Combined monodimensional chromatographic approaches to monitor the presence of D-amino acids in cheese

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Abstract

The presence of D-amino acids (D-AAs) as a consequence of natural or artificial interventions such as ageing, microorganism action, preservative and conservative processes (alkali or heat treatment), is a scarcely treated aspect from the scientific community. It is also fully documented that even a minor degree of racemisation on the proteins’ AAs is the cause of a reduced digestion of such proteins. Besides interfering with the regular metabolism of L-AAs, D-AAs can also contribute to the development of pathological conditions in humans. So far, nearly all the most important chromatographic techniques were applied to quantify D-AAs in foodstuffs. However, most of them rely upon pre- or post-column derivatization procedures, often combined with sophisticated analytical equipments. Differently, in this paper we propose an easy-to-set up combination of monodimensional chromatographic methods to monitor the variation of the D-Ala, D-Asp and D-Glu content in two commercially available Spanish cheese samples prepared from the same milk mixture and characterized by a different maturity time: no ripening and six months ripening. After the free amino acid mixture was extracted from the two cheese samples, an ion-pairing RP-HPLC achiral protocol was firstly optimized with the objective to avail of a method enabling the complete distinction of Ala, Asp, and Glu from all the other aminoacidic species in the two extracts. An ion-exchange-based chromatographic method was also optimized, thus allowing a profitable fractionation of the two aminoacidic mixtures. With such a procedure, less complex samples to be analyzed with a chiral ligand-exchange chromatography (CLEC) stationary phase based on S-trityl-L-cysteine (L-STC) units were obtained. The optimized CLEC conditions were then applied to the previously identified Ala, Asp and Glu containing fractions as well as to those including all the remaining species. For all the three compounds the enantiomeric excess (ee) was found to decrease passing from the ripened to the fresh cheese. As expected, the largest difference was found for Ala (ee value from 83.0% down to 20.5%), followed progressively by Asp (ee value from 90.5 to 75.0%) and Glu (ee value from 99.0 to 91.8%).
Keywords

Achiral ion-pairing chromatography; Strong anion-exchange resin; Chiral ligand-exchange chromatography; Enantioseparation; D-amino acids; Food control.
1. Introduction

The identification, characterization and quantisation of naturally occurring amino acids (AAs) are the subject matter of continuing study and interest (Friedman, 1999; Friedman & Levin, 2012). The presence of L-amino acids (L-AAs) in foodstuffs has attracted a remarkable attention due to the relevant involvements with the taste properties and the nutritional and nutraceutical value they determine (Friedman, 2010; Friedman & Levin, 2012). Conversely, the presence of D-amino acids (D-AAs) as a consequence of natural or artificial interventions such as ageing, microorganism action, preservative and conservative processes (alkali or heat treatment), is a remarkably neglected aspect from the scientific community, as shown by the paucity of publications in this field.

Frequently, the presence of free D-AAs is an indication of microbial contamination, making these compounds as indicators of food quality (Albert, Pohn, Lóki, & Csapó, 2009; Brückner, Jack, Langer, & Godel, 1992; Friedman, 2010). Indeed, D-alanine (D-Ala), D-aspartic acid (D-Asp) and D-glutamic acid (D-Glu) are present in peptidoglycan, a fundamental constituent of the bacterial cell walls (Csapó, Albert, & Csapó-Kiss, 2009). Therefore, the study and the evaluation of the presence of D-AAs in edible products constitute an interesting and reliable approach in the field of the food safety, that enters the frame of the plurality of strategies aimed at assessing the product quality (Friedman, 1999).

It is also fully documented that even a minor degree of racemisation on the proteins’ AAs is the cause of a reduced digestion of such proteins (Csapó et al., 2009). Reduced protein digestibility depends on the fact that racemised AAs are not suitable substrates for proteolytic enzymes, and also exert a deleterious effect on the capacity for liberation of adjacent non-racemised amino acids (Hayashi & Kameda, 1980; Rosen-Levin, Smithson, & Gray, 1980). Thus, the racemisation of some AAs can impair the nutritional quality of an edible product.

Besides interfering with the regular metabolism of L-AAs, D-AAs can contribute to the development of pathological conditions in humans (Friedman, 1999; Friedman, 2010; Friedman &
Nevertheless, beneficial nutritional and health-related aspects were also described for the D-isomer of some aminoacidic compound, thus suggesting the potential use as nutraceuticals of the foods where they are concentrated (Friedman & Levin, 2012).

