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MINI-REVIEW

D-amino acids in foods

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Abstract

With the only exception of glycine, all amino acids exist in two specular structures which are mirror images of each other, called D-(dextro) and L-(levo) enantiomers. During evolution, L-amino acids were preferred for protein synthesis and main metabolism; however, the D-amino acids (D-AAs) acquired different and specific functions in different organisms (from playing a structural role in the peptidoglycan of the bacterial cell wall to modulating neurotransmission in mammalian brain). With the advent of sophisticated and sensitive analytical techniques, it was established during the past few decades that many foods contain considerable amounts of D-AAs: we consume more than 100 mg of D-AAs every day. D-AAs are present in a variety of foodstuffs, where they fulfill a relevant role in producing differences in taste and flavor and in their antimicrobial and antiaging properties from the corresponding L-enantiomers. In this review, we report on the derivation of D-AAs in foods, mainly originating from the starting materials, fermentation processes, racemization during food processing, or contamination. We then focus on leading-edge methods to identify and quantify D-AAs in foods. Finally, current knowledge concerning the effect of D-AAs on the nutritional state and human health is summarized, highlighting some positive and negative effects. Notwithstanding recent progress in D-AA research, the relationships between presence and nutritional value of D-AAs in foods represent a main scientific issue with interesting economic impact in the near future.

Keywords D-amino acids · Fermented food · Food contamination · Food processing · Detection of D-amino acids

Introduction

Amino acids have an α-carbon connected to four functional groups: an amine group, a carboxyl group, a hydrogen group, and a side chain. The α-carbon is a stereocenter (or chiral center) of the molecule since, depending on the spatial arrangement of these four different groups, two stereoisomers exist that are mirror images of each other (Fig. 1): levorotatory (L) and dextrorotatory (D). It is likely that more than one physical-chemical event in the primordial environment resulted in the generation of homochirality, favoring L-amino acids (L-AAs): natural proteins are built from L-AAs. Indeed, the D-amino acids (D-AAs) were identified in the past 30 years as natural biomolecules that play interesting and specific roles and that are components of our diet. Interestingly, D-AAs are formed during food processing and originate from microbial sources and from aqueous, soil, and other environments. D-AAs (mainly D-Ala and D-Glu) are key components of the peptidoglycan (PG) in the bacterial cell wall. PG synthesis is carried out in cytosol and periplasm/extra-cytoplasm compartments by a number of enzymes. In most bacteria, racemases convert L-Ala and L-Asp into the corresponding D-enantiomer; a lyase generates the D-alanyl-D-alanine dipeptide; MurA-MurE enzymes generate UDP-muramyl-L-Ala-γ-D-Glu-*meso*-diaminopimelate, which is then linked to D-Ala-D-Ala to give UDP-MurNAc-pentapeptide and an additional enzyme allows its binding to lipid I yielding lipid II, the lipid-disaccharylpentapeptide. On the extracellular compartment, D,D-transpeptidases link the D-Ala residues at position 4 in one peptide to the *meso*-diaminopimelate portion of a second peptide. PG is a dynamic structure: periplasmic enzymes edit peptidoglycan by introducing further D-AAs during stationary phase, principally into the terminus of the peptide moiety of muropeptides. For a review, see Cava et al. (2011), Horcajo et al. (2012), and Pidgeon et al. (2015).

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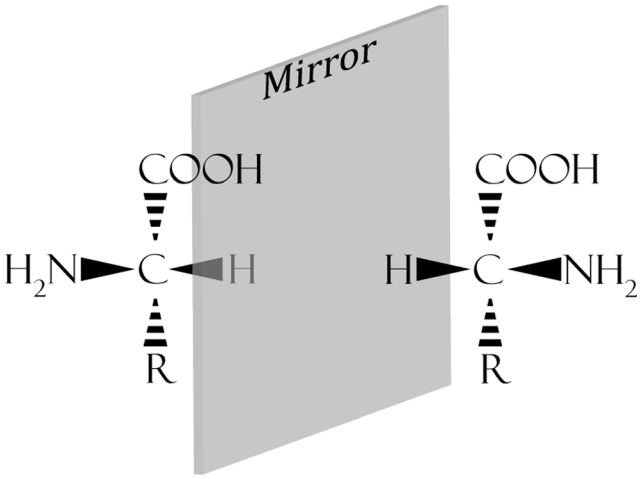

Fig. 1 The L- and the D-enantiomer of a general amino acid (R = side chain)

D-AAs make the cell wall resistant to most proteases and the presence of alternative D-AAs (i.e., D-Asp or D-Ser) at the terminal position of the stem peptide provides resistance to some antibiotics, such as vancomycin (De Jonge et al. 2002; Reynolds and Courvalin 2005; Marcone and Marinelli 2014). The bacterial flora of the human body and infections constitute an additional permanent, endogenous source of D-AAs.

In mammals, absorption does not constitute a main issue in digesting D-AAs since amino acid transporters have a relatively broad specificity, whereby the preference for D-enantiomer transport is lower than for L-AAs. Two pathways are available for the biological utilization of D-AAs: racemases or epimerases convert D-AAs directly to L-enantiomer; degrading enzymes such as D-amino acid oxidase (DAAO) and D-aspartate oxidase (DASPO or DDO) catalyze the oxidative deamination of D-AAs into α-keto acids (Pollegioni et al. 2007; Pollegioni and Sacchi 2010; Sacchi et al. 2012), which can then be specifically aminated to the L-enantiomer (Brückner and Fujii 2010). In mammals, conversion by the oxidases predominates over the racemases (which occur primarily in bacteria). In the organism, D-AAs are excreted in the urine: the transformation takes place principally in the kidneys.

D-AAs are common constituents of our diet. Grocery stores are selling increasing quantities of foods (fruit juices and pulp, cereals, potatoes, tomato sauces, milk, etc.), which in some cases contain substantial quantities of D-AAs (Csapó et al. 2009). Indeed, during food processing, which is usually done to improve flavor, consistency, or nonperishability, the L-AAs may be racemized to their D-enantiomers (Masters and Friedman 1979; Friedman et al. 1981). D-AAs can also be generated as a consequence of adulteration, as for hydrolyzed proteins added to foodstuffs to hide low nutrient content. Notably, more than half of all dairy products and fermented foods contain D-Ala, D-Asp, and D-Glu. Every day, more than 100 mg D-AAs are likely ingested: for example, consumption of 100 g Emmental cheese corresponds to an intake of 70–80 mg D-AAs and 100 mL of instant coffee to one of > 20 mg.

D-amino acids in foods

In recent years, it has been shown that, in addition to L-AAs normally present in food proteins, foods can also contain D-AAs. D-AAs are found in a free or bound state, in a wide variety of foods and beverages, either naturally (such as in molluscs or in fermented food) (see section "D-amino acids in fermented foods") or are formed artificially (during food processing or food adulteration) (see section "D-amino acids due to technological processes"). D-AAs have properties that differ from those of L-enantiomers, in terms of taste, flavor, and antimicrobial or anti-aging properties (Mutaguchi et al. 2016). Up to now, little is known about the role of D-AAs in foodstuffs; this is also because it is complicated to detect D-AAs in amino acid racemic mixtures. For this reason, an increasing number of studies have focused on methods to identify and quantify D-AAs in food (see section "Assay of D-amino acids in foods").

