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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%).

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, Jaek, 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

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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, 2010; Friedman & Levin, 2012). 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 et al., 1992; Pätzold & Brückner, 2005; Qi, Chen, Xie, Guo, & Wang, 2008; Van de Merbel et al., 1995; Voss & Galensa 2000). 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 et al., 2010; Natalini, Sardella, Carbone, Macchiarulo, & Pellicciari, 2009; 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 (NH_4OH), 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 was 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. Proteins were 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, an 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

Table 1
Main characteristics of the employed columns.

Column	Packing #	Characteristics				
		Dimensions (mm)	Particle size (μm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)
Luna C-18	I	250 × 4.6	5	100	400	17.8
Grace Smart RP-18	II	250 × 4.6	5	100	220	10.0
Ultra II Aqueous C18	III	250 × 4.6	5	100	290	15.0