Basing on the above assumptions, the qualitative and quantitative knowledge of the enantiomeric content of free amino acids in foodstuffs is of prior importance. So far, nearly all the most important chromatographic techniques were applied to quantify D-AAs in foodstuffs (Brückner & Hausch, 1990; Carlavilla, Moreno-Arribas, Fanali, & Cifuentes, 2006; Gandolfi, Palla, Delprato, De Nisco, Marchelli, & Salvatori, 1992; Qi, Chen, Xie, Guo, & Wang, 2008; Van de Merbel, Stenberg, Öste, Marko-Varga, Gorton, Lingeman, & Brinkman, 1995; Voss & Galensa 2000; Pätzold & Brückner, 2005). However, most of them rely upon pre- or post-column derivatization procedures, often combined with sophisticated analytical equipments.

We recently described (Sardella, Ianni, Natalini, Blanch, & del Castillo, 2012) the direct employment of a chiral ligand-exchange chromatography (CLEC) stationary phase achieved through the dynamic coating of an octadecylsilica-based material with S-trityl-L-cysteine (L-STC) units (Natalini, Sardella, Carbone, Macchiarulo, & Pellicciari, 2009; Natalini, Sardella, Giacchè, Palmiotto, Camaioni, Marinozzi, Macchiarulo, & Pellicciari, 2010; Natalini, Sardella, Macchiarulo, & Pellicciari, 2008), as an effective way to evaluate the presence of D-AAs in six cheese samples of different milk composition and ripening time. However, owing to the limited peak resolution provided by the selected chiral system in the first 10 min of analysis, only identifying information could be gained with the proposed chromatographic protocol. Remarkable improvements of the enantioresolution quality of the same coated chiral stationary phase (C-CSP) system have been obtained by optimizing dedicated pre-analysis purification procedures. Accordingly, in this paper we describe the optimization of the achiral chromatographic methods preceding the CLEC analysis and of a series of parameters improving the quality of enantioseparation with the L-STC-based CSP. In order to prove the practical utility of the proposed combination of monodimensional methods, an application has been carried out to evaluate the presence and the incidental variation of
the D-aminoacidic content into two commercially available Spanish cheese samples. The two cheese samples were prepared from the same cow, sheep, and goat milk mixture, and characterized by a different maturity time: no ripening and 6 months ripening.

2. Materials and methods

2.1. Chemicals

Water for HPLC analysis was purified with a New Human Power I Scholar water purification system (Human Corporation, Seoul, Korea). All standard amino acids along with copper(II) nitrate pentahemihydrate and the chiral selector S-trityl-L-cysteine (L-STC) were of high analytical purity and purchased from Sigma-Aldrich (Milan, Italy). Methanol (MeOH), acetonitrile (MeCN), heptafluorobutyric acid (HFBA), sodium hydroxide (NaOH), aqueous ammonia solution (NH₄OH), glacial acetic acid (AcOH), hydrochloric acid (HCl), trichloroacetic acid, and the Dowex 1X8-200 ion-exchange resins were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Extraction of cheese amino acids

A 100 g weight of each of the two cheese samples were first separately lyophilized and pulverized. A 20 mL volume of 0.1 M HCl was added to 5 g of each lyophilized cheese. Each suspension was stirred for 3 h by using a magnetic stirrer and then left at 5 °C overnight to settle. The two-phase system was then shaken-up again, and then centrifuged at 500 g and 8 °C for 10 min. Protein was precipitated from the supernatant, with equal volume of 25% (w/v) trichloroacetic acid solution with the final concentration of trichloroacetic acid of 12.5%. The suspension was again centrifuged at 500 g and 8 °C for 10 min after 30 min standing. Subsequently, a 8 mL volume of supernatant was placed into a 10 mL vial and then neutralized with 4 M NaOH solution following dilution with
distilled water. The extract was filtered through a 0.45 µm filter, frozen and then lyophilized. Extracts from 6 months ripened and fresh cheeses are indicated throughout the text as samples S1 and S2, respectively.

2.3. Instrumentation

The HPLC measurements were made on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a 20 µl stainless steel loop. A Varian 385-LC evaporative light scattering detector (ELSD) (Agilent Technologies, Santa Clara, CA, USA) was specifically utilized for the achiral analyses. The analog-to-digital conversion of the output signal from the ELSD was allowed by a common interface device. The adopted ELSD conditions for the analysis were: 30 °C nebulization temperature, 50 °C evaporation temperature, 1 L/min gas flow rate (air) and 1 as the gain factor.