D-AAs are naturally present in some plants, fruits, and vegetables (Brückner and Westhauser 2003). The natural amounts of D-AAs found in fruits and vegetables are usually lower than 3.4% and 0.7%, respectively. The highest amount of individual D-AA found was 3.4% D-Asn and 1.9% D-Asp in grapefruit, 2.7% D-Ala and 1.7% D-Ser in apples, and 1.3% D-Glu in clementines. Apples (Golden Delicious) contain 0.8 μmol/kg of *N*-malonyl-D-tryptophan (Brückner and Westhauser 1994). In commercially available fruit juices, high amounts of D-Ala (10–42%) can be found as result of bacterial contamination [see section "D-amino acids as a markers of contamination (in unfermented foods)"].

The natural occurrence of D-AAs in higher plants in a free or conjugated form has been widely debated. Such studies highlighted at least three ways that may have contributed to the development of D-AAs in plants, including vegetables and fruits. The first process establishes the involvement of enzymes in the de novo synthesis of free and conjugated D-AAs via different metabolic pathways (Fukuda et al. 1973; Ogawa and Fukuda 1973; Kawasaki et al. 1982; Rekoslavskaya et al. 1999; Rozan et al. 2000). The second, demonstrated by Brückner and coworkers, presupposes that D-AAs were nonenzymatically formed by the reaction of L-AAs and reactive carbonyl compounds (Brückner et al. 2001). The third assumes that D-AAs originate from exogenous sources such as bacteria (i.e., principally due to mycorhizal associations between the roots of plants and fungi), which offer an abundant reservoir of D-AAs in the peptidoglycan-bound form as well as free molecules (Schleifer and Kandler

Table 1 Distribution of D-AAs in foods. The reported percentage of D-AA is relative to the total amino acid amounts

Food	D-AAs	Relative amount (%)	Analytical method	Reference
Milk and milk powder				
Raw milk	D-Asp	2–3	GC	Palla et al. 1989
	D-Glu	2–3		
	D-Ala	3–4		
UTH-milk	D-Asp	2–3	GC	Palla et al. 1989
	D-Glu	3–5		
	D-Ala	4–6		
Infant formula	D-Asp	10–12	GC	Palla et al. 1989
	D-Glu	3–5		
	D-Ala	2–3		
Milk powder	D-Asp	4–5	GC	Palla et al. 1989
	D-Glu	3–5		
	D-Ala	8–12		
Dairy/cheese				
Yogurt	D-Asp	20.9	GC	Brückner and Hausch 1990
	D-Glu	12.4		
	D-Ala	61.3		
Yakult	D-Glu	24.2	HPLC	Jin et al. 1999
	D-Asp	40.3		
	D-Ala	53.8		
Kefir	D-Asp	17.6	GC	Brückner and Hausch 1989
	D-Glu	4.9		
	D-Ala	37.4		
	D-Leu	22.6		
	D-Val	5.6		
	D-Lys	4.7		
	D-Ser	3.4		
Sour milk	D-Asp	14.1	GC	Brückner and Hausch 1990
	D-Glu	4.0		
	D-Ala	38.6		
	D-Leu	18.4		
	D-Val	6.8		
	D-Lys	3.1		
Gorgonzola	D-Glu	1.5	GC	Brückner and Hausch 1989
	D-Ala	33.2		
	D-Leu	0.5		
Emmentaler	D-Asp	2.9	GC	Brückner and Hausch 1989
	D-Glu	6.2		
	D-Ala	42.2		
	D-Leu	1.0		
	D-Pro	14.7		
	D-Lys	1.8		
Parmigiano Reggiano (24 months ripened)	D-Asp	27	GC	Marchelli et al. 2007
	D-Glu	15		
	D-Pro	32		
	D-Ala	26		
Vegetables				
Red cabbage	D-Ala	2.5	GC	Brückner and Westhauser 1994
	D-Asn	1.5		
	D-Val	1.0		
	D-Glu	0.3		
Green cabbage	D-Ala	3.8	GC	Brückner and Westhauser 1994
	D-Asn	0.9		
	D-Leu	1.0		
	D-Glu	0.4		
Pickled cabbage	D-Ala	9.4	GC	Brückner and Westhauser 1994
	D-Asn	3.7		
	D-Glu	11.0		
	D-Leu	5.2		
	D-Lys	8.7		
Tomato	D-Asp	0.2	GC	Brückner and Westhauser 1994

Table 1 (continued)

Food	D-AAs	Relative amount (%)	Analytical method	Reference
	D-Glu	0.1		
	D-Asn	0.2		
	D-Ala	0.7		
Carrot	D-Asp	0.2	GC	Brückner and Westhauser 1994
	D-Ser	0.1		
	D-Asn	0.1		
	D-Ala	0.6		
Garlic	D-Ala	1.3	GC	Brückner and Westhauser 1994
	D-Asn	0.8		
	D-Glu	0.5		
	D-Leu	0.6		
	D-Val	1.4		
Vegetable juice				
Carrot	D-Asp	0.9	GC	Brückner and Hausch 1989
	D-Phe	4.9		
	D-Glu	5.0		
	D-Ala	13.5		
	D-Leu	13.8		
	D-Val	8.2		
Tomato	D-Glu	1.9	GC	Brückner and Westhauser 1994
Red beet	D-Asp	1.3	GC	Brückner and Hausch 1989
	D-Glu	1.0		
	D-Ala	8.2		
	D-Leu	5.5		
	D-Val	1.9		
Celery	D-Asp	0.6	GC	Brückner and Hausch 1989
	D-Glu	0.8		
	D-Ala	22.1		
Fruits				
Apple	D-Ala	2.7	GC	Brückner and Westhauser 1994
	D-Ser	1.7		
	D-Asn	0.7		
	D-Glu	0.5		
	D-Asp	0.4		
Pineapple	D-Leu	2.4	GC	Brückner and Westhauser 1994
	D-Val	1.1		
	D-Asp	0.9		
	D-Asn	0.8		
	D-Glu	0.4		
Papaya	D-Asp	1.4	GC	Brückner and Westhauser 1994
	D-Ala	0.9		
	D-Arg	0.9		
	D-Ser	0.8		
	D-Asn	0.7		
	D-Gln	0.4		
Mango	D-Asp	1.5	GC	Brückner and Westhauser 1994
	D-Ala	0.4		
	D-Glu	0.4		
	D-Arg	0.3		
	D-Gln	0.3		
Clementine	D-Asp	1.0	GC	Brückner and Westhauser 1994
	D-Asn	0.5		
	D-Glu	1.3		
	D-Ala	0.8		
	D-Arg	0.8		
Orange	D-Asp	0.4	GC	Brückner and Westhauser 1994
	D-Asn	0.5		
	D-Glu	1.2		
	D-Ala	1.3		
	D-Arg	0.4		
Pear	D-Asp	0.5	GC	Brückner and Westhauser 1994

Table 1 (continued)

