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Analytical Methods

Chiral nano-liquid chromatography–mass spectrometry applied to amino acids analysis for orange juice profiling

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Abstract

Determination of amino acid enantiomers is a very important topic in food analysis, since the presence of D-isomers may indicate, e.g., adulteration, microbiological contamination, uncontrolled fermentation processes, etc. In fact, the D- and L-enantiomers contents can be a useful marker for several elements such as quality control, contamination detection, processing monitoring, etc. Here we studied the potentiality of nano-liquid chromatography (nano-LC) coupled with mass spectrometry for the enantiomeric separation of several D- and L-amino acids that can be found in food products. Analytes were derivatized with fluorescein isothiocyanate (FITC). The mixture was injected and compounds focused on a C18 cartridge, then nano-LC analysis was carried out in a capillary column (75 μm i.d.) packed with vancomycin-modified silica–diol particles. The effect of some experimental parameters, such as pH and buffer concentration on enantioresolution and retention factors, was studied for method optimization. The chromatographic separation system was coupled with an ion-trap mass spectrometer through a nano spray interface. It provided a final evaluation on analytes detected in all investigated samples with LOD values as low as 8 ng/mL. That method was applied to the comparative analysis of two different orange juice samples (fresh natural vs. commercial one). Obtained profiles confirmed expected high quality standards. In fact, they mainly contained L-amino acids forms and not their antipodes.

Keywords: Amino acids; Derivatization; Chiral; Nano-liquid chromatography; Mass spectrometry

1. Introduction

During the last decade, separation techniques coupled with mass spectrometry became an essential tool in molecular biochemistry, especially in the analysis of chiral compounds of pharmaceutical, clinical, environmental and/or agro-chemical interest (Fanali, Aturki, Kašicka, Raggi, & D'Orazio, 2005). The use of enantioselective separation procedures can be very important in Food Science and Technology for several purposes such as : (i) identification of adulterated foods and beverages, (ii) a more precise control and monitoring on fermentation processes and products, (iii) microbial contamination's evaluation and identification, (iv) treatment and storage effects, (v) a more precise evaluation of some flavours and fragrance components, (vi) fingerprinting complex mixtures, and (vii) analysis of chiral metabolites of many foods and beverages' chiral and prochiral constituents (Armstrong, Chang, & Li, 1990; Simó, Barbas, & Cifuentes, 2003).

On the other hand, fruit juice industry has become one of the most important agricultural business in the world. Trade amounts to $10 billions per year being dominated by citrus juice (Robards & Antolovich, 1995). Because of this economic impact, orange juice's adulteration is an

Abbreviations: nano-LC, nano-liquid chromatography; CSP, chiral stationary phase; GABA, γ-aminobutyric acid; FITC, fluorescein isothiocyanate.

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important issue that demands development of new analytical procedures which are able to detect the increasingly sophisticated adulteration procedures tailored to defeat detection methods.

In this context, analysis of amino acid enantiomers is a valuable tool that provides relevant information about food and beverages' quality including orange juices (Gandolfi, Palla, Dossena, Puelli, & Salvadori, 1994; Ooghe, Kasteleyn, Temmerman, & Sandra, 1984; Robards & Antolovich, 1995). Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the techniques used until now to carry out that type of separations (Gandolfi et al., 1994; Ooghe et al., 1984). In fact, they provide, unequivocal results in many cases. On the other hand procedures for sample preparation prior to GC analysis are frequently laborious and time consuming (Gandolfi et al., 1994) and in HPLC expensive chiral columns are used. In addition, in GC procedures the derivatizing reaction cannot be applied for some basic amino acids (Bruckner & Lupke, 1991). New actions have been recently developed. They are mainly based on capillary electrophoresis' use (Cifuentes, 2006; Simó, Barbas, & Cifuentes, 2002; Simó, Martin-Alvarez, Barbas, & Cifuentes, 2004; Simó, Rizzi, Barbas, & Cifuentes, 2005). By the way, new analytical strategies have to be implemented in order to help assessing citrus juice's quality.

Recently, a great attention was paid in developing miniaturized liquid chromatographic systems that can provide higher sensitivity than classic HPLC. In fact, sensitivity will increase reducing the i.d. of the column and keeping constant all other experimental parameters. It has been reported that sensitivity is raising in proportion to the square column's radius. When it passes from 4.6 mm to 0.100 mm sensitivity will increase of about 2000 (Chervet, Ursen, & Salzmann, 1996).