The analytical columns were: Luna C18(2) (Phenomenex, Torrance, CA, USA) (packing I), GraceSmart RP 18 (Grace, Lokeren, Belgium) (packing II) and Ultra II Aqueous C18 (Restek, Bellefonte, PA, USA) (packing III). Specific column characteristics are reported in Table 1. Unless otherwise reported, the flow rate was fixed at 1 mL/min. For the achiral analyses the column temperature was fixed at 25 °C, while chiral analyses were carried out at 20 °C. Column temperature was controlled through a Grace (Sedriano, Italy) heater/chiller (Model 7956R) thermostat.

HPLC/MS experiments were performed using an Agilent Infinity Series LC system (Agilent Technologies, Palo Alto, CA, USA). The LC system was interfaced to an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS detector, also from Agilent Technologies and equipped with an Agilent Dual Jet Stream Technology ESI source. The mass spectrometer system was controlled by the Agilent MassHunter Workstation software. Mass spectra were recorded from m/z 100 to 1000.
Continuous lock mass infusion included \( m/z \) 121.05087300 and 922.00979800. A FWHM resolution ranging from 10000 to 25000 was used. The operating conditions were: gas temperature, 300 °C; drying gas, nitrogen at 9 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 320 °C; sheath gas flow, nitrogen at 9 L/min; capillary voltage, 4000 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; nozzle voltage, 0 V; fragmentor voltage, 100 V.

The separation of underivatized amino acids was performed with Kinetex 1.7 μm C8, 50 x 2.1 mm ID column in positive mode. The following gradient program, obtained from eluent A (7 mM HFBA in water) and eluent B (net MeCN), was applied: 0-3 min, 100% A; 3-9 min, linear gradient to 75% A; 9-12 min, linear gradient to 70% A; 12-12.1 min, gradient back to 100% A; 12.1-30 min, 100% A to equilibrate the column before a new injection. The chromatographic run for the analysis on the DNS-Ala containing fraction was instead carried out with the same Kinetex C8 column, by slightly modifying a known elution method (Timperio, Fagioni, Grandinetti, & Zolla, 2007). The injection volume was 1 µL. Eluent flow rate was 0.2 mL/min and column temperature was fixed at 30 °C.

2.4. Preparation of the L-STC-based stationary phase and column evaluation

A GraceSmart RP 18 (packing II) analytical column was dynamically coated with L-STC units. The chiral selector (250 mg) was solubilised into a water/MeOH solution (250 mL, 50:50, v/v), carefully filtered through a 0.22 μm Millipore filter and degassed with 10 min sonication. The optimal adsorption of the selector was achieved by recycling the prepared solution for 5 days at 0.5 mL/min. With this procedure, approximately 0.05 g of the selector were established to be hydrophobically bonded to the RP-18 sorbent surface. After washing with a water/MeOH solution (50 mL, 98:2, v/v) in order to displace the excess of chiral discriminating agent and MeOH, a Cu(II) nitrate solution was flowed through the column and used as the mobile phase after 2 h of equilibration. The first vacancy peak of a blank injection of mobile phase components was used to calculate the \( t_0 \) value.
Column performance was assessed by periodic injection of racemic proline (rac-Pro). The dynamic CSP used in this study was found to be stable and uniformly effective in the chiral separation of amino acids for at least 30 days.

2.5. CLEC mobile phase preparation and experimental conditions

The mobile phase for the CLEC runs was prepared by dissolving Cu(II) nitrate (at a 0.25, 0.5 or 1 mmol/L concentration) in HPLC-grade water. The resulting solution was filtered through a 0.22 μm Millipore filter and degassed by sonication for 20 min. The sample solutions were prepared at concentrations between approximately 0.1 and 0.5 mg/mL in filtered mobile phase components and sonicated until completely dissolved. The UV detection wavelengths were set at 254 and 210 nm, and the flow rate was changed among the values 0.1, 0.3, 0.5 or 1 mL/min, according to the specific application.