Food	D-AAs	Relative amount (%)	Analytical method	Reference
	D-Asn	0.4		
	D-Glu	0.9		
	D-Ala	2.1		
	D-Ser	1.1		
Lemon	D-Asp	0.5	GC	Brückner and Westhauser 1994
	D-Asn	0.7		
	D-Glu	1.1		
	D-Ala	0.9		
	D-Ser	0.4		
Passion fruit	D-Ala	1.2	GC	Brückner and Westhauser 1994
	D-Arg	0.8		
Grapefruit	D-Asn	3.4	GC	Brückner and Westhauser 1994
	D-Asp	1.9		
Water melon	D-Ala	0.6	GC	Brückner and Westhauser 1994
	D-Asp	0.5		
	D-Arg	0.4		
	D-Gln	0.2		
Fruit juice				
Apple	D-Ala	17	GC	Brückner and Westhauser 1994
Grapefruit	D-Ala	22–33	GC	Brückner and Westhauser 1994
Pineapple	D-Ala	42	GC	Brückner and Westhauser 1994
Orange	D-Ala	21	GC	Brückner and Westhauser 1994
Pears	D-Ala	11	GC	Brückner and Westhauser 1994
Alcoholic beverages				
Wheat beer	D-Asp	36	GC	Brückner and Hausch 1989
	D-Phe	0.6		
	D-Glu	16.2		
	D-Ala	13.2		
Beer	D-Asp	3.6	GC	Brückner and Hausch 1989
	D-Glu	1.9		
	D-Ala	1.1		
White wine	D-Asp	2	GC	Brückner and Hausch 1989
	D-Glu	0.7		
	D-Ala	1.2		
	D-Leu	0.5		
Red wine	D-Asp	4.1	GC	Brückner and Hausch 1989
	D-Glu	3.0		
	D-Ala	3.3		
	D-Pro (starting from 5-years old wines)	10		
Sake	D-Asp	1.7	GC	Brückner and Hausch 1989
	D-Glu	1.1		
	D-Ala	0.6		
Vinegar				
Rice	D-Asp	2.7	UPLC	Mutaguchi et al. 2013
	D-Ala	2.3		
Apple	D-Asp	3.5	UPLC	Mutaguchi et al. 2013
	D-Ala	13.4		
	D-Glu	2		
	D-Asn	13.1		
White wine	D-Ala	3.3	UPLC	Mutaguchi et al. 2013
Tomato	D-Asp	0.4	UPLC	Mutaguchi et al. 2013
	D-Ala	0.3		
	D-Glu	0.2		
	D-Asn	0.5		
Balsamic	D-Asp	5.7	UPLC	Mutaguchi et al. 2013
	D-Ala	3.4		
	D-Glu	2.4		
	D-Arg	0.7		
Coffee				

Table 1 (continued)

Food	D-AAs	Relative amount (%)	Analytical method	Reference
Instant	D-Asp	43	GC	Brückner and Hausch 1989
	D-Glu	27.4		
	D-Pro	14.4		
Roasted	D-Asp	23–38	GC	Palla et al. 1989
	D-Glu	32–41		
	D-Phe	9–12		
Green	D-Glu	< 0.2	GC	Palla et al. 1989
Ham/meat				
Raw beef	D-Asp	6.2	GC	Bunjapamai et al. 1982
	D-Phe	2.8		
	D-Ala	3.2		
	D-Leu	3.1		
	D-Val	1.6		
Roasted hamburger	D-Asp	5.5	GC	Bunjapamai et al. 1982
	D-Phe	2.7		
	D-Ala	2.8		
	D-Leu	3.2		
	D-Val	1.5		
Cooked chicken muscle	D-Asp	22.4	GC	Liardon and Hurrel 1983
	D-Ala	0.5		
	D-Phe	0.4		
	D-Leu	0.1		
Raw chicken	D-Asp	2.9–4.4	GC	Bunjapamai et al. 1982
	D-Phe	2.5		
	D-Ala	1.9		
	D-Leu	2.5		
	D-Glu	2.7		
	D-Pro	1.5		
Cooked bacon	D-Asp	10.7	GC	Liardon and Hurrel 1983
	D-Ala	2.4		
	D-Phe	3.1		
	D-Leu	3.1		
	D-Val	1.6		
Unheated bacon	D-Asp	2.4	GC	Liardon and Hurrel 1983
	D-Phe	1.8		
	D-Leu	3.3		
	D-Val	0.7		
Fish				
Raw mackerel	D-Ala	5.2	HPLC	Opstvedt et al. 1984
	D-Asp	8.0		
	D-Glu	12.9		
Cooked mackerel (95 °C)	D-Ala	6.2	HPLC	Opstvedt et al. 1984
	D-Asp	10.0		
	D-Glu	14.7		
Raw pollock	D-Ala	6.0	HPLC	Opstvedt et al. 1984
	D-Arg	5.4		
	D-Glu	15.6		
Cooked pollock (95 °C)	D-Ala	6.2	HPLC	Opstvedt et al. 1984
	D-Arg	6.0		
	D-Glu	16.5		
Flour-based products				
Extruded soy flour	D-Asp	7.6	GC	Bunjapamai et al. 1982
	D-Ala	2.2		
	D-Phe	2.4		
	D-Leu	2.7		
	D-Val	0.8		
Soy flour	D-Asp	4.4	GC	Bunjapamai et al. 1982
	D-Ala	2.5		
	D-Phe	2.8		
	D-Leu	1.4		
	D-Val	1.0		

Table 1 (continued)

Food	D-AAs	Relative amount (%)	Analytical method	Reference
Treated soy bean protein	D-Asp	27.7	GC	Bunjapamai et al. 1982
	D-Ala	9.9		
	D-Phe	19.7		
	D-Leu	3.1		
	D-Val	1.0		
	D-Met	18.2		
Untreated soy bean protein	D-Asp	0.5	GC	Bunjapamai et al. 1982
	D-Ala	0.2		
	D-Phe	0.5		
	D-Leu	0.2		
	D-Val	0.03		
	D-Met	0.3		
Taco shells	D-Asp	5.8	GC	Bunjapamai et al. 1982
	D-Ala	3.2		
	D-Leu	3.8		
	D-Val	1.5		
	D-Met	1.1		
Corn meal	D-Asp	5.2	GC	Bunjapamai et al. 1982
	D-Ala	2.3		
	D-Leu	2.5		
	D-Val	1.3		
	D-Met	2.4		
Treated corn protein (zein)	D-Asp	40.2	GC	Friedman and Liardon 1985
	D-Ala	17.6		
	D-Phe	31.3		
	D-Leu	5.0		
	D-Val	2.9		
	D-Met	19.5		
Unheated corn protein	D-Asp	3.4	GC	Friedman and Liardon 1985
	D-Ala	0.7		
	D-Phe	2.2		
	D-Leu	0.7		
	D-Val	0.4		
	D-Met	0.9		
Toast	D-Asp	10.5	GC	Bunjapamai et al. 1982
	D-Phe	2.4		
	D-Ala	2.8		
	D-Leu	2.7		
	D-Val	1.1		
Untreated toast (bread)	D-Asp	5.6	GC	Bunjapamai et al. 1982
	D-Phe	2.3		
	D-Ala	2.4		
	D-Leu	3.2		
	D-Val	0.9		
Other products				
Fermented black beans	D-Asp	7.8	GC	Brückner and Hausch 1989
	D-Ala	10.6		
	D-Pro	4.9		
Liquid spice	D-Asp	6.9	GC	Brückner and Hausch 1989
	D-Phe	3.7		
	D-Glu	3.0		
	D-Ala	2.7		
	D-Leu	10.0		
	D-Val	1.5		
	D-Pro	2.0		
	D-Ser	1.4		
Peanut butter	D-Ala	3.1	GC	Bunjapamai et al. 1982
	D-Leu	2.2		
	D-Pro	1.5		
	D-Met	2.0		
	D-Glu	3.7		
Raw peanuts	D-Ala	2.8	GC	

