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1 # Combined monodimensional chromatographic approaches to monitor the
2 # 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.

82 **1. Introduction**

83

84 The identification, characterization and quantisation of naturally occurring amino acids (AAs) are
85 the subject matter of continuing study and interest (Friedman, 1999; Friedman & Levin, 2012). The
86 presence of L-amino acids (L-AAs) in foodstuffs has attracted a remarkable attention due to the
87 relevant involvements with the taste properties and the nutritional and nutraceutical value they
88 determine (Friedman, 2010; Friedman & Levin, 2012). Conversely, the presence of D-amino acids
89 (D-AAs) as a consequence of natural or artificial interventions such as ageing, microorganism
90 action, preservative and conservative processes (alkali or heat treatment), is a remarkably neglected
91 aspect from the scientific community, as shown by the paucity of publications in this field.
92 Frequently, the presence of free D-AAs is an indication of microbial contamination, making these
93 compounds as indicators of food quality (Albert, Pohn, Lóki, & Csapó, 2009; Brückner, Jaek,
94 Langer, & Godel, 1992; Friedman, 2010). Indeed, D-alanine (D-Ala), D-aspartic acid (D-Asp) and
95 D-glutamic acid (D-Glu) are present in peptidoglycan, a fundamental constituent of the bacterial
96 cell walls (Csapó, Albert, & Csapó-Kiss, 2009). Therefore, the study and the evaluation of the
97 presence of D-AAs in edible products constitute an interesting and reliable approach in the field of
98 the food safety, that enters the frame of the plurality of strategies aimed at assessing the product
99 quality (Friedman, 1999).
100 It is also fully documented that even a minor degree of racemisation on the proteins' AAs is the
101 cause of a reduced digestion of such proteins (Csapó et al., 2009). Reduced protein digestibility
102 depends on the fact that racemised AAs are not suitable substrates for proteolytic enzymes, and also
103 exert a deleterious effect on the capacity for liberation of adjacent non-racemised amino acids
104 (Hayashi & Kameda, 1980; Rosen-Levin, Smithson, & Gray, 1980). Thus, the racemisation of some
105 AAs can impair the nutritional quality of an edible product.
106 Besides interfering with the regular metabolism of L-AAs, D-AAs can contribute to the
107 development of pathological conditions in humans (Friedman, 1999; Friedman, 2010; Friedman &

108 Levin, 2012). Nevertheless, beneficial nutritional and health-related aspects were also described for
109 the D-isomer of some aminoacidic compound, thus suggesting the potential use as nutraceuticals of
110 the foods where they are concentrated (Friedman & Levin, 2012).
111 Basing on the above assumptions, the qualitative and quantitative knowledge of the enantiomeric
112 content of free amino acids in foodstuffs is of prior importance.
113 So far, nearly all the most important chromatographic techniques were applied to quantify D-AAs
114 in foodstuffs (Brückner & Hausch, 1990; Carlavilla, Moreno-Arribas, Fanali, & Cifuentes, 2006;
115 Gandolfi, Palla, Delprato, De Nisco, Marchelli, & Salvatori, 1992; Qi, Chen, Xie, Guo, & Wang,
116 2008; Van de Merbel, Stenberg, Öste, Marko-Varga, Gorton, Lingeman, & Brinkman, 1995; Voss
117 & Galensa 2000; Pätzold & Brückner, 2005). However, most of them rely upon pre- or post-column
118 derivatization procedures, often combined with sophisticated analytical equipments.
119 We recently described (Sardella, Ianni, Natalini, Blanch, & del Castillo, 2012) the direct
120 employment of a chiral ligand-exchange chromatography (CLEC) stationary phase achieved
121 through the dynamic coating of an octadecylsilica-based material with S-trityl-L-cysteine (L-STC)
122 units (Natalini, Sardella, Carbone, Macchiarulo, & Pellicciari, 2009; Natalini, Sardella, Giacchè,
123 Palmiotto, Camaioni, Marinozzi, Macchiarulo, & Pellicciari, 2010; Natalini, Sardella, Macchiarulo,
124 & Pellicciari, 2008), as an effective way to evaluate the presence of D-AAs in six cheese samples of
125 different milk composition and ripening time. However, owing to the limited peak resolution
126 provided by the selected chiral system in the first 10 min of analysis, only identifying information
127 could be gained with the proposed chromatographic protocol. Remarkable improvements of the
128 enantioresolution quality of the same coated chiral stationary phase (C-CSP) system have been
129 obtained by optimizing dedicated pre-analysis purification procedures. Accordingly, in this paper
130 we describe the optimization of the achiral chromatographic methods preceding the CLEC analysis
131 and of a series of parameters improving the quality of enantioseparation with the L-STC-based
132 CSP. In order to prove the practical utility of the proposed combination of monodimensional
133 methods, an application has been carried out to evaluate the presence and the incidental variation of