This effect can be ascribed to both a reduction of analyte chromatographic dilution (Vissers, 1999) and an increase of efficiency (Hsieh & Jorgenson, 1996; Kennedy & Jorgenson, 1989). Because of the low column volumes due to the small i.d. (<100 μm), in nano-LC the injected sample volume is as low as 10 nL. Therefore, the system cannot offer high sensitivity, so large volume injection methods have been successfully investigated and applied. This technique is based on peak compression both on-column and extra-column. In the first approach, solutes are dissolved in a solvent of lower eluting power compared to the mobile phase (Claessens & Kuyken, 1987; Heron, Tchapla, & Chervet, 2000; Mills, Maltas, & Lough, 1997; Vissers, de Ru, Ursem, & Chervet, 1996). The second solution consists of using micro pre-columns combined with a switching system (Meiring, Van der Heeft, ten Hove, & de Jong, 2002; Saarinen, Sirén, & Riekkola, 1995).

An alternative/complementary approach used to increase sensitivity in nano-LC, is the coupling of separation system with mass spectrometry. Hyphenation is easy to obtain because of the relatively low flow rate involved in the separation process. Indeed, when the electrospray ionization (ESI) is used as the continuous-flow ionization technique, a decrease of the flow rate will increase ions' number in the gas phase and as a consequence sensitivity will increase (Legido-Quigley, Smith, & Mallet, 2002).

Different chiral selectors have been used in separation science for enantiomeric resolution of many compounds. Among them macrocyclic antibiotics firstly introduced by Armstrong et al. (1994) have been used for that aim.

The high resolution capability of this type of chiral selectors is due to the presence of a large number of chiral centres and functional groups that allow useful interactions for chiral enantioresolution. Several studies testify the wide applicability of these chiral stationary phases (CSPs) to the chiral resolution of different amino acids by liquid chromatography (Berthod, Liu, Bagwill, & Armstrong, 1996; Cavazzini et al., 2004; Chen, 2006; Ilisz, Berkecz, & Peter, 2006; Petrusevska et al., 2006).

Amino acid enantiomers' separation in their native form by nano-LC presents same drawbacks encountered in classical HPLC. It is mainly due to: (i) absence/reduced chromophores in the structure, and (ii) reduced interactions with the chiral selectors. Therefore, derivatization procedures can be helpful to overcome all the drawbacks. They will provide better results in terms of sensitivity and enantioselectivity. Several derivatizing reagents have been used for LC analysis of amino acid enantiomers. Among them, fluorescein isothiocyanate (FITC) resulted to be useful for LC separations.

This paper describes our results on enantiomeric separation of amino acid enantiomers after derivatization with FITC by using a capillary column of 75 μm i.d. packed with a CSP containing silica modified with vancomycin. A column-switching system was used before the nano-LC column. It was equipped with a pre-column cartridge, in order to achieve both focusing and clean up. After optimization, the method was applied to the analysis of fruit juice samples.

2. Materials and methods

2.1. Chemicals and samples

All chemicals were of HPLC grade and they were used as received. Methanol (MeOH), acetonitrile, acetone, acetic and formic acids were purchased from Carlo Erba reagenti Spa (Rodano, Milano, Italy). Ammonia solution (30%) was from Riedel-de Häen (Seelze, Germany), while sodium hydroxide, boric acid and sodium hydrogen carbonate were from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

γ-Aminobutyric acid (GABA), fluorescein isothiocyanate (FITC) and the enantiomers (D- and L-isomers) of arginine (Arg), proline (Pro), alanine (Ala), leucine (Leu), serine (Ser), phenylalanine (Phe), asparagine (Asn), glutamic acid (Glu), aspartic acid (Asp) were from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions at

1 mg/mL were prepared by dissolving the same amount of each enantiomer in methanol and stored at 4 °C.

Two different orange juice samples were studied in this work: a fresh prepared by ourselves from natural oranges and a commercial pasteurized one ("Santal 100% Aranciata") bought in a supermarket. The latter sample did not contain any additive or colorant as declared in the label.

2.2. Instrumentation

2.2.1. Nano-LC

Chromatographic experiments were carried out using a Ultimate Chromatographic System (LCPackings/Dionex, Sunnyvale, CA, USA) connected with μ-LC pump (Switchos™ II Advance Microcolumn Switching Unit) through a μ-Precolumn™ Cartridge (C_{18} 100 Å 5 μm, 300 μm ID × 5 mm), 6-port valve and at finally 10-port low dispersion switching valve (VICI VALCO Instrumentations Co. Inc. – Houston, TX, USA). The 6-port valve was equipped with an external loop of 5 μL for injection. The complete set-up used for nano-LC analysis of amino acid enantiomers is shown in Fig. 1.

The capillary column was directly connected to a 10-port valve in order to avoid dead volume and useless longitudinal dispersion of the injected plug sample.

UV detection was carried out using a single-wavelength UV–Vis detector (LC Packings, CA, USA) equipped with a Z flow cell with a volume of approximately 3 nL and operating at 205 nm. CHROMLEON software by Dionex for Windows 2000 (Microsoft, USA) was used for controlling instruments and for data collection.