2.6. Anion-Exchange Chromatography

A Dowex 1x 8-200 ion-exchange resin, poured into a 400 x 10 mm i.d. glass column, was successfully used for the amino acid mixture (S1 or S2) fractionation. Before being utilized, the resin was submitted to treatment aimed at flushing out the constitutive Cl⁻ anions. This action was carried out by means of a 0.3 N NaOH solution until the eluate became basic. Chloride anions were then replaced with acetate by flowing a 0.3 N AcOH solution until the eluate assumed an acidic character. A following wash out with water allowed the resin to reach a neutral pH. At this point, the sample dissolved in ammonia solution (pH ~ 9.0) was first chromatographed with water in order to elute other compounds than amino acids, along with basic amino acids. Subsequently, an ionic-strength gradient elution with a progressive increase of the AcOH solution concentration, allowed the fractionation of the original S1 or S2 mixture into groups of amino acids. Within a 0.1-2 N
range, the AcOH concentration was progressively varied as follows: 0.1, 0.2, 0.5, 1 and 2 N. The chromatographic process was followed via ion pairing (IP)-RP HPLC analysis. The obtained fractions (of around 1-2 mL) were carefully evaporated until dryness.

2.7. Chromatographic parameters considered in the CLEC analyses

All the following chromatographic parameters were calculated according to the German Pharmacopeia (DAB). The retention factor ($k$) values were computed by taking the retention time ($t_R$) at the peak maximum. Enantioseparation factor ($\alpha$), resolution factor ($R_s$) and column efficiency (expressed as reduced plate height, $h$) were computed from the following Eqs. 1-4:

$$\alpha = \frac{k_2}{k_1} \quad (1)$$

$$R_s = 1.18 \frac{t_R - t_{R_p}}{W_{0.5} + W_{P_{0.5}}} \quad (2)$$

$$h = \frac{1000L}{N_d_p} \quad (3)$$

$$N = 5.54 \left( \frac{t_R}{W_{0.5}} \right)^2 \quad (4)$$

where $k_1$ is the retention factor of the first eluted enantiomer, $k_2$ is the retention factor of the second eluted enantiomer, $W_{0.5}$ is the width of the peak at the position of 50% peak height, $W_{P_{0.5}}$ is the width of the peak at the position of previous 50% peak height and $t_{R_p}$ is the retention time of the first eluted peak within each enantiomer couple. $N$ is the number of theoretical plates, $L$ is the length of the column (mm) and $d_p$ is the stationary phase particle diameter.
3. Results and Discussion

The free amino acid mixture was extracted from the two cheese samples according to the procedure described in section 2.2. Since now and in the course of the text, the extract from the fresh cheese sample will be referred as S1 while that from the six-months maturated one will be labelled as S2.

The work has been developed according to the consecutive steps summarized in Scheme 1. An ion-pairing (IP) RP-HPLC achiral protocol was firstly optimized with the objective to avail of a method enabling the complete distinction of Ala, Asp, and Glu from all the other aminoacidic species in S1 and S2 (STEP 1). An ion-exchange-based chromatographic method was also optimized, thus allowing a profitable fractionation of S1 and S2 aminoacidic mixture (STEP 2). With such a procedure, less complex samples to be analyzed with the CLEC-CSP medium were obtained. A C-CSP operating according to the principle of the ligand-exchange chromatography was useful to quantify the variation of the enantiomeric ratio of Ala, Asp, and Glu in the two selected cheeses (STEP 3).

A description of all these phases is fully detailed in the following sections.

< Please insert Scheme 1 near here>

3.1. Optimization of the IP-RP-HPLC method and its application to S1 and S2

Due to its high purity, volatility and limited cost, HFBA was selected as the IP reagent (Petritis, de Person, Elfakir, & Dreux, 2004). Nonetheless, HFBA-based eluents give also the advantage to avoid prolonged re-equilibration times between consecutive runs (Petritis et al., 2004).

With the use of nine proteinogenic amino acids, the performance achieved with different HFBA concentrations was compared by running a linear gradient obtained by simultaneously increasing the concentration of net MeCN, and decreasing the IP reagent concentration in the overall mobile
phase. The pool of nine model compounds was assembled so as to include representative polar 
[serine (Ser), threonine (Thr)], acidic (Asp, Glu), basic (Lys), aliphatic [Ala, leucine (Leu)] and 
aromatic [Phe, tryptophan (Trp)] compounds. A C18 phase with trimethylsilyl (TMS) end-capping 
treatment of the based-silica support (packing I, Table 1) was chosen with the aim of reducing the 
effect of non-specific secondary interactions with free silanols.