134 the D-aminoacidic content into two commercially available Spanish cheese samples. The two
135 cheese samples were prepared from the same cow, sheep, and goat milk mixture, and characterized
136 by a different maturity time: no ripening and 6 months ripening.
137
138 **2. Materials and methods**
139
140 *2.1. Chemicals*
141
142 Water for HPLC analysis was purified with a New Human Power I Scholar water purification
143 system (Human Corporation, Seoul, Korea). All standard amino acids along with copper(II) nitrate
144 pentahemihydrate and the chiral selector S-trityl-L-cysteine (L-STC) were of high analytical purity
145 and purchased from Sigma-Aldrich (Milan, Italy). Methanol (MeOH), acetonitrile (MeCN),
146 heptafluorobutyric acid (HFBA), sodium hydroxide (NaOH), aqueous ammonia solution (NH_4OH),
147 glacial acetic acid (AcOH), hydrochloric acid (HCl), trichloroacetic acid, and the Dowex 1X8-200
148 ion-exchange resins were purchased from Sigma-Aldrich (Milan, Italy).
149
150 *2.2. Extraction of cheese amino acids*
151
152 A 100 g weight of each of the two cheese samples were first separately lyophilized and pulverized.
153 A 20 mL volume of 0.1 M HCl was added to 5 g of each lyophilized cheese. Each suspension was
154 stirred for 3 h by using a magnetic stirrer and then left at 5 °C overnight to settle. The two-phase
155 system was then shaken-up again, and then centrifuged at 500 g and 8 °C for 10 min. Protein was
156 precipitated from the supernatant, with equal volume of 25% (w/v) trichloroacetic acid solution
157 with the final concentration of trichloroacetic acid of 12.5%. The suspension was again centrifuged
158 at 500 g and 8 °C for 10 min after 30 min standing. Subsequently, a 8 mL volume of supernatant
159 was placed into a 10 mL vial and then neutralized with 4 M NaOH solution following dilution with

160 distilled water. The extract was filtered through a 0.45 μm filter, frozen and then lyophilized.
161 Extracts from 6 months ripened and fresh cheeses are indicated throughout the text as samples S1
162 and S2, respectively.

163

164 *2.3. Instrumentation*

165

166 The HPLC measurements were made on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped
167 with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A
168 photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a
169 20 μl stainless steel loop. A Varian 385-LC evaporative light scattering detector (ELSD) (Agilent
170 Technologies, Santa Clara, CA, USA) was specifically utilized for the achiral analyses. The analog-
171 to-digital conversion of the output signal from the ELSD was allowed by a common interface
172 device. The adopted ELSD conditions for the analysis were: 30 °C nebulization temperature, 50 °C
173 evaporation temperature, 1 L/min gas flow rate (air) and 1 as the gain factor.
174 The analytical columns were: Luna C18(2) (Phenomenex, Torrance, CA, USA) (packing I),
175 GraceSmart RP 18 (Grace, Lokeren, Belgium) (packing II) and Ultra II Aqueous C18 (Restek,
176 Bellefonte, PA, USA) (packing III). Specific column characteristics are reported in Table 1. Unless
177 otherwise reported, the flow rate was fixed at 1 mL/min. For the achiral analyses the column
178 temperature was fixed at 25 °C, while chiral analyses were carried out at 20 °C. Column
179 temperature was controlled through a Grace (Sedriano, Italy) heater/chiller (Model 7956R)
180 thermostat.
181 HPLC/MS experiments were performed using an Agilent Infinity Series LC system (Agilent
182 Technologies, Palo Alto, CA, USA). The LC system was interfaced to an Agilent 6540 UHD
183 Accurate-Mass Q-TOF LC/MS detector, also from Agilent Technologies and equipped with an
184 Agilent Dual Jet Stream Technology ESI source. The mass spectrometer system was controlled by
185 the Agilent MassHunter Workstation software. Mass spectra were recorded from m/z 100 to 1000.

