	40
D-Pro	116→70	$y = 0.2241x + 0.0011$	1	50
L-Val	118→72	$y = 0.4617x + 0.0039$	0.9999	3.5
D-Val	118→72	$y = 0.4848x + 0.006$	1	5
L-Thr	120→74	$y = 0.1377x + 0.033$	0.9998	40
D-Thr	120→74	$y = 0.1808x + 0.059$	0.9998	40
L-Leu	132→86	$y = 0.6161x + 0.0151$	1	2.5
D-Leu	132→86	$y = 0.5907x + 0.0130$	1	3.5
L-Ile	132→86	$y = 0.6822x + 0.0095$	1	3
D-Ile	132→86	$y = 0.5504x + 0.0004$	1	4
L-α-mLeu	146→100	$y = 0.7286x + 0.0040$	1	4
D-α-mLeu	146→100	$y = 0.9018x + 0.0015$	0.9999	4
L-Met	150→104	$y = 0.1582x + 0.0022$	1	10
D-Met	150→104	$y = 0.1609x + 0.0006$	1	10
L-Phe	166→120	$y = 0.5950x + 0.0054$	1	4
D-Phe	166→120	$y = 0.5987x + 0.016$	0.9999	5
L-Tyr	182→165	$y = 0.2049x + 0.0074$	0.9999	10
D-Tyr	182→165	$y = 0.1955x + 0.0124$	0.9995	11

by LC–ISP-MS–MS [48]. This may be due to the post-column acidic liquid addition and the total absence of ion pairing reagent, as well as to the higher concentration of ACN in the mobile phase which improves mobile-phase evaporation and spray stability. Furthermore, the injection-to-injection stability of the MS response was excellent, as can be seen from the RSD of the peak of L-Gln, which was only 0.68%.

4. Conclusion

In this study, the high selectivities of the teicoplanin column for enantiomers and positional isomers of amino acids are combined with the specificity of mass spectrometry to overcome the problem of co-elution among different amino acids and increase the number of chiral underivatized amino acids that can be analysed in a single run.

Signal stability, linearity and injection-to-injection stability of the MS response were excellent. Limits of detection were very good, but still 10–100 times higher than for fluorescence methods. However, the new generation of mass spectrometers is reported to be about 10 times more sensitive than that used in this study. It is possible, however, that a combination of micro-HPLC with turboionspray as ion source and new generation mass spectrometers could reach or even overcome (for only some amino acids) fluorescence detection sensitivity.

The calculated limits of detection for this method are sufficiently low to be able to analyse the amino acid composition of 50 to 100 mg extracts from the Murchison meteorite. Moreover, HPLC systems and mass spectrometers have been impressively reduced in weight and volume over the last 10 years, which makes it feasible for them to be included in a spacecraft and used for the in situ analysis of amino acids, where sample availability is not a problem. Moreover, the lack of derivatization simplifies instrumentation and eliminates problems such as the long-term stability (several years are necessary to reach most planets of interest, e.g. Mars) and enantiomeric purity of chiral reagents. In addition, the high specificity of mass spectrometry reduces the necessary purification steps. Finally, the number of different compounds that can be analysed without previous derivatization is much greater than with conventional UV or fluorescence detection methods, allowing the analysis of other important compounds present in extraterrestrial materials.

The future of this work will be the application of this method to the analysis of underivatized amino acids in meteorite extracts as well as the investigation of a universal method for the chiral and achiral analysis of underivatized amino acids by coupling, in tandem, chiral and achiral columns for their separation and MS for their detection.

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