To run the analyses with a progressive increase of MeCN was also required to allow the elution of 
the most hydrophobic Phe and Trp and avoid the stable adsorption of the IP reagent molecules onto 
the stationary phase (Chaimbault, Petritis, Elfakir, & Dreux, 1999). Moreover, MeCN was preferred 
over MeOH for its higher eluotropic strength and the lower column back-pressure generated.

The mobile phase gradient was obtained from eluent A (3, 5, or 7 mM HFBA in water) and eluent B 
(net MeCN) as follows: 0-5 min, 100% A; 5-25 min, linear gradient to 70% A; 25-27 min, gradient 
back to 100% A; 27-50 min, 100% A to equilibrate the column before a new injection. All the 
HPLC/ELSD analyses were carried out with the experimental conditions reported in section 2.3.

Fig. 1 shows the chromatographic traces recorded with a 3 mM (Fig. 1A), 5 mM (Fig. 1B) and 7 
mM (Fig. 1C) perfluorinated carboxylic acid concentration. In all the cases, the eluent A pH was 
not modified in order to avoid the possible occurrence of less volatile salts (Petritis, Chaimbault, 
Elfakir, & Dreux, 1999).

Analyte retention progressively increased as the HFBA concentration was increased in the eluent 
aqueous component, which is in line with experimental observation by other authors (Chaimbault, 
Petritis, Elfakir, & Dreux, 2000). Moreover, especially for the first five eluted compounds (Ala,
Asp, Glu, Ser, Thr), also selectivity improved upon the increase in the HFBA concentration, while the elution order remained unchanged (Chaimbault et al., 1999; Chaimbault et al., 2000). The observed elution order (Fig. 1) is not readily explained as it depends on analyte charge and polarity at once (Chaimbault et al., 1999).

With a 7 mM HFBA concentration, the base-line separation of all the selected nine amino acids was achieved, while Asp and Ser experienced co-elution with a reduction of the eluent A ionic strength. The presence of system peaks in Fig. 1 can be plausibly ascribed to the desorption of HFBA units from the previously saturated C18 phase and their migration along the column, as the net MeCN content is increased during the run (Chaimbault et al., 1999). Worth to be pointed out is that HFBA concentrations higher than 7 mM were avoided since too acidic (pH < 2) mobile phases could irreversibly damage the RP packing material (Chaimbault et al., 2000). With the identified best performing HFBA concentration, three commercially available RP packings (I, II and III, Table 1) were then compared by applying the same gradient elution profile for the analysis of the same model amino acidic mixture. Due to their wide diffusion, also packings II and III were octadecyl-bonded silica stationary phases. However, while packing II was a common C18 stationary phase, packing III was still selected among those manufactured in a way to reduce the free silanol activity. The main physico-chemical parameters of the three packings are summarized in Table 1. The chromatographic traces obtained with packings II and III are shown in Fig. 2A and B, respectively.

In accordance with literature data (Chaimbault et al., 2000), the most efficient packing (that is packing I) was the most hydrophobic C18 silica-based material, being packings II and III unsuited to distinguish Asp from Ser, and Ala from Glu (Figs. 1 and 2). Therefore, packing I was used in the course of the following optimization steps.
On the basis of the results achieved by other authors, different combinations and amounts of almost all the proteinogenic amino acids can be present as free species in dairy products (Albert et al., 2009; Csapó et al., 2009; Csapó, Csapó-Kiss, & Stefler, 1995; Csapó, Varga-Visi, Lóki, & Albert, 2007; Friedman, 1999; Friedman, 2010; Friedman & Levin, 2012; Gandolfi et al., 1992). Hence, the objective of the following gradient profile optimization step was to get as many standard proteinogenic amino acids as possible separated within a single run. However, in this framework, focused efforts were spent to identify suitable conditions mainly allowing Ala, Asp and Glu peaks to be fully distinguishable from other chromatographic signals.

With the use of the heuristic “trial and error method”, the following gradient program, obtained from eluent A (7 mM HFBA in water) and eluent B (net MeCN), was found to produce the best chromatographic performance towards the separation of the most representative underivatized proteinogenic amino acids in cheeses: 0-10 min, 100% A; 10-30 min, linear gradient to 75% A; 30-38 min, linear gradient to 70% A; 38-39 min, gradient back to 100% A; 39-65 min, 100% A to equilibrate the column before a new injection. Co-elution of Asn, Gly and Ser and of His and Lys was observed, while the peaks corresponding to Ala, Asp, and Glu were completely resolved from the remaining chromatographic signals.