2.2.2. Mass spectrometry

Mass spectrometry (MS) chromatograms were acquired with a LCQ quadrupole ion-trap mass spectrometer, Thermo-Finnigan (S. Josè, CA, USA) and Xcalibur™ 1.3 software (Thermo-Finnigan). The end of the chiral column was joined to probe through the nano-electrospray ion source (nano-ESI) Thermo-Finnigan. Emitter tips were prepared in our laboratory; a fused silica capillary (25 μm i.d. × 375 μm O.D × 10.7 cm) etc. (Composite Metal Services, Ilkley, UK) was shaped using sand paper or emery paper on a rotating disk. The tip was washed with water and methanol. It was set on the nano-ESI interface positioned at 1–2 mm from the MS orifice.

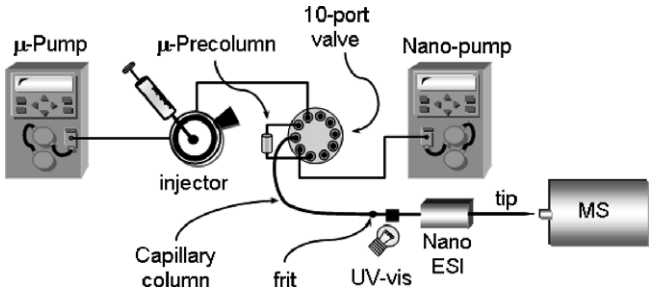

Fig. 1. Schematic representation of the column-switching system coupled with mass spectrometry.

The analytes were detected in the positive ion mode; capillary voltage was 30 V while the ion-spray voltage was 2.0 kV; capillary temperature was set at 170 °C.

2.3. Packed capillary column preparation

Fused silica capillary (75 μm i.d. × 375 μm O.D.) was purchased from Composite Metal Service (Hallow, UK) and packed in our laboratory with Vancomycin-modified diol–silica particles as Chiral Stationary Phase (CSP).

CSP was synthesized in our laboratory using a previously published method (Desiderio, Aturki, & Fanali, 2001). The oxidation of diol–silica particles (LiChrospher DIOL silica 5 μm 100 Å, Merck, Darmstadt, Germany) to aldehyde groups and reduction of amine groups of Vancomycin were able to chemically bind the glycopeptide antibiotic on the silica surface.

The capillary chiral column (CSP length, 25.0 cm) was prepared using the slurry packed method previously described elsewhere (D'Orazio et al., 2005).

2.4. Derivatization procedure with FITC

The derivatization procedure was performed following a previous published method (Simó et al., 2005). Briefly, a solution of FITC (3.75 mM in acetone) and amino acid solutions (6–11 mM, 1 mg/mL in water) were prepared and stored at 4 °C. One hundred micro liters of amino acid standard solutions were mixed with 100 μL of 0.2 M ammonium carbonate pH 9.0 and 200 μL of FITC solution. This mixture was kept at 24 °C in darkness for 2 h in order to allow derivatization and then analyzed by nano-LC–MS.

The orange juice sample was centrifugated for 15 min at 2500 rpm. Then, the supernatant solution was treated with 5 M NaOH, adjusting the pH at 9.0. One hundred micro liters of this solution was reacted with FITC as reported above for standard samples.

2.5. Chromatographic conditions

After injection of 5 μL samples, a 5 mM ammonium formate at pH 3.5 buffer solution was employed to elute analytes from the pre-column at a flow rate of 6 μL/min. The transfer time was set at 8 min, as a result of several experiments. That time allowed an optimum recovery of analytes into the separation column. Mobile phases of different compositions were tested in order to find optimum experimental conditions to separate amino acid derivatives in their enantiomers. Ammonium formate (500 mM), pH 3.5/water/methanol 4/11/85 (v/v/v) was the mobile phase selected and used at a flow of 300 nL/min.

3. Results and discussion

Due to amino acids' native chemical structure, their enantiomeric resolution is known to be a challenging task.

It is true because poor interaction between analytes and chiral selectors makes complex to find appropriate CSPs. Therefore, an initial modification of the amino acids structure via their derivatization with FITC was considered. Derivatization frequently facilitates amino acids' chiral separation, since inclusion and interaction with chiral selectors become more discriminating. This is frequently not only due to the increase in size but also to new interactions involving label (Simó et al., 2005). Moreover, derivatization provides a better sensitivity for their analysis by ESI–MS. In fact, the resulting larger molecules can be ionized with a higher yield and their molecular mass increases until a mass range (>150 *m*/*z*) where MS background noise is usually lower (Simó et al., 2005). Based on our experience and on data reported in the literature, vancomycin-CSP was selected for our experiments to separate D- and L-FITC-amino acids because it offers high enantioselectivity towards an affinity enantioresolution mechanism. Experimental parameters, such as buffer pH and its concentration, were studied in order to find the optimum conditions to achieve the enantiomeric resolution of the selected amino acids.