Table 1 (continued)

Food	D-AAs	Relative amount (%)	Analytical method	Reference
	D-Leu	2.4		Bunjapamai et al. 1982
	D-Pro	1.7		
	D-Met	2.8		
	D-Glu	4.3		
Heated casein	D-Asp	31	GC	Hayase et al. 1973
	D-Ala	12		
	D-Leu	7.0		
	D-Val	4.4		
Unheated casein	D-Asp	3.1	GC	Hayase et al. 1973
	D-Ala	1.5		
	D-Leu	1.9		
Honey (from mixed flowers)	D-Pro	0.2	HPLC	Pawlowska and Armstrong 1994
	D-Phe	3.1		
	D-Leu	3.6		

1972; Brückner et al. 1993). In this latter case, plants readily take up microbial D-AAs (Aldag et al. 1971).

For a list of foods containing D-AAs, see Table 1.

Origin of D-amino acids

D-amino acids in fermented foods

The presence of D-AAs can be considered "natural" in fermented foods. D-AA formation in fermented products can arise from the starting materials or from microbial activity during fermentation. Different fermentation processes are involved in the production of fermented foods (i.e., alcoholic, acetic, and lactic fermentation), and each process could make a different contribution to the formation of D-AAs. A study on D-AAs in fermented foodstuffs produced from the same lot of materials stated that lactic fermentation represents the main means by which D-AAs are generated. For example, lactic fermentation is mainly responsible for producing high levels of D-Asp, D-Ala, and D-Glu in tomatoes and vinegar (Mutaguchi et al. 2013), in kefir, yogurt, curdled milk, and goat fresh cheese, soy sauce, etc., and in particular in beer, wine, and sake (Brückner and Hausch 1990; Erbe and Brückner 2000; Gogami et al. 2011). The behaviors of metabolites such as organic acids and amino acids formed during fermentation are used as indicators of progress during the fermentation process.

D-AAs such as D-Ala, D-Glu, D-Asp, D-Leu, and D-Ile are present in wine (Brückner and Hausch 1989; Kato et al. 1995; Jin et al. 1999) and might affect its flavor (i.e., D-Ala is known to have a sweet taste) (see section "D-amino acid taste") (Schiffman et al. 1981). In some cases, the amount of D-AAs seems to be due to the presence of particular bacterial species: in red and white wine fermentation, an increase in amounts of D-Ala, D-Glu, and D-Lys correlates well with the presence of Gram-positive *Oenococcus oeni* (Kato et al. 2011), a beneficial bacterium as it performs malolactic fermentation whose end products are responsible for specific flavors. In the past few years, the presence of D-Pro in wine was used as an indicator for age dating (Chiavaro et al. 1998), but more recently, others found no correlation between aging and D-AAs content in wines (Ali et al. 2010). Instead, this study suggested that D-AA formation mainly depends on the bacteria employed for the fermentation: alterations in D-AA composition during fermentation are of great interest for winemakers, especially when related to commercial value and sensory qualities.

In some cases, fermentation is necessary to make a raw material more workable or to acquire characteristics fundamental for an excellent product. For example, sourdough fermentation makes the flour suitable for baking, controls the development of flour components, and inhibits fermentation by undesired bacteria or yeasts. The use of lactic acid bacteria and yeasts in sourdough fermentation before baking produces free D-Ala and D-Glu in the dough (Gobbetti et al. 1994). Large amounts of D-Ala, D-Asp, and D-Glu are also present in well-aged cheeses such as Parmigiano Reggiano and Grana Padano (up to 5 g/kg): the D-AA content varies among cheeses and during their production and ripening (Table 1) (Marchelli et al. 1997; Innocente and Palla 1999; Marchelli et al. 2007).

D-amino acids due to technological processes

The food industry is producing increasing quantities of foods (baked potatoes, fruit juices and fruit pulp, breakfast cereals, tomato sauces, milk, etc.) that can contain substantial quantities of D-AAs. In these foods, the racemization process is mainly responsible for the formation of D-AAs. Although it has been demonstrated that a detectable racemization could appear also at low storage temperature (25 °C) in selected

foods, such as Korean kimchi (Taniguchi et al. 2017), the principal factors influencing racemization are usually alkaline or acid pH values, treatment duration, heat treatment, and duration of heating (Palla et al. 1989; Genchi 2017).

The amino acid racemization process can be promoted in the course of preparation of foods that normally contain low quantities of D-AAs, such as milk, meat, and some fruit juices. For example, D-AAs level does not increase in samples following heat treatments at a high temperature for a short time (such as pasteurization and ultra-high temperature processes), while amino acid racemization is apparent in sterilized or powdered milk (Table 1). In those cases, the technological process increased D-Ala level from 3 to 4% (the reference concentration) up to 12% (Gandolfi et al. 1992). In commercially available milk, an increased quantity of D-AAs might represent a marker of inflammatory disease of the cows producing it. Actually, substantial quantities of D-Asp, D-Glu, D-Ala, and D-*allo*-isoleucine (D-*allo*-Ile) are present in samples derived from cows with mastitis, and a positive correlation was observed between the D-AA increase and disease severity (Csapò et al. 1995) (see also section "D-amino acids as markers of contamination").

A recent study concerning protein modification in infant milk formula reported significant D-AAs levels as a consequence of the thermal treatment required for the safety and prolonged shelf life of the products. The analyzed amino acids showed different values of racemization: D-Arg, the most abundant, increased up to 32% of the total amount of Arg (Chen et al. 2019).

D-Met can be chemically modified during food processing and L-Phe and L-Tyr can rapidly racemize into the corresponding D-enantiomer upon alkali or heat treatment (Csapò et al. 2008). The effect of temperature on the D-Ser and D-Thr content of soybean protein was reported by Friedman (1999). Effects of alkali treatment and high temperature on racemization have also been reported in commercial, ripe olives: after heating, D-Ser and D-Ala showed the highest racemization values, reaching 20% and 11%, respectively. In addition, D-Ser, D-Ala, and D-Asn levels increased at high pH (Casado et al. 2007). An interesting study conducted on different food wastes from the agrifood industry reported that a D-Asp increase could be mainly related to the harshness of the process (pH and temperature stress condition) and the percentage of D-Ala might more likely be due to the fermentation process, while D-Glu could be related to both conditions (Prandi et al. 2019). Moreover, elevated temperatures are responsible for a significant increase in D-AAs in honey: samples subjected to high temperatures possessed a greater quantity of D-AAs, where values increased proportionally to the temperature. An increase in D-AA content following roasting and alkaline treatment was also observed in cocoa beans (Pätzold and Brückner 2006). Other processing conditions might also influence D-AA production, such as the percentage of sodium chloride in fermented fish sauce. A study conducted on 60 fish sauce samples, divided into 10%- and 20%-salt preparations, reported that a higher salt concentration could hamper D-AA production (Abe et al. 1999).