The established method was then applied to S1 and S2. As a result of the IP-RP analyses, the two samples seemed to contain the same amino acidic composition. In Fig. 3, the chromatographic trace of S2 along with that of a standard mixture is exemplarily shown. The presence of a wide peak in the correspondence of the Asp retention time prevented its identification in S2. Apart from Trp, the following analyses on the fractionized sample as well as the LE-based ones, confirmed the presence of all the remaining compounds and the “hidden” Asp.

< Please insert Figure 3 near here >
3.2. Fractionation of the amino acidic mixture S1 and S2

The fractionation of the amino acidic mixture was carried out with a strong anion-exchange (SAX) resin. After the resin was opportunely pre-treated (see section 2.6. for details), around 120 mg of S1 or S2 were loaded onto the column.

The amino acids were displaced through the column by application of an ionic strength gradient-based method. The process was performed starting from net water, then progressively increasing the eluent concentration of AcOH (from 0.1 N up to 2 N), which was selected as the displacement developer. Amino acids were eluted out from the column into a series of mixed bands which overlapped one another to a different extent.

Owing to the recognized high chemoselectivity of the chosen C-CSP, no other following fractionation was carried out. Each collected fraction contained no more than 2 mL eluent.

Fraction composition was examined through the previously established IP-RP HPLC/ELSD method, after being concentrated by vacuum evaporation. For a number of selected fractions to be analyzed in the following CLEC step, species identity was confirmed through HPLC/MS analysis (see section 2.3. for details). Accordingly, the m/z ratio value for [M+H]^+ was found equal to 134.0450 and 148.0606 for free Asp and Glu, respectively. The difficult ionization of Ala required its dansylation according to a standard procedure (Mazzucco, Gosetti, Bobba, Marengo, Robotti, & Gennaro, 2010). The m/z ratio value for [M+H]^+ was found equal to 323.1065 for the Dns-Ala derivative.

Chromatograms of the fractions containing Ala, Asp, and Glu, are shown in Fig. 4.
3.3. Optimization of the CLEC method and its application to fractions separately containing Ala, Asp and Glu

Due to its excellent performance, a ligand-exchange (LE)-based CSP obtained through the dynamic coating of packing II with L-STC units (Fig. 5) (Natalini et al., 2008; Natalini et al., 2009; Sardella et al., 2012), was selected to monitor the variation of the Ala, Asp and Glu enantiomeric ratio in the two cheese samples (S1 and S2).

A series of preliminary analyses was carried out to achieve the base-line separation ($R_S > 1.5$) between the enantiomeric peaks of the three species. Accordingly, mobile phase systems with increasing Cu(II) nitrate concentrations were initially evaluated at a 0.5 mL/min flow-rate and with a 20 °C column temperature. As expected (Davankov, Bochkov, Kurganov, Roumeliotis, & Unger, 1980; Hyun, Yang, Kim, & Ryoo, 1994; Natalini et al., 2008), an increase in retention was generally observed with decreasing Cu(II) concentrations (Fig. 6). However, $\alpha$ values remained nearly unchanged (Fig. 7) thus revealing that the thermodynamics of retention is almost equally affected for the two enantiomers by such changes in the mobile phase ionic strength.

For both Ala and Asp (Fig. 7A and B, respectively) the $R_S$ value underwent a progressive improvement as the Cu(II) concentration was reduced down to 0.25 mM (Fig. 7), which can be
readily explained with the concurrent increase in retention and column efficiency (data not shown).

Differently, only subtle changes in the $R_S$ value turned out for Glu (Fig. 7C).

With the aim of obtaining the base-line resolution of Asp and Glu enantiomeric peaks, a flow-rate study was then performed with the lowest concentrated Cu(II) solution. Very profitably, for both compounds a relevant amelioration in terms of column efficiency was gained by decreasing the eluent flow-rate down to 0.1 mL/min (Fig. 8A and B). This trend also reflected on $R_S$ being the value equal to 1.88 and 1.82 for Asp and Glu, respectively (Fig. 8C and D). In accordance to previous studies (Natalini et al., 2010), the variation of mobile phase velocity did not modify the strength of the stereoselective contacts for the two enantiomers to different extents, which is suggested by the enantioseparation factor being nearly unaffected (Fig. 8C and D).