3.1. Column switching

Preliminary experiments were done analyzing derivatized amino acid mixtures directly injected into CSP column. By the way, those tests did not provide satisfactory results; it is because of FITC excesses' presence that strongly influenced capillary column and MS signal's performances (reduction of signal/noise ratio, S/N). Therefore, a trapping RP18 column was used together with the switching system in order to eliminate the excess of reagent achieving good baseline and amino acids separation.

Some test experiments have been carried out modifying composition of the mobile phase in order to avoid the elution of the FITC-amino acids and therefore trapping analytes. As a consequence, large volumes of sample could be injected without overloading effects (Mills et al., 1997).

5 mM ammonium formate at pH 3.5 was the mobile phase used in the first step. It allowed to retain analytes and to elute other interfering polar components including FITC in excess. After switching the 10-port valve, the mobile phase used for the analytical separation (described under Section 2.4) eluted trapped compounds and chiral separation started.

3.2. Method optimization

As mentioned above, different mobile phases were tested to find optimum experimental conditions for separating amino acid derivatives in their enantiomers. For that reason, methanol and aqueous solutions of ammonium formate and acetate (pH range, 2.5–6.5) were investigated studying influence of parameters such as buffer concentration and organic modifier concentration on retention time and enantioresolution.

Table 1 shows effects of buffer pH on retention and enantioresolution factors of the studied FITC derivatized amino acid enantiomers. Data obtained at pH 6.5 have not been reported in this table because no resolution was achieved for the analyzed compounds. As can be observed, a growth of retention factors was recorded by increasing the pH for Leu, Pro, Phe, Arg, Ser, Ala while the *k* value of Asp, Glu and Asn after an increase passing from pH 2.5 to 3.5, decreased at higher pHs. pH changes also influenced enantioresolution factors of analyzed compounds. In fact, raising the buffer pH caused a general decrease of R_s with the exception of Asp and Arg derivatives that exhibited only a poor enantiomeric resolution at pH 3.5. The highest enantiomeric resolution of main studied compounds was observed at the lowest pH 2.5.

In order to explain the trend of the studied analytes using a vancomycin CSP it is useful to take into account that pH buffer's change is influencing the charge of both analytes and chiral selector. In fact, in their chemical structure amino and/or carboxylic substituents can be recognized. Concerning the interactions analyte/selector, it has

Table 1
Effect of pH on the enantioresolution (R_s) and retention factor (k_i)

	pH											
	2.5			3.5			4.5			5.5		
	k_1	k_2	R_s	k_1	k_2	R_s	k_1	k_2	R_s	k_1	k_2	R_s
Asp	7.49	7.49	NR	7.74	8.67	0.39	2.52	2.52	NR	1.73	1.73	NR
Glu	5.03	6.60	1.43	5.66	7.21	1.01	3.78	3.78	NR	2.64	2.64	NR
Asn	4.27	6.23	2.48	5.02	7.21	1.66	2.48	3.13	0.80	2.27	2.64	NR
Phe	2.31	3.22	1.30	2.66	3.38	1.25	3.00	3.42	0.55	3.55	3.55	NR
Ser	3.33	4.59	1.66	3.56	4.85	1.49	4.17	4.17	NR	4.45	4.45	NR
Leu	0.98	1.75	1.23	1.68	2.40	1.25	2.49	3.12	1.13	3.55	3.55	NR
Ala	2.44	5.20	3.94	2.83	5.66	2.36	3.35	3.35	NR	3.91	3.91	NR
Pro	2.04	2.68	0.75	2.62	2.95	0.47	3.14	3.46	0.31	3.36	3.36	NR
Arg	2.78	2.78	NR	5.33	5.50	0.20	7.79	7.79	NR	8.09	8.09	NR

Experimental conditions: mobile phase, mixture of 500 mM ammonium formate buffer (for pH 2.5 and 3.5) or ammonium acetate (for pH 4.5 and 5.5)/H_2O/MeOH 1/14/85 v/v/v; flow rate: 300 nL/min; injected volume: 5 μL; sample concentration of all racemic (D + L) amino acids and GABA was 250 ng/mL. For further informations see text.

been reported that several interactions are involved in chiral separation of derivatized amino acids, e.g., ionic interactions among charged amino/carboxylic groups (Ding, Liu, Cong, & Wang, 2001), hydrogen bonds between phenolic moieties (in amino acid) and amide groups on the CSP (Cavazzini et al., 2004), interaction with unreacted silanol groups onto the CSP (Peyrin et al., 2001). Additional interactions that can take place in chiral recognition mechanism also include Van der Waals interactions, steric repulsions and inclusion–complexation.