186 Continuous lock mass infusion included *m/z* 121.05087300 and 922.00979800. A FWHM
187 resolution ranging from 10000 to 25000 was used. The operating conditions were: gas temperature,
188 300 °C; drying gas, nitrogen at 9 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 320 °C;
189 sheath gas flow, nitrogen at 9 L/min; capillary voltage, 4000 V; skimmer, 65 V; octopole
190 radiofrequency voltage, 750 V; nozzle voltage, 0 V; fragmentor voltage, 100 V.
191 The separation of underivatized amino acids was performed with Kinetex 1.7 μm C8, 50 x 2.1 mm
192 ID column in positive mode. The following gradient program, obtained from eluent A (7 mM
193 HFBA in water) and eluent B (net MeCN), was applied: 0-3 min, 100% A; 3-9 min, linear gradient
194 to 75% A; 9-12 min, linear gradient to 70% A; 12-12.1 min, gradient back to 100% A; 12.1-30 min,
195 100% A to equilibrate the column before a new injection. The chromatographic run for the analysis
196 on the DNS-Ala containing fraction was instead carried out with the same Kinetex C8 column, by
197 slightly modifying a known elution method (Timperio, Fagioni, Grandinetti, & Zolla, 2007). The
198 injection volume was 1 μL. Eluent flow rate was 0.2 mL/min and column temperature was fixed at
199 30 °C.
200
201 *2.4. Preparation of the L-STC-based stationary phase and column evaluation*
202
203 A GraceSmart RP 18 (packing II) analytical column was dynamically coated with L-STC units. The
204 chiral selector (250 mg) was solubilised into a water/MeOH solution (250 mL, 50:50, v/v), carefully
205 filtered through a 0.22 μm Millipore filter and degassed with 10 min sonication. The optimal
206 adsorption of the selector was achieved by recycling the prepared solution for 5 days at 0.5 mL/min.
207 With this procedure, approximately 0.05 g of the selector were established to be hydrophobically
208 bonded to the RP-18 sorbent surface. After washing with a water/MeOH solution (50 mL, 98:2, v/v)
209 in order to displace the excess of chiral discriminating agent and MeOH, a Cu(II) nitrate solution
210 was flowed through the column and used as the mobile phase after 2 h of equilibration. The first
211 vacancy peak of a blank injection of mobile phase components was used to calculate the t_0 value.

212 Column performance was assessed by periodic injection of racemic proline (*rac*-Pro). The dynamic
213 CSP used in this study was found to be stable and uniformly effective in the chiral separation of
214 amino acids for at least 30 days.
215
216 *2.5. CLEC mobile phase preparation and experimental conditions*
217
218 The mobile phase for the CLEC runs was prepared by dissolving Cu(II) nitrate (at a 0.25, 0.5 or 1
219 mmol/L concentration) in HPLC-grade water. The resulting solution was filtered through a 0.22 µm
220 Millipore filter and degassed by sonication for 20 min. The sample solutions were prepared at
221 concentrations between approximately 0.1 and 0.5 mg/mL in filtered mobile phase components and
222 sonicated until completely dissolved. The UV detection wavelengths were set at 254 and 210 nm,
223 and the flow rate was changed among the values 0.1, 0.3, 0.5 or 1 mL/min, according to the specific
224 application.
225
226 *2.6. Anion-Exchange Chromatography*
227
228 A Dowex 1x 8-200 ion-exchange resin, poured into a 400 x 10 mm i.d. glass column, was
229 successfully used for the amino acid mixture (S1 or S2) fractionation. Before being utilized, the
230 resin was submitted to treatment aimed at flushing out the constitutive Cl^- anions. This action was
231 carried out by means of a 0.3 N NaOH solution until the eluate became basic. Chloride anions were
232 then replaced with acetate by flowing a 0.3 N AcOH solution until the eluate assumed an acidic
233 character. A following wash out with water allowed the resin to reach a neutral pH. At this point,
234 the sample dissolved in ammonia solution (pH ~ 9.0) was first chromatographed with water in order
235 to elute other compounds than amino acids, along with basic amino acids. Subsequently, an ionic-
236 strength gradient elution with a progressive increase of the AcOH solution concentration, allowed
237 the fractionation of the original S1 or S2 mixture into groups of amino acids. Within a 0.1-2 N

238 range, the AcOH concentration was progressively varied as follows: 0.1, 0.2, 0.5, 1 and 2 N. The
239 chromatographic process was followed *via* ion pairing (IP)-RP HPLC analysis. The obtained
240 fractions (of around 1-2 mL) were carefully evaporated until dryness.