With the exception of Asp, whose enantiomeric elution order was found to be $k_L < k_D$, the L-enantiomers of the other two compounds resulted more retained than their speculars ($k_D < k_L$). We already proposed a chiral recognition model (Natalini, Sardella, Macchiarulo, & Pellicciari, 2006) accounting for the observed elution profile, which is consistent with the formation of the two energetically different diastereomeric ternary complexes. In this model, the chiral selector is hydrophobically adsorbed onto the original C18 packing through its trityl portion and sulfur atom; Cu(II) is then coordinated by the loaded chiral selector and the analyte enantiomer, thus producing a mixed ternary complex (Fig. 9). The first coordination sphere of the central ion can be either completed by achiral components of the eluent (water molecules, salt anions) or adjunctive functionalities in the analyte structure.

While the combination of L-STC with the L-enantiomer produces a cisoid ternary complex, that we referred as the ‘closed model’, the coupling with the D-enantiomer gives rise to a transoid ternary complex named as the ‘open model’. In Fig. 9A and B, the exemplary case
with the Ala enantiomers as the analyte, is shown. In the cisoid configuration (Fig. 9A), the analyte α-radical is oriented towards the modified stationary phase, thus stabilizing hydrophobic interactions that lead to a longer retention of the embedded sample enantiomer. Conversely, in the transoid complex (Fig. 9B), the analyte side-chain points towards the bulk eluent.

An adjunctive axial coordination by the side-chain carboxy group of D-Asp (Fig. 9C) can be called into play to account for the reversed enantiomeric elution order of this compound (that is $k_L < k_D$ instead of $k_D < k_L$). The additional ‘point of attach’ in the L-STC/Cu(II)/D-Asp complex can be thought to favour its stabilization with respect to the corresponding diastereomeric adduct in which the hydrophilic side-chain residual of L-Asp tends to be oriented towards the hydrophobic layer (Fig. 9D).

As a result of an additional methylene unit embedded within its α-radical, D-Glu is hampered to undergo an axial coordination with the distal carboxylic moiety, which reflects into a “canonical” enantiomeric elution order (namely $k_D < k_L$) (Sardella et al., 2012).

The optimized CLEC conditions were then applied to the previously identified Ala, Asp and Glu containing fractions as well as to those including all the remaining species. Chromatograms in Fig. 10 clearly highlight a different enantiomeric excess of the three compounds in the two investigated cheese samples. More in details and in line with other observations, for all compounds the ee was found to decrease passing from S1 to S2, as indicated by the values in Fig. 10. The largest difference was found for Ala (ee value from 83.0% down to 20.5%) (Fig. 10A), followed progressively by Asp (ee value from 90.5 to 75.0%) (Fig. 10B) and Glu (ee value from 99.0 to 91.8%) (Fig. 10C).
Except for Ala, Asp, and Glu, no other D-amino acids were revealed. No information can be instead gained for Lys and Ser, being the employed C-CSP unable to distinguish the corresponding enantiomers.

4. Conclusions

With the use of a CLEC system based on L-STC units adsorbed onto a conventional ODS packing, and a 0.25 mM Cu(II) nitrate solution as the metal source into the eluent, we were able to quantify the variation of the free D-Ala, D-Asp and D-Glu content in two cheese samples of different ripening time. Among the advantages of the CLEC approach, worth to be mentioned are: the generation of UV/vis-active metal complexes, which allows the detection of even UV-transparent molecules, the use of commercially available and cost-effective chiral enantiodiscriminating agents, combined with rather unexpensive RP columns and, the “eco-friendly” character of the whole chromatographic process, due to the frequent exclusive use of water-based eluents.

As expected, a particularly relevant increase was observed for D-Ala (ee value from 83.0% down to 20.5%) as a result of a maturation time of six months; while progressively lower variations were revealed for the D-Asp (ee value from 90.5 to 75.0%) and D-Glu (ee value from 99.0 to 91.8%). The prior fractionation of the aa mixture from both cheese samples with a SAX resin and a ionic strength gradient elution facilitated the goodness of the CLEC analysis. Moreover, an optimized gradient IP-RP-HPLC/ELSD method, based on the use of HFBA as the IP reagent, was successfully applied to identify Ala, Asp and Glu in the collected fractions.