Arg and Asp derivatives were both influenced by changes of mobile phase pH exhibiting an opposite trend. In fact, $k_{Asp} > k_{Arg}$ at low pH 2.5 while $k_{Arg} > k_{Asp}$ at pH 5.5. Besides relatively high values of k were recorded, their enantiomeric resolution was very poor at every studied pH. The increase of pH caused a dissociation of carboxylic groups in both vancomycin and analytes. As a consequence of that phenomenon, in Arg there is an electrostatic interaction between its amino group and vancomycin carboxylic substituent. It causes an increase of k values. This is also confirmed by the behavior of Glu and Asn. Presence of COO^- groups on the structure of the studied derivatized compounds must also have an important role in the interaction with the CSP. In fact, carboxylated groups are strong hydrogen-bond acceptor that may interact with amide groups (donors) (Cavazzini et al., 2004) in the vancomycin cavity.

Observing k values of Ser, Ala, Phe and Pro derivatives, we can remark that their trend was similar but less remarkable than that one of Arg, i.e., k increased by raising the pH. Considering that these compounds do not contain chargeable amino groups; at the end we can confirm that electrostatic interaction cannot take place and, as a consequence, Van der Waals and/or π–π, hydrogen bonds can be considered in the separation mechanism. Besides CSP exhibited a high affinity for those compounds by increasing pH of the mobile phase, those interactions were not stereoselective causing poor or none enantiomeric resolution.

3.3. Effect of buffer concentration

The buffer concentration was modified in 5–30 mM range. It was possible keeping constant ratio methanol/aqueous phase for studying buffer concentration's effects on enantioselectivity and enantioresolution factors. Buffer's pH was selected at 3.5 (ammonium formate) because it allowed enantioresolution of all studied derivatized amino acids.

Fig. 2a and b shows buffer concentration's effects on enantioresolution and retention factors, respectively. Raising buffer concentration, caused a general decrease of k shortening the analysis time due to the competition of the ion buffer with analytes for the stationary phase. Enantiomeric resolution was also influenced by the change of that parameter; the effect was more remarkable for Ala and Asn. The highest enantioresolution values were obtained at the highest buffer concentration (20–30 mM). Changes of the mobile phase composition influenced the electrospray signal, too. This effect was remarkable at buffer concentrations lower than 30 mM. In fact, at this concentration the signal was not stable with a corresponding loss of efficiency and serious broadening effects.

3.4. Separation and analysis of FITC-amino acid enantiomers

Optimum experimental conditions were selected from previous experiments in order to reach high standards of enantioresolution and selectivity.

Therefore, the mobile phase containing 500 mM ammonium formate pH 3.5/water/MeOH, 4/11/95 (v/v/v) was selected in order to elute the mixture in vancomycin based CSP column. Fig. 3 shows nano-LC–MS chromatograms (extracted ion mode) of a FITC-amino acid enantiomers and GABA standard mixture. As can be observed, studied enantiomers were separated in all cases, except for Arg and, for Asp. Two additional peaks were detected in Asn trace's case at 28.95 and 32.81 min. They correspond to Leu derivative ($m/z = 521.9$) which cannot be resolved by the ion-trap. However combination of nano-LC and MS makes possible a correct identification of D- and L-forms of the investigated amino acids.

Some relevant parameters were studied to assess performances of the optimized nano-LC–MS system and method. In particular, we analyzed: limit of detection and quantification (LOD and LOQ, respectively), intra- and inter-day

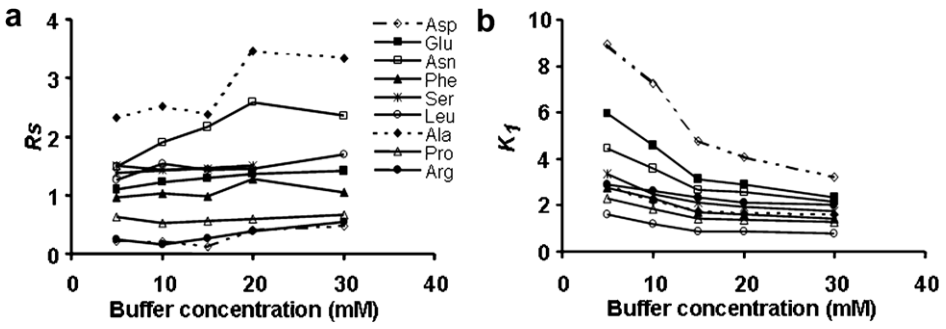

Fig. 2. Influence of the buffer concentration on retention (k_1) and enantioresolution (R_s) factors. Chromatographic conditions: mobile phases composed by different concentrations of ammonium formate (from 5 to 30 mM) at pH 3.5 in 85% MeOH. For other experimental condition see Fig. 1.