241

242 *2.7. Chromatographic parameters considered in the CLEC analyses*

243

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

248 $$\alpha = \frac{k_2}{k_1} \quad (1)$$

249 $$R_S = 1.18 \frac{t_R - t_{Rp}}{W_{0.5} + Wp_{0.5}} \quad (2)$$

250 $$h = \frac{1000L}{Nd_P} \quad (3)$$

251 $$N = 5.54 \left(\frac{t_R}{W_{0.5}} \right)^2 \quad (4)$$

252

253 where k_1 is the retention factor of the first eluted enantiomer, k_2 is the retention factor of the second
254 eluted enantiomer, $W_{0.5}$ is the width of the peak at the position of 50% peak height, $Wp_{0.5}$ is the
255 width of the peak at the position of previous 50% peak height and t_{Rp} is the retention time of the
256 first eluted peak within each enantiomer couple. N is the number of theoretical plates, L is the
257 length of the column (mm) and d_P is the stationary phase particle diameter.

258

259

260

261 **3. Results and Discussion**

262

263 The free amino acid mixture was extracted from the two cheese samples according to the procedure
264 described in section 2.2. Since now and in the course of the text, the extract from the fresh cheese
265 sample will be referred as S1 while that from the six-months maturated one will be labelled as S2.
266 The work has been developed according to the consecutive steps summarized in Scheme 1.
267 An ion-pairing (IP) RP-HPLC achiral protocol was firstly optimized with the objective to avail of a
268 method enabling the complete distinction of Ala, Asp, and Glu from all the other aminoacidic
269 species in S1 and S2 (STEP 1). An ion-exchange-based chromatographic method was also
270 optimized, thus allowing a profitable fractionation of S1 and S2 aminoacidic mixture (STEP 2).
271 With such a procedure, less complex samples to be analyzed with the CLEC-CSP medium were
272 obtained. A C-CSP operating according to the principle of the ligand-exchange chromatography
273 was useful to quantify the variation of the enantiomeric ratio of Ala, Asp, and Glu in the two
274 selected cheeses (STEP 3).
275 A description of all these phases is fully detailed in the following sections.

276

277 **< Please insert Scheme 1 near here>**

278

279 *3.1. Optimization of the IP-RP-HPLC method and its application to S1 and S2*

280

281 Due to its high purity, volatility and limited cost, HFBA was selected as the IP reagent (Petritis, de
282 Person, Elfakir, & Dreux, 2004). Nonetheless, HFBA-based eluents give also the advantage to
283 avoid prolonged re-equilibration times between consecutive runs (Petritis et al., 2004).
284 With the use of nine proteinogenic amino acids, the performance achieved with different HFBA
285 concentrations was compared by running a linear gradient obtained by simultaneously increasing
286 the concentration of net MeCN, and decreasing the IP reagent concentration in the overall mobile

287 phase. The pool of nine model compounds was assembled so as to include representative polar
288 [serine (Ser), threonine (Thr)], acidic (Asp, Glu), basic (Lys), aliphatic [Ala, leucine (Leu)] and
289 aromatic [Phe, tryptophan (Trp)] compounds. A C18 phase with trimethylsilyl (TMS) end-capping
290 treatment of the based-silica support (packing I, Table 1) was chosen with the aim of reducing the
291 effect of non-specific secondary interactions with free silanols.
292
293 **< Please insert Table 1 near here>**
294
295 To run the analyses with a progressive increase of MeCN was also required to allow the elution of
296 the most hydrophobic Phe and Trp and avoid the stable adsorption of the IP reagent molecules onto
297 the stationary phase (Chaimbault, Petritis, Elfakir, & Dreux, 1999). Moreover, MeCN was preferred
298 over MeOH for its higher eluotropic strength and the lower column back-pressure generated.
299 The mobile phase gradient was obtained from eluent A (3, 5, or 7 mM HFBA in water) and eluent B
300 (net MeCN) as follows: 0-5 min, 100% A; 5-25 min, linear gradient to 70% A; 25-27 min, gradient
301 back to 100% A; 27-50 min, 100% A to equilibrate the column before a new injection. All the
302 HPLC/ELSD analyses were carried out with the experimental conditions reported in section 2.3.
303 Fig. 1 shows the chromatographic traces recorded with a 3 mM (Fig. 1A), 5 mM (Fig. 1B) and 7
304 mM (Fig. 1C) perfluorinated carboxylic acid concentration. In all the cases, the eluent A pH was
305 not modified in order to avoid the possible occurrence of less volatile salts (Petritis, Chaimbault,
306 Elfakir, & Dreux, 1999).
307
308 **<Please insert Figure 1 near here >**
309
310 Analyte retention progressively increased as the HFBA concentration was increased in the eluent
311 aqueous component, which is in line with experimental observation by other authors (Chaimbault,
312 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.