In conclusion, the proposed combination of easy-to-realize monodimensional chromatographic approaches can be fruitfully applied to assess the impact of natural or artificial interventions on the product quality, thus contributing to ensure food safety.
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5. References


**Figure captions**

Scheme 1 Block diagram showing the different stages in this work.

**Fig. 1.** Chromatographic traces of the preliminary standard amino acid mixture recorded with a (A) 3 mM, (B) 5 mM, or (C) 7 mM HFBA eluent A concentration. Experimental conditions: column, packing I; mobile phase, eluent A (HFBA in water) and eluent B (net MeCN); gradient, 0-5 min, 100% A; 5-25 min, linear gradient to 70% A; 25-27 min, gradient back to 100% A; 27-50 min, 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap} 50 °C, gas flow rate 1 L/min, gain 1.

**Fig. 2.** Chromatographic traces of the preliminary standard amino acid mixture obtained with (A) packing II and (B) packing III. Experimental conditions: mobile phase, eluent A (7 mM HFBA in water) and eluent B (net MeCN); gradient, 0-5 min, 100% A; 5-25 min, linear gradient to 70% A; 25-27 min, gradient back to 100% A; 27-50 min, 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap} 50 °C, gas flow rate 1 L/min, gain 1.

**Fig. 3.** Chromatographic traces of S2 and the corresponding standard amino acid mixture, with the optimised RP elution conditions. Experimental conditions: column, packing I; mobile phase, eluent A (7 mM HFBA in water) and eluent B (net MeCN); gradient, 0-10 min, 100% A; 10-30 min, linear gradient to 75% A; 30-38 min, linear gradient to 70% A; 38-39 min, gradient back to 100% A; 39-65 min, 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap} 50 °C, gas flow rate 1 L/min, gain 1.

**Fig. 4.** Chromatographic traces of S2 fractions containing (A) Ala, (B) Asp, and (C) Glu with the optimised RP elution conditions. Experimental conditions: column, packing I; mobile phase, eluent
A (7 mM HFBA in water) and eluent B (net MeCN); gradient, 0-10 min, 100% A; 10-30 min, linear gradient to 75% A; 30-38 min, linear gradient to 70% A; 38-39 min, gradient back to 100% A; 39-65 min, 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, $T_{\text{neb}}$ 30 °C, $T_{\text{vap}}$ 50 °C, gas flow rate 1 L/min, gain 1.

**Fig. 5.** Chiral selector employed in this study (S-trityl-L-cysteine, L-STC).

**Fig. 6.** Influence of Cu(II) nitrate concentration on the enantiomeric retention of (A) Ala, (B) Asp, and (C) Glu. Experimental conditions: column, packing II coated with L-STC units; mobile phase, Cu(II) nitrate (0.25 mM or 0.5 mM or 1 mM); flow rate, 0.5 mL/min, column temperature, 20 °C, detection wavelength, 254 nm.

**Fig. 7.** Influence of Cu(II) nitrate concentrations on the enantioselectivity ($\alpha$) and enantioresolution (RS) of (A) Ala, (B) Asp, and (C) Glu. Experimental conditions: column, packing II coated with L-STC units; mobile phase, Cu(II) nitrate (0.25 mM or 0.5 mM or 1 mM); flow rate, 0.5 mL/min, column temperature, 20 °C, detection wavelength, 254 nm.

**Fig. 8.** Influence of the eluent flow rate on the column efficiency ($h$), enantioselectivity ($\alpha$) and enantioresolution (RS) in the elution of (A, C) Asp, and (B, D) Glu. Experimental conditions: column, packing II coated with L-STC units; mobile phase, 0.25 mM Cu(II) nitrate; flow rate, 0.1 or 0.3 or 0.5 mL/min, column temperature, 20 °C, detection wavelength, 254 nm.

**Fig. 9.** The proposed structures of the diastereomeric ternary complexes formed from the fixed ligand (L-STC), Cu(II) and (A) L-Ala (closed model) or (B) D-Ala (open model) or (C) D-Asp (open model) or (d) L-Asp (closed model).
Fig. 10. Chromatographic traces of S1 and S2 fractions containing (A) Ala, (B) Asp, and (C) Glu.

Experimental conditions: column, packing II coated with L-STC units; mobile phase, 0.25 mM Cu(II) nitrate; flow rate, 0.1 mL/min, column temperature, 20 °C, detection wavelength, 254 nm.