Today, the food industry is mindful of the possible risks of treating proteinaceous food harshly: actually, new biotechnological processes are increasingly being adopted to avoid drastic reaction conditions, preferring the use of enzymes working at neutral pH, e.g., soy protein hydrolysates are produced enzymatically nowadays. In some cases, however, such as making gelatins or recovering proteins from cereals, milling by-products or oilseeds, drastic conditions are necessary (Lüpke and Brückner 1998; Seo et al. 2008). Alkali treatments are employed to give foods a special texture (e.g., tofu), to peel fruits and vegetables, and to prepare canned, dried, or frozen fish (Friedman 1999).

Altogether, the presence of D-AAs in foods can be used as a biomarker for excessive thermal and alkaline treatments and of inflammatory disease affecting the animals producing the raw materials. It can also be useful to evaluate protein quality of food wastes generated from the agrifood industry in view of a possible re-use.

D-amino acids as markers of contamination (in unfermented foods)

Dairy products have been reported to contain amounts of D-Ala, D-Asp, and D-Glu (1–3 mg/L) that are generally attributed to bacterial activity rather than processing technologies (Gandolfi et al. 1992): no other D-AAs were detected up to 0.01 ppm. A 3–4% D/(D + L) ratio of the three free D-AAs was detected, a value that could be considered physiological.

The D-Ala content of raw milk samples increases after storage at 4 °C for 1 month: the D/(D + L) ratio reached 50–55% with a D-Ala content of 3–5 mg/L. The increase in D-Ala levels was related to the presence of psychrotrophic bacteria (Gandolfi et al. 1992). Since the amount of D-Ala did not increase from milk treatment (see above), D-Ala can be considered as an indicator of bacterial milk contamination. Actually, microbial contamination contributes to the free D-AA content because of microbial racemase enzymes and cell wall lysis. The D-AA levels were quantified in cow, sheep, and goat milk (Albertini et al. 1996): a higher amount of D-Ala, D-Asp, and D-Lys was detected in goat than in cow and sheep milk samples. Analyses of goat milk detected a ratio of D/(D + L) Ala in the 3.5–36.7% range in comparison to a 1.2–4.5% and 1.2–6.5% range for cow and sheep milk samples, respectively (Albertini et al. 1996). These results can be attributed to a higher microbial contamination of the goat milk, due to less controlled storage conditions.

Significant amounts of D-Ala were also found in fruit juices (Gandolfi et al. 1994). Grapefruit juices stored for 40 days showed an increase in the D-Ala content up to 5–

Table 2 HPLC methods used to detect D-AAs in foodstuffs

Derivatization agent (pre- or post-column)	Column	Detected D-AAs	Foodstuff	Detection limit	Reference
(a) Indirect chiral methods					
OPA-NAC (pre)	Chromasil C-18	Ala, Leu, Ser, Thr, Trp, Tyr, Val	Milk, oyster	LOD = 0.02–0.17 ng	Rubio-Barroso et al. 2006
OPA-NAC (pre) FLEC-ADAM (pre)	Develosil ODS-UG-5 Develosil ODS-UG-5	Ala, Asp, Glu, Gly, Ile, Leu, Phe, Ser, Thr, Trp, Val Arg, Asn, Gln, His, Lys, Pro	Sake	LOQ = 0.006–20 μM LOQ = 0.01–20 μM	Gogami et al. 2011
OPA-NAC (post)	Shim-pac amino-Na	Cys		LOQ = 1.5 μM	
DBD-PyNCS (pre)	Wakosil-II-3C18RS	Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	Milk, cream, fermented dairy products, fermented beverages	LOD = 0.16–0.75 pmol	Jin et al. 1999
NBD-F (pre)	Monolithic ODS; Sumichiral OA-2500 (or 3200)	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	Black vinegars		Miyoshi et al. 2014
L-PGA-OSu (pre)	ACQUITY UPLC BEH C18	Ala, Ile, Leu, Met, Phe, Pro, Trp, Tyr, Val	Yogurt	LOD = 0.5–3.2 fmol	Mochizuki et al. 2014
NBD-F (pre)	ODS Ascentis Express C18	Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	Black vinegars, fermented milk drinks, yogurt	LOD = 11–64 pmol	Eto et al. 2011
(S)-NIFE (pre)	ACQUITY UPLC BEH C18	Ala, Arg, Asn, Asp, Cys, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	Milk	LOD = 0.01–6.14 ng/mL	Tian et al. 2017
(R)-BiAC (pre)	Triart Phenyl	Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, Cys, Gly	Black vinegar, lactic acid bacteria beverage	LOD = 7–127 amol	Harada et al. 2019
(b) Direct chiral methods					
/	CROWNPAK CR-I(+) and CR-I(−)	Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, Val	Milk, vinegar	LOD = 1–40 nmol/mL	Konya et al. 2017
/	CROWNPAK CR-I(+) and CR-I(−)	Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, Val	Vinegar	LOD = 0.005–0.5 nmol/mL	Nakano et al. 2017

Table 3 Overview of different analytical methods to detect D-AAs in foods

Analytical method	Reference	Sample preparation	Detector	Time of analysis	Detection limit	Operational cost[a]
HPLC	Rubio-Barroso et al. 2006	OPA-NAC derivatization (pre-column)	Fluorescence	25 min	LOD = 0.02–0.17 ng (approx. 0.15–1.3 pmol)	$/$$$
	Gogami et al. 2011	OPA-NAC derivatization (pre-column)	Fluorescence	90 min	LOQ = 0.006–20 μM	
		FLEC-ADAM derivatization (pre-column)		60 min	LOQ = 0.01–20 μM	
		OPA-NAC derivatization (post-column)		20 min	LOQ = 1.5 μM	
	Jin et al. 1999	DBD-PyNCS derivatization (pre-column)	Fluorescence	80 min	LOD = 0.16–0.75 pmol	
2D-HPLC	Miyoshi et al. 2014	NBD-F derivatization (pre-column)	Fluorescence	20 min	–	$$$
UPLC	Mochizuki et al. 2014	L-PGA-OSu derivatization (pre-column)	ESI-MS/MS	12 min	LOD = 0.5–3.2 fmol	$$$
	Eto et al. 2011	NBD-F derivatization (pre-column)	Circular dichroism	10 min	LOD = 11–64 pmol	
	Tian et al. 2017	(S)-NIFE derivatization (pre-column)	IM-HRMS	24 min	LOD = 0.01–6.14 ng/mL (approx. 0.08–48 pmol)	
LC-MS	Harada et al. 2019	(R)-BiAC derivatization (pre-column)	MS/MS	11.5 min	LOD = 7–127 amol	$$
	Konya et al. 2017	Dissolved in the mobile phase	TOFMS	10 min	LOD = 1–40 nmol/mL	
	Nakano et al. 2017	Dissolved in the mobile phase	MS/MS	8 min	LOD = 0.005–0.5 nmol/mL	
GC	Brückner and Haush 1989; Brückner and Haush 1990; Brückner and Westhauser 1994; Patzold and Brückner 2006	Conversion into volatile *N(O)*-pentafluoropropionyl 2-propyl esters	FID	40 min	–	$$$
	Ali et al. 2010		MS	40 min	–	
	Erbe and Brückner 2000	Conversion into volatile *N(O)*-pentafluoropropionyl 2-propyl esters or *N(O)*-trifluoroacetyl 1-propyl esters	FID MS	70 min	LOQ = 0.57–1.49 mg/L (approx. 4.5–12 μM) LOQ = 0.06–0.39 mg/L (approx. 0.5–3 μM)	
CE	Martinez-Giron et al. 2009	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization	Diode array	20 min	LOD = 6.4–8.2 μM LOQ = 21–25 μM	$$
	Herrero et al. 2007	FITC derivatization	LIF	25 min	LOD = 160–790 nM LOQ = 0.53–2.6 μM	
	Carlavilla et al. 2006			20 min	LOD = 16.6–45.7 nM	
	Simó et al. 2004			18 min	–	
	Giuffrida et al. 2009		TOFMS	19 min	LOD = 0.16–1.87 μM	
Biosensors	Sacchi et al. 1998	Directly added into sensor system	Amperometric	15 min	LOD = 0.15 mM	$
	Varadi et al. 1999			1 min	LOD = 0.1–0.2 mM	
	Inaba et al. 2003			12 min	LOD = 0.05 mM	
	Sarkar et al. 1999			4 min	LOD = 0.15–0.47 mM	
	Weislo et al. 2007			10 min	LOD = 1–30 μM	
	Lata et al. 2012			3 s	LOD =1 μM	
	Lata et al. 2012			2 s	LOD = 0.2 μM LOQ = 0.03 μM	
	Rosini et al. 2008			15 min	LOD = 0.25 mM	