< Please insert Figure 2 near here >

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.

338 On the basis of the results achieved by other authors, different combinations and amounts of almost
339 all the proteinogenic amino acids can be present as free species in dairy products (Albert et al.,
340 2009; Csapó et al., 2009; Csapó, Csapó-Kiss, & Stefler, 1995; Csapó, Varga-Visi, Lóki, & Albert,
341 2007; Friedman, 1999; Friedman, 2010; Friedman & Levin, 2012; Gandolfi et al., 1992). Hence, the
342 objective of the following gradient profile optimization step was to get as many standard
343 proteinogenic amino acids as possible separated within a single run. However, in this framework,
344 focused efforts were spent to identify suitable conditions mainly allowing Ala, Asp and Glu peaks
345 to be fully distinguishable from other chromatographic signals.
346 With the use of the heuristic "trial and error method", the following gradient program, obtained
347 from eluent A (7 mM HFBA in water) and eluent B (net MeCN), was found to produce the best
348 chromatographic performance towards the separation of the most representative underivatized
349 proteinogenic amino acids in cheeses: 0-10 min, 100% A; 10-30 min, linear gradient to 75% A; 30-
350 38 min, linear gradient to 70% A; 38-39 min, gradient back to 100% A; 39-65 min, 100% A to
351 equilibrate the column before a new injection. Co-elution of Asn, Gly and Ser and of His and Lys
352 was observed, while the peaks corresponding to Ala, Asp, and Glu were completely resolved from
353 the remaining chromatographic signals.
354 The established method was then applied to S1 and S2. As a result of the IP-RP analyses, the two
355 samples seemed to contain the same amino acidic composition. In Fig. 3, the chromatographic trace
356 of S2 along with that of a standard mixture is exemplarily shown. The presence of a wide peak in
357 the correspondence of the Asp retention time prevented its identification in S2. Apart from Trp, the
358 following analyses on the fractionized sample as well as the LE-based ones, confirmed the presence
359 of all the remaining compounds and the "hidden" Asp.
360

361 **< Please insert Figure 3 near here >**

362

363

364 *3.2. Fractionation of the amino acidic mixture S1 and S2*

365

366 The fractionation of the amino acidic mixture was carried out with a strong anion-exchange (SAX)
367 resin. After the resin was opportunely pre-treated (see section 2.6. for details), around 120 mg of S1
368 or S2 were loaded onto the column.
369 The amino acids were displaced through the column by application of an ionic strength gradient-
370 based method. The process was performed starting from net water, then progressively increasing the
371 eluent concentration of AcOH (from 0.1 N up to 2 N), which was selected as the displacement
372 developer. Amino acids were eluted out from the column into a series of mixed bands which
373 overlapped one another to a different extent.
374 Owing to the recognized high chemoselectivity of the chosen C-CSP, no other following
375 fractionation was carried out. Each collected fraction contained no more than 2 mL eluent.
376 Fraction composition was examined through the previously established IP-RP HPLC/ELSD
377 method, after being concentrated by vacuum evaporation. For a number of selected fractions to be
378 analyzed in the following CLEC step, species identity was confirmed through HPLC/MS analysis
379 (see section 2.3. for details). Accordingly, the *m/z* ratio value for $[M+H]^+$ was found equal to
380 134.0450 and 148.0606 for free Asp and Glu, respectively. The difficult ionization of Ala required
381 its dansylation according to a standard procedure (Mazzucco, Gosetti, Bobba, Marengo, Robotti, &
382 Gennaro, 2010). The *m/z* ratio value for $[M+H]^+$ was found equal to 323.1065 for the Dns-Ala
383 derivative.
384 Chromatograms of the fractions containing Ala, Asp, and Glu, are shown in Fig. 4.