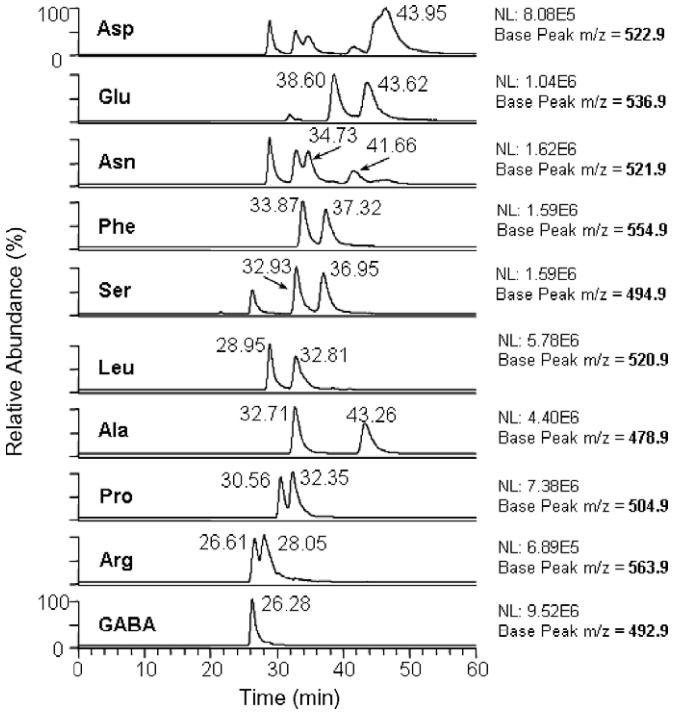

Fig. 3. Nano-LC–MS chromatograms in EIC mode of a FITC derivatized - AAs mixture at a concentration of 250 ng/mL. MS conditions: positive ion mode; capillary voltage: 30 V; ion-spray voltage: 2.0 kV; capillary temperature: 170 °C. For chromatographic conditions see Fig. 1.

repeatability of retention times and peak areas. Measured LOD values were in the range 3–8 ng/mL with the lowest and the highest value for GABA and Asp/Glu/Arg, respectively. LOQ values were between 5 and 15 ng/mL. Six different analysis were carried out injecting a mixture of FITC-amino acids at a concentration of 250 ng/mL of GABA and each racemic compound measuring retention times and peak areas. Excellent repeatability of retention time was recorded for all studied compounds, within the same day ($n = 6$), 1.0–1.9% and for different days with %RSD values for four different days of 3.0–3.8%. As expected, repeatability of peak areas was acceptable according to the MS used ($n = 4$ days, %RSD values were in the range 11–16%).

In order to show the applicability of the optimized nano-LC–MS method, a fresh orange juice and a pasteurized one, were comparatively studied. It has been reported that high quality orange juices contain exclusively L-amino acids; low concentrations of D-isomers have been found in low quality orange juice (Robards & Antolovich, 1995) although, some D-amino acids can naturally be found in some orange juices (Simó et al., 2002). Fig. 4a and b shows nano-LC–MS chromatograms of the analysis of a fresh and a commercial orange juice, respectively. The two profiles are quite similar and assuming that the fresh juice was evidently not adulterated, we can conclude that the commercial sample is of good quality because it does not contain D-enantiomers and their similar L-amino acids profile. However, as can be observed in Fig. 4a (first trace) the amount of L-Asp is very low while in the commercial sample (Fig. 4b) this compound can be easily quantified. In order to explain this unexpected result, it is important to state that the concentration of each amino acid is influenced by cultivation type, climate, growing areas and ripening of fruit. In fact, some authors indicated that the level of aspartic acid concentration decreases with advancing season (Robards & Antolovich, 1995).