[a] Arbitrary scale based on the cost of sample preparation and analysis

20 mg/L and a bacterial contamination corresponding to a concentration > 10^7 CFU/mL. As already found in milk samples (Gandolfi et al. 1992), the D-Ala content in fruit juices did not increase with thermal treatments (i.e., pasteurization or sterilization processes) or during the products' shelf life.

Assay of D-amino acids in foods

Detection of D-AAs in food provides valuable information about quality, authenticity, or microbial contamination. The amino acid assay consists of several steps: the release of the AAs from the food matrix, the separation of the individual AA, detection, and quantification. The established analytical techniques based on high-performance liquid chromatography (HPLC; see Table 2) and gas chromatography (GC) have recently been supplemented by a number of new methods, such as capillary electrophoresis (CE) and ultra-performance HPLC (UPLC) combined with novel derivatization reagents and different detectors, see Table 3. Various methods are continuously being developed that are driven by the need to improve speed of analysis, sensitivity, robustness, and reproducibility.

HPLC and UPLC methods

HPLC is the most frequently used separation technique in bioanalysis. Two main approaches can be followed: (i) an indirect method based on chiral derivatizing reagents to give diasteroisomers separated on an achiral, reversed-phase HPLC column (Buck and Krummen 1987; Brückner et al. 1995; Erbe and Brückner 2000), and (ii) a direct chiral method based on a chiral stationary phase, or achiral, derivatizing reagents followed by separation on a chiral stationary or mobile phase (Guillén-Casla et al. 2010; Konya et al. 2017; Nakano et al. 2017; Hamase et al. 2010). See Table 2.

Ninhydrin, phenyl isothiocyanate (PITC), and *o*-phthaldialdehyde (OPA) have been used most frequently for the pre- and post-column derivatization of AAs: OPA reacts with primary amines, giving unstable derivatives with Gly and Lys (Bidlingmeyer et al. 1984), and PITC reacts with both primary and secondary amino acids, giving unstable derivatives with Glu and Asp. To avoid these drawbacks, new derivatizing reagents have been developed in the past few years (Thippeswamy et al. 2006): the best solution is represented by a stable and very sensitive reagent that reacts rapidly and at room temperature. The indirect chiral method based on OPA-NAC reaction has been optimized to detect seven free D-AAs (D-Ser, D-Thr, D-Ala, D-Tyr, D-Val, D-Trp, and D-Leu) in milk and oyster samples in 25 min (Rubio-Barroso et al. 2006), the level of detection (LOD, defined as 3 times the signal-to-noise ratio) being between 0.02 and 0.17 ng (Table 2). A method that combined two pre-column derivatization reactions with OPA-NAC and (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC)/1-aminoadamantane (ADAM) (Einarsson et al. 1987) and one post-column derivatization reaction with OPA-NAC (Ishida et al. 1981) was used to detect and quantify D-AAs in 141 different sake samples (Gogami et al. 2011). In particular, Gly and D- and L-forms of Ala, Asp, Glu, Ile, Leu, Phe, Ser, Thr, Trp, and Val were detected by using the OPA-NAC pre-column derivatization method and separated by applying the gradient elution mode; Arg, Asn, Gln, His, Lys, and Pro were analyzed with the FLEC/ADAM pre-column derivatization method and separated by the gradient elution mode (with the exception of Pro separated by isocratic elution mode); Cys was detected by using the OPA-NAC post-column derivatization method (Table 2).

A number of chiral derivatizing reagents are reported in Ilisz et al. (2008). The use of 2,7-dimethyl-3,8-dinitrodipyrazolo[1,5-a:1′,5′-d]pyrazine-4,9-dione (Gioia et al. 2006) or 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde (Gatti et al. 2010), combined with a reversed-phase HPLC system, resulted in a detection limit range of 20–80 pmol and 3–11 pmol, respectively. Higher selectivity and sensibility were achieved using the fluorescent derivatization reagent 2-[2-(7H-dibenzo[a,g]carbazol-7-yl)-ethoxy] ethyl chloroformate: a LOD value of 0.19–1.17 fmol/μL was obtained in a HPLC-fluorimetric detection-tandem MS system (Li et al. 2011).

The fluorescent chiral reagents R(−)- and S(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazoles (DBD-PyNCS) react rapidly and quantitatively with both primary and secondary amino acids to give stable derivatives that can be detected with a high sensitivity. The resolution of 17 D,L-AAs was achieved by employing an isocratic (for hydrophilic AAs) or gradient elution (for hydrophobic AAs) method on a reversed-phase HPLC system (Jin et al. 1999). The detection limits were in the 0.16- to 0.75-pmol range (Table 2). The method was also applied to identify D-AAs in milk, cream, fermented dairy products, tomato products, and fermented beverages, obtaining a recovery of the internal standards of 92–94%.

A two-dimensional HPLC (2D-HPLC) method combining a pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) followed by a reversed-phase separation (by means of a microbore monolithic ODS column) and a chiral separation (by a narrow-bore enantioselective column), made it possible to detect the enantiomers of all proteinogenic amino acids as well as of *allo*-Thr and *allo*-Ile in different Japanese Kurozu vinegar samples (Miyoshi et al. 2014).

A highly sensitive detection method (detection sensitivity = 0.5–3.2 fmol, Table 2) involves using light and heavy L-pyroglutamic acid succinimidyl ester (L-PGA-OSu) reagents combined with a UPLC method and the detection by ESI-MS/MS (Mochizuki et al. 2014). The pairs of 9 amino acids were separated in a single chromatographic run, and this method

was applied to detect D-AAs in two yogurt samples (Mochizuki et al. 2014).