385

386 **< Please insert Figure 4 near here >**

387

388

389 *3.3. Optimization of the CLEC method and its application to fractions separately containing Ala,*
390 *Asp and Glu*
391

392 Due to its excellent performance, a ligand-exchange (LE)-based CSP obtained through the dynamic
393 coating of packing II with L-STC units (Fig. 5) **(Natalini et al., 2008; Natalini et al., 2009;**
394 **Sardella et al., 2012)**, was selected to monitor the variation of the Ala, Asp and Glu enantiomeric
395 ratio in the two cheese samples (S1 and S2).
396

397 **< Please insert Figure 5 near here >**
398

399 A series of preliminary analyses was carried out to achieve the base-line separation ($R_S > 1.5$)
400 between the enantiomeric peaks of the three species. Accordingly, mobile phase systems with
401 increasing Cu(II) nitrate concentrations were initially evaluated at a 0.5 mL/min flow-rate and with
402 a 20 °C column temperature. As expected (Davankov, Bochkov, Kurganov, Roumeliotis, & Unger,
403 1980; Hyun, Yang, Kim, & Ryoo, 1994; Natalini et al., 2008), an increase in retention was
404 generally observed with decreasing Cu(II) concentrations (Fig. 6). However, α values remained
405 nearly unchanged (Fig. 7) thus revealing that the thermodynamics of retention is almost equally
406 affected for the two enantiomers by such changes in the mobile phase ionic strength.
407

408 **< Please insert Figure 6 near here >**
409

410 **< Please insert Figure 7 near here >**
411

412 For both Ala and Asp (Fig. 7A and B, respectively) the R_S value underwent a progressive
413 improvement as the Cu(II) concentration was reduced down to 0.25 mM (Fig. 7), which can be

414 readily explained with the concurrent increase in retention and column efficiency (data not shown).

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

416 With the aim of obtaining the base-line resolution of Asp and Glu enantiomeric peaks, a flow-rate

417 study was then performed with the lowest concentrated Cu(II) solution. Very profitably, for both

418 compounds a relevant amelioration in terms of column efficiency was gained by decreasing the

419 eluent flow-rate down to 0.1 mL/min (Fig. 8A and B). This trend also reflected on R_S being the

420 value equal to 1.88 and 1.82 for Asp and Glu, respectively (Fig. 8C and D). **In accordance to**

421 **previous studies (Natalini et al., 2010), the variation** of mobile phase velocity did not modify the

422 strength of the stereoselective contacts for the two enantiomers to different extents, which is

423 suggested by the enantioseparation factor being nearly unaffected (Fig. 8C and D).

424

425 **< Please insert Figure 8 near here >**

426

427 With the exception of Asp, whose enantiomeric elution order was found to be $k_L < k_D$, the L-

428 enantiomers of the other two compounds resulted more retained than their speculars ($k_D < k_L$). We

429 already proposed a chiral recognition model (Natalini, Sardella, Macchiarulo, & Pellicciari, 2006)

430 accounting for the observed elution profile, which is consistent with the formation of the two

431 energetically different diastereomeric ternary complexes. In this model, the chiral selector is

432 hydrophobically adsorbed onto the original C18 packing through its trityl portion and sulfur atom;

433 Cu(II) is then coordinated by the loaded chiral selector and the analyte enantiomer, thus producing a

434 mixed ternary complex (Fig. 9). The first coordination sphere of the central ion can be either

435 completed by achiral components of the eluent (water molecules, salt anions) or adjunctive

436 functionalities in the analyte structure.

437 **While the combination of L-STC with the L-enantiomer produces a *cisoid* ternary complex,**

438 **that we referred as the 'closed model', the coupling with the D-enantiomer gives rise to a**

439 ***transoid* ternary complex named as the 'open model'. In Fig. 9A and B, the exemplary case**