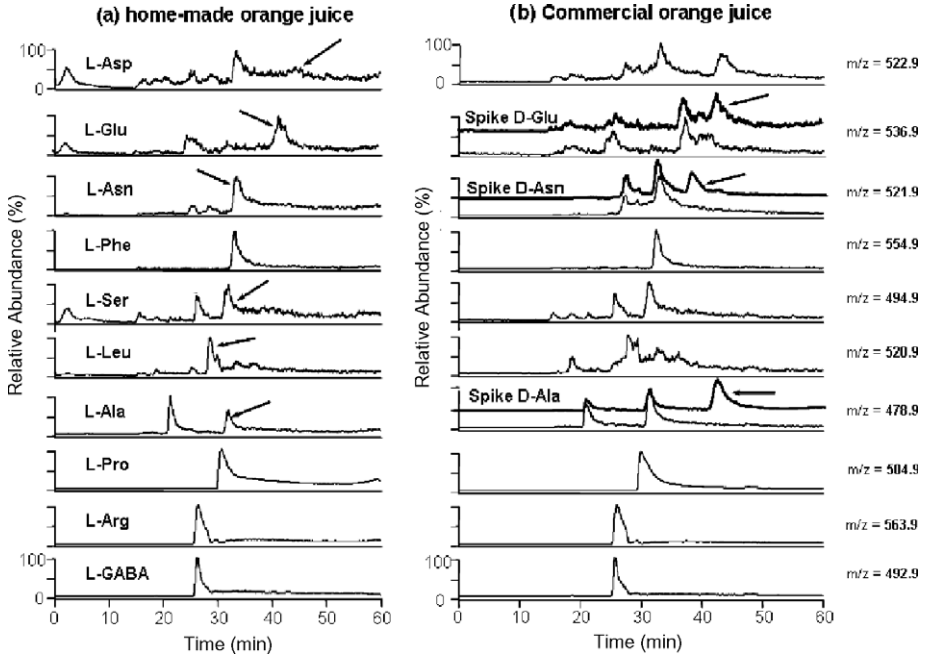

Fig. 4. Comparison of two different nano-LC–MS profiles from orange juice: fresh squeezed and commercial juice. For experimental conditions see Figs. 3 and 1.

In order to demonstrate the capability of this nano-LC-method to detect D-amino acids, commercial orange juice was spiked with some D-amino acids (D-Asn, D-Ala and D-Glu), giving rise to the nano-LC–MS chromatograms shown in Fig. 4b. As can be seen, the D-amino acids were not detected in the real samples either because absent or being at concentration levels lower than the LOD (1.5–4 ng/mL).

4. Conclusions

A capillary column packed with CSP based on vancomycin bonded silica, set into a nano-LC system, was capable of achieving a good enantioseparation of eight couples of derivatized amino acid enantiomers plus the achiral amino acid GABA in a single chromatographic run.

The column-switching system equipped with pre-column cartridge, provided both the on-line clean-up of sample from the excess of derivatizing reagent and the pre-concentration of sample. LODs as low as 8 ng/mL were obtained by injecting 5 μL of sample with good repeatability for the same day and different days. The chromatographic miniaturized system was coupled with a MS ion-trap detector and the separated analytes monitored.

The optimized nano-LC–MS method was applied to the comparative analysis of orange juice samples of different types (fresh natural vs. commercial one). Chromatographic profiles revealed the absence of D-isomers confirming juices' good quality. Lower concentration of L-aspartic acid was monitored in fresh juice probably due to the origin and season of the collected fruits.

Acknowledgements

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References

Armstrong, D. W., Chang, C. D., & Li, W. L. (1990). Relevance of enantiomeric separations in food and beverage analyses. *Journal of Agricultural and Food Chemistry, 38*, 1674–1677.

Armstrong, D. W., Tang, Y., Chen, S., Zhou, Y., Bagwill, C., & Chen, J.-R. (1994). Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography. *Analytical Chemistry, 66*, 1473–1484.

Berthod, A., Liu, Y., Bagwill, C., & Armstrong, D. W. (1996). Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase. *Journal of Chromatography A, 731*, 123–137.

Bruckner, H., & Lupke, M. (1991). Determination of amino acid enantiomers in orange juices by chiral phase capillary gas chromatography. *Chromatographia, 31*, 123–128.

Cavazzini, A., Nadalini, G., Dondi, F., Gasparrini, F., Ciogli, A., & Villani, C. (2004). Study of mechanisms of chiral discrimination of amino acids and their derivatives on a teicoplanin-based chiral stationary phase. *Journal of Chromatography A, 1031*, 143–158.

Chen, S. (2006). The enantioseparation of amino acids on a teicoplanin chiral stationary phase using non-aqueous mobile phases after pre-column derivatization with sulfur-containing reagents: the considerations of mobile phase composition and analyte structure variation on resolution enhancement. *Biomedical Chromatography, 20*, 718–728.

Chervet, J. P., Ursen, M., & Salzmann, J. P. (1996). Instrumental requirements for nanoscale liquid chromatography. *Analytical Chemistry, 68*, 1507–1512.

Cifuentes, A. (2006). Recent advances in the application of capillary electromigration methods for food analysis. *Electrophoresis, 27*, 283–303.

Claessens, H. A., & Kuyken, M. A. J. (1987). A comparative study of large volume injection techniques for microbore columns in HPLC. *Chromatographia, 23*, 331–336.

Desiderio, C., Aturki, Z., & Fanali, S. (2001). Use of vancomycin silica stationary phase in packed capillary electrochromatography I. Enantiomer separation of basic compounds. *Electrophoresis, 22*, 535–543.

Ding, G.-S., Liu, Y., Cong, R.-Z., & Wang, J.-D. (2001). Chiral separation of enantiomers of amino acid derivatives by high-performance liquid chromatography on a norvancomycin-bonded chiral stationary phase. *Talanta, 62*, 997–1003.

D'Orazio, G., Aturki, Z., Cristalli, M., Quaglia, M. G., & Fanali, S. (2005). Use of vancomycin chiral stationary phase for the enantiomeric resolution of basic and acidic compounds by nano-liquid chromatography. *Journal of Chromatography A, 1081*, 105–113.