A high-throughput analytical method based on UPLC equipped with a circular dichroism (CD) detector was developed to detect the 20 proteinogenic AAs. Interestingly, rapid analysis was performed since the CD detector does not require separation of optical isomers to evaluate the enantiomeric ratio; the analysis time was within 5.5 min with a detection limit of 11–64 pmol/injection using a pre-column derivatization technique with NBD-F. The method was applied to assay AAs in Japanese black vinegars, fermented milk drinks, and yogurt samples (Eto et al. 2011).

A higher sensitivity can be achieved by combining pre-column derivatization methods with MS detection. A specific and sensible quantification of L- and D-AAs, excluding the interference of co-eluting isomers or matrix ions with identical *m/z*, was achieved by employing a UPLC method coupled to ion mobility high-resolution MS (IM-HRMS) (Tian et al. 2017). Indeed, 18 different chiral AAs were characterized in human, cow, yak, buffalo, goat, and camel milk. Using the (S)-NIFE derivatization method (Visser et al. 2011) to separate L- and D-AAs, the extraction recovery was in the 82–105% range and the LOD values from 0.01 ng/mL for methionine to 6.14 ng/mL for serine (Table 2) (Tian et al. 2017).

Axial chiral derivatizing reagents are of utmost relevance: recently, the complete chiral separation of 19 proteinogenic amino acids was achieved within 11.5 min with Rs > 1.9 using a new axial chiral reagent derived from 6,6′-dimethyl-2,2′-biphenyldiamine ((R)-BiAC) and a LC-MS/MS system (Harada et al. 2019). By employing the optimized method, D-AAs in black vinegar and in a lactic acid bacteria beverage were detected (Table 2).

Derivatization methods are time consuming and often the source of analytical bias when performed simultaneously on different AAs. Actually, real samples often contain more than 100-fold higher L-AA concentrations than the corresponding D-forms: a nonquantitative conversion of the analytes to their derivatives represents a possible source of analytical bias (Harada et al. 2019). Indeed, high temperatures and acidic conditions can result in amino acid racemization and can induce hydrolysis of the amide group of Asn and Gln to Asp and Glu (Waldhier et al. 2010). All these issues can be overcome using direct chiral methods. Here, the simultaneous detection of 18 chiral proteinogenic AAs, combining a chiral column and liquid chromatography time-of-flight mass spectrometry (LC-TOFMS), made it possible to obtain an excellent peak resolution without any derivatization steps (Konya et al. 2017). The analytical separation (run of 10 min) by means of the CROWNPAK CR-I(+) and CR-I(−) chiral columns achieved a LOD value ranging from 1 to 40 nmol/mL (Table 2). The method was validated using water, milk, and vinegar as matrices; the recovery rate of all D-AAs in milk and vinegar samples was 72–90%, the only exception being D,L-Pro (not separated). Using the same chiral columns, a highly sensitive and selective detection of trace amino acids was achieved by using a versatile and quantitative method based on multiple reaction monitoring (MRM) mode applied to tandem mass spectrometry analysis (MS/MS) (Nakano et al. 2017). The simultaneous detection of 18 D-AAs without a derivatization process was obtained; the LOD value ranged from 0.005 to 0.5 nmol/mL (Table 2). The method was used on three vinegar samples.

A comparison between performance, time, and cost of D-AAs analysis by HPLC-based techniques and alternative methods is reported in Table 3.

GC-MS and GC-FID methods

Gas chromatography (GC) was one of the first separation techniques used to detect amino acids. Similar to HPLC methods, direct and indirect chiral analyses can be performed, although direct analysis is the preferred method for D-AA assays. Usually, two different chiral stationary phases are used: the Chirasil-L-Val capillary column and cyclodextrin-based chiral stationary phases. Bruckner and coworkers widely used the capillary column to quantify D-AAs. Capillary GC equipped with a flame ionization detector (GC-FID) assayed free D-AAs in fermented and roasted cocoa beans, cocoa powder, chocolate, and cocoa shells. AAs were isolated using a Dowex cation exchanger, converted into volatile *N(O)*-pentafluoropropionyl amino acid 2-propyl esters, adding 1% antioxidant 2,6-di-tert-butyl-p-cresol, and analyzed on a Chirasil-L-Val column (Pätzold and Brückner 2006). The same method was used to detect free D-AAs in dairy products (kefir and Gorgonzola cheese), fermented sausages, and vegetable juices, alcoholic beverages (beer, white wine, and sake), milk and sour milk products, coffee, and fruits (Brückner and Hausch 1989; Brückner and Hausch 1990; Brückner and Westhauser 1994). D-AAs were determined in beers and raw materials on a GC instrument equipped with FID or a mass spectrometer (GC-MS) (Erbe and Brückner 2000): the limit of quantification (LOQ) values ranged from 0.57 to 1.49 mg/L and from 0.06 to 0.39 mg/L, using the FID and the MS detection system, respectively (Table 3). Worthy of note is that the same food samples were analyzed by applying the HPLC method, giving LOQ values from 0.04 to 0.68 mg/L. The RSD values ranged from 1.1% to 9.2% using FID to quantify D-AAs and 1.1–7.2% using the MS detector. The method based on GC-MS equipped with the silica capillary column Chirasil-L-Val was also used to detect L- and D-AAs in 26 wines, comprising white, red, and sparkling wines (Ali et al. 2010). The Arg, His, Cys, and Trp content could not be determined by employing derivatization chemistry, nor could Asn and Gln be determined as they are hydrolyzed to Asp and Glu under the acidic derivatization conditions used (Erbe and Brückner 2000).

Capillary electrophoresis method

Capillary electrophoresis (CE) is a powerful separation method that combines electromigration and chromatographic techniques. For derivatization, the chiral selectors most widely used are naphthalene dicarboxaldeyde (NDA), NBD-F, and FITC, combined with capillary zone electrophoresis or micellar electrokinetic chromatography (MEKC) separation analyses. The in-capillary derivatization procedure is fully automated and minimizes consumption of sample and derivatizing reagents. A fast in-capillary derivatization method with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate was successfully applied to determine the enantiomers of Arg, Lys, and ornithine in wines and dietary supplement samples (Table 3) (Martínez-Girón et al. 2009). A linear concentration range of 20–400 μM for Arg and Lys and 10–200 μM for ornithine was obtained. By employing a chiral MEKC method, including FITC derivatization and laser-induced fluorescence detection (MEKC-LIF), the L- and D-forms of Arg, Ser, Ala, Glu, and Asp could be separated in 25 min, with LOD values in the nM range (Table 3) (Herrero et al. 2007). The MEKC-LIF method proved to be a fast (run of 20 min) and sensitive method to analyze and quantify enantiomers of Pro, Ala, Arg, Glu, and Asp in vinegars (Carlavilla et al. 2006). With the same procedure, three types of commercial orange juices could be classified, providing the separation of the main 15 L- and D-AAs found in orange juices (Simó et al. 2004).

In capillary zone electrophoresis, cyclodextrins are the most frequently used chiral selectors. In particular, the use of modified 3-monodeoxy-3-monoamino-β-CD cyclodextrin to separate five chiral amino acids (Glu, Asp, Ala, Asn, and Arg) was investigated for the CE-TOF-MS separation technique. The time of analysis was of 19.2 min, with LOD values in the nM range (Table 3). The method was used to detect D- and L-AAs in vinegar and in wild and transgenic soybeans (Giuffrida et al. 2009).