440 **with the Ala enantiomers as the analyte, is shown. In the *cisoid* configuration (Fig. 9A), the**
441 **analyte α-radical is oriented towards the modified stationary phase, thus stabilizing**
442 **hydrophobic interactions that lead to a longer retention of the embedded sample enantiomer.**
443 **Conversely, in the *transoid* complex (Fig. 9B), the analyte side-chain points towards the bulk**
444 **eluent.**
445
446 **< Please insert Figure 9 near here >**
447
448 **An adjunctive axial coordination by the side-chain carboxy group of D-Asp (Fig. 9C) can be**
449 **called into play to account for the reversed enantiomeric elution order of this compound (that**
450 **is $k_L < k_D$ instead of $k_D < k_L$). The additional 'point of attach' in the L-STC/Cu(II)/D-Asp**
451 **complex can be thought to favour its stabilization with respect to the corresponding**
452 **diastereomeric adduct in which the hydrophilic side-chain residual of L-Asp tends to be**
453 **oriented towards the hydrophobic layer (Fig. 9D).**
454 **As a result of an additional methylene unit embedded within its α-radical, D-Glu is hampered**
455 **to undergo an axial coordination with the distal carboxylic moiety, which reflects into a**
456 **"canonical" enantiomeric elution order (namely $k_D < k_L$) (Sardella et al., 2012).**
457 The optimized CLEC conditions were then applied to the previously identified Ala, Asp and Glu
458 containing fractions as well as to those including all the remaining species. Chromatograms in Fig.
459 10 clearly highlight a different enantiomeric excess of the three compounds in the two investigated
460 cheese samples. More in details and in line with other observations, for all compounds the ee was
461 found to decrease passing from S1 to S2, as indicated by the values in Fig. 10. The largest
462 difference was found for Ala (ee value from 83.0% down to 20.5%) (Fig. 10A), followed
463 progressively by Asp (ee value from 90.5 to 75.0%) (Fig. 10B) and Glu (ee value from 99.0 to
464 91.8%) (Fig. 10C).
465

< Please insert Figure 10 near here >

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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618 **Figure captions**

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

621

622 **Fig. 1.** Chromatographic traces of the preliminary standard amino acid mixture recorded with a (A)
623 3 mM, (B) 5 mM, or (C) 7 mM HFBA eluent A concentration. Experimental conditions: column,
624 packing I; mobile phase, eluent A (HFBA in water) and eluent B (net MeCN); gradient, 0-5 min,
625 100% A; 5-25 min, linear gradient to 70% A; 25-27 min, gradient back to 100% A; 27-50 min,
626 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap} 50 °C,
627 gas flow rate 1 L/min, gain 1.

628

629 **Fig. 2.** Chromatographic traces of the preliminary standard amino acid mixture obtained with (A)
630 packing II and (B) packing III. Experimental conditions: mobile phase, eluent A (7 mM HFBA in
631 water) and eluent B (net MeCN); gradient, 0-5 min, 100% A; 5-25 min, linear gradient to 70% A;
632 25-27 min, gradient back to 100% A; 27-50 min, 100% A; flow rate, 1 mL/min; column
633 temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap} 50 °C, gas flow rate 1 L/min, gain 1.

634

635 **Fig. 3.** Chromatographic traces of S2 and the corresponding standard amino acid mixture, with the
636 optimised RP elution conditions. Experimental conditions: column, packing I; mobile phase, eluent
637 A (7 mM HFBA in water) and eluent B (net MeCN); gradient, 0-10 min, 100% A; 10-30 min, linear
638 gradient to 75% A; 30-38 min, linear gradient to 70% A; 38-39 min, gradient back to 100% A; 39-
639 65 min, 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap}
640 50 °C, gas flow rate 1 L/min, gain 1.

641

642 **Fig. 4.** Chromatographic traces of S2 fractions containg (A) Ala, (B) Asp, and (C) Glu with the
643 optimised RP elution conditions. Experimental conditions: column, packing I; mobile phase, eluent

644 A (7 mM HFBA in water) and eluent B (net MeCN); gradient, 0-10 min, 100% A; 10-30 min, linear
645 gradient to 75% A; 30-38 min, linear gradient to 70% A; 38-39 min, gradient back to 100% A; 39-
646 65 min, 100% A; flow rate, 1 mL/min; column temperature, 25 °C; ELSD setting, T_{neb} 30 °C, T_{vap}
647 50 °C, gas flow rate 1 L/min, gain 1.

648

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

650

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

655

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

660

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

665

666 **Fig. 9.** The proposed structures of the diastereomeric ternary complexes formed from the fixed
667 ligand (L-STC), Cu(II) and (A) L-Ala (closed model) or (B) D-Ala (open model) or (C) D-Asp
668 (open model) or (d) L-Asp (closed model).

669

670 **Fig. 10.** Chromatographic traces of S1 and S2 fractions containg (A) Ala, (B) Asp, and (C) Glu.
671 Experimental conditions: column, packing II coated with L-STC units; mobile phase, 0.25 mM
672 Cu(II) nitrate; flow rate, 0.1 mL/min, column temperature, 20 °C, detection wavelength, 254 nm.