Fanali, S., Aturki, Z., Kašicka, V., Raggi, M. A., & D'Orazio, G. (2005). Enantiomeric separation of mirtazapine and its metabolites by nano-liquid chromatography with UV-absorption and mass spectrometric detection. *Journal of Separation Science, 28*, 1719–1728.

Gandolfi, I., Palla, G., Dossena, A., Puelli, S., & Salvadori, C. (1994). D-Alanine in fruit juices: A molecular marker of bacterial activity, heat treatments and shelf-life. *Journal of Food Science, 59*, 152–154.

Heron, S., Tchapla, A., & Chervet, J.-P. (2000). Influence of injection parameters on column performance in nanoscale high-performance liquid chromatography. *Chromatographia, 51*, 495–498.

Hsieh, S., & Jorgenson, J. W. (1996). Preparation and evaluation of slurry-packed liquid chromatography microcolumns with inner diameters from 12 to 33 μm. *Analytical Chemistry, 68*, 1212–1217.

Ilisz, I., Berkecz, R., & Peter, A. (2006). HPLC separation of amino acid enantiomers and small peptides on macrocyclic antibiotic-based chiral stationary phases: A review. *Journal of Separation Science, 29*, 1305–1321.

Kennedy, R. T., & Jorgenson, J. W. (1989). Preparation and evaluation of packed capillary liquid chromatography columns with inner diameters from 20 to 50 micrometers. *Analytical Chemistry, 61*, 1128–1135.

Legido-Quigley, C., Smith, N. W., & Mallet, D. (2002). Quantification of the sensitivity increase of a micro-high-performance liquid chromatography ionization mass spectrometry system with decreasing column diameter. *Journal of Chromatography A, 976*, 11–18.

Meiring, H. D., Van der Heeft, E., ten Hove, G. J., & de Jong, A. P. J. M. (2002). Nanoscale LC–$MS^{(n)}$: Technical design and applications to peptide and protein analysis. *Journal of Separation Science, 25*, 557–568.

Mills, M. J., Maltas, J., & Lough, W. J. (1997). Assessment of injection volume limits when using on-column focusing with microbore liquid chromatography. *Journal of Chromatography A, 759*, 1–11.

Ooghe, W., Kasteleyn, H., Temmerman, I., & Sandra, P. (1984). Amino acid enantiomer separation for the detection of adulteration in fruit juices. *Journal of High Resolution Chromatography, 7*, 284–285.

Petrusevska, K., Kuznetsov, M. A., Gedicke, K., Meshko, V., Staroverov, S. M., & Seidel-Morgenstern, A. (2006). Chromatographic enantioseparation of amino acids using a new chiral stationary phase based on a macrocyclic glycopeptide antibiotic. *Journal of Separation Science, 29*, 1447–1457.

Peyrin, E., Ravalet, C., Nicolle, E., Villet, A., Grosset, C., Ravel, A., & Alary, J. (2001). Dansyl amino acid enantiomer separation on a teicoplanin chiral stationary phase: Effect of eluent pH. *Journal of Chromatography A, 923*, 37–43.

Robards, K., & Antolovich, M. (1995). Methods for assessing the authenticity of orange juice. A review. *Analyst, 120*, 1–28.

Saarinen, T., Sirén, & H., & Riekkola, M.-L. (1995). Screening and determination of beta-blockers, narcotic analgesics and stimulants in urine by high performance liquid chromatography with column switching. *Journal of Chromatography B, 664*, 341–346.

Simó, C., Barbas, C., & Cifuentes, A. (2002). Sensitive micellar electrokinetic chromatography-laser-induced fluorescence method to analyze chiral amino acids in orange juices. *Journal of Agricultural and Food Chemistry, 50*, 5288–5293.

Simó, C., Barbas, C., & Cifuentes, A. (2003). Chiral electromigration methods in food analysis. *Electrophoresis, 24*, 2431–2441.

Simó, C., Martin-Alvarez, P. J., Barbas, C., & Cifuentes, A. (2004). Application of stepwise discriminant analysis to classify commercial orange juices using chiral micellar electrokinetic chromatography-laser induced fluorescence data of amino acids. *Electrophoresis, 25*, 2885–2891.

Simó, C., Rizzi, A., Barbas, C., & Cifuentes, A. (2005). Chiral capillary electrophoresis-mass spectrometry of amino acids in foods. *Electrophoresis, 26*, 1432–1441.

Vissers, J. P. C. (1999). Recent developments in microcolumn liquid chromatography. *Journal of Chromatography A, 856*, 117–143.

Vissers, J. P. C., de Ru, A. H., Ursem, M., & Chervet, J.-P. (1996). Optimised injection techniques for micro and capillary liquid chromatography. *Journal of Chromatography A, 746*, 1–7.