Biosensors

Chromatographic methods require time-consuming sample pretreatment, expensive instruments, and trained personnel. Biosensors overcome these drawbacks, allowing amino acids to be detected simply, fast, and specifically. Actually, biosensors work optimally within the 2 to 900 s and 5.3 to 9.5 pH range and a temperature range of 25–45 °C, with LOD values between 0.02 and 1250 μM and a working potential from −0.05 to 0.45 V (Pundir et al. 2018). A number of D-AA biosensors have been reported based on immobilization of the enzyme DAAO from different sources by applying several immobilization methods, such as adsorption, cross-linking, and covalent immobilization (Table 3) (Sarkar et al. 1999; Sacchi et al. 1998; Compagnone and Trojanowicz 2007; Rosini et al. 2008).

A flow electrochemical device based on DAAO from *Rhodotorula gracilis* adsorbed on the graphite electrode was used to detect D-AAs in dairy products: this system showed the complete recovery of 1 mM D-Ala when added to milk samples (Sacchi et al. 1998). Samples from different stages of brewing were investigated by a flow injection analysis (FIA) system consisting of a thin-layer enzyme cell made of porcine DAAO immobilized on a membrane: the hydrogen peroxide produced by the enzymatic reaction was determined by the amperometric detector. With an optimal flow rate of 0.7 mL/min, 50–60 samples were measured in 1 h. The working concentration range was between 0.2–3 mM, with a RSD value of 2–2.7% (Table 3) (Varadi et al. 1999). A different FIA system consisting of immobilized porcine DAAO and pyruvate oxidase was developed to detect D-Ala: pyruvic acid formed by DAAO was further oxidized by pyruvate oxidase and the oxygen consumed was proportional to D-Ala concentration (Inaba et al. 2003). A linear response was obtained in the range of 0.1–1 mM D-Ala with a LOD value of 0.05 mM, one assay requiring 12 min (Table 3). The performance of the biosensor was tested on fish sauces samples, yielding values in good agreement with those obtained by conventional methods (Inaba et al. 2003).

To facilitate the hydrogen peroxide oxidation, a screen-printed rhodinized carbon working electrode that used immobilized *Crotalus adamateus* L-amino acid oxidase (LAAO) and porcine DAAO was developed (Sarkar et al. 1999). The device responded to all amino acids (except Pro), exhibiting stability over 56 days: a linear response was obtained for Gly, L-Leu, and L-Phe with LOD values of 0.47, 0.15, and 0.2 mM, respectively. In 4 min, the bi-enzymatic sensor monitored changes in amino acid content in milk, fruit juice, and urine samples, giving values similar to those obtained by a ninhydrin-based photometric assay (Table 3).

The cross-linking of porcine DAAO and bovine serum albumin with glutaraldehyde on a screen-printed graphite working electrode modified with Prussian Blue and Naflon layers was able to reach LOD values in the range 1–30 μM, with a linear response for D-Ala between 5 and 200 μM; it was used to assay commercial fruit juices (Weislo et al. 2007). A highly sensitive D-AA biosensor (LOD value = 1 μM; Table 3) was obtained by the covalent immobilization of goat DAAO onto polyindole 5-carboxylic acid/zinc sulfide nanoparticles hybrid film electrodeposited on an Au electrode (Lata et al. 2012). The biosensor was used to quantify D-AAs in fruit juices. The same research group developed an improved biosensor by covalent immobilization of the enzyme onto carboxylated multi-walled carbon nanotube/copper nanoparticles/polyaniline hybrid film electrodeposited on a gold electrode, reaching a LOD value of 0.2 μM with a LOQ of 0.03 μM and a linear response on D-Ala in the range 0.001–0.7 mM (Lata et al. 2012).

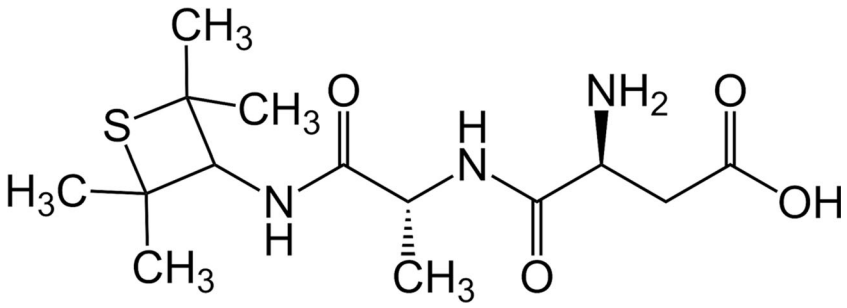

Fig. 2 Formula of the sweetener Alitame

A quantitative system for the enantiomeric determination of L- and D-AAs was developed by combining the efficiency of HPLC method, enzymatic specificity, and electrochemical sensitivity (Voss and Galensa 2000); different D-AAs were detected in beer, sherry, port wine, wine, and fruit juice without performing an evaporation or derivatization step. In this system, *Crotalus durissus* venom LAAO and porcine DAAO were immobilized on pore glass activated with glutaraldehyde and filled into reactor cartridges. The oxidative deamination of different amino acids, separated isocratically, produced hydrogen peroxide that was detected electrochemically. The method was optimized for the detection of D-Ala, allowing a very sensitive detection of bacterial contamination: less than 0.1 mg/L D-Ala was detected in 32 fruit concentrates and purees, each analysis requiring 55 min (Voss and Galensa 2000).

Worthy of note is that the biosensor performance depends on the substrate specificity of the employed enzyme and thus the total D-AA content cannot be measured. A low variability of response as a function of the D-AAs composition was achieved by using the Amberzyme-immobilized T60A/Q144R/K152E and M213G variants of DAAO from *Rhodotorula gracilis*: a limited dependence on the solution composition was apparent when at least 20% of the D-AAs was made up of D-Ala (Rosini et al. 2008). The entire D-AAs content was detected, with a LOD value of 0.25 mM in 10–15 min (Table 3). By using this device, the content of D-AAs in Grana Padano cheese could be quantified.

D-amino acid taste

Work published in 1965 reported that D-His, D-Leu, D-Phe, D-Trp, and D-Tyr have a sweet taste while the corresponding L-enantiomers possess a bitter taste (Solms et al. 1965). Although it is not a general rule, D-AAs frequently taste sweeter than L-enantiomers: this represents a case of correlation between stereochemistry and flavor. In some cases, the sweetening power of D-Val, D-Phe, and D-Trp is higher than that of sucrose (Linden and Lorient 1999). Alitame (L-α-asparty l-*N*-(2,2,4,4-tetramethyl-3-thioethanyl)-D-alaninamide; Fig. 2), an artificial dipeptide sweetener containing L-Asp and D-Ala, is of commercial interest because it is about 2000 times sweeter than sucrose, about 10 times sweeter than aspartame, and six times sweeter than saccharin (Chattopadhyay et al. 2014). A principal component analysis of sake taste and D-AA concentrations identified the strong taste as the most important component and the sweet taste as second most important (Okada et al. 2013). The high score for the first component is apparent at a D-Ala concentration > 100 μM, as well as for D-Asp and D-Glu. The origin of these D-AAs is due to lactic acid bacteria during storage.

By using a cellular model overexpressing sweet and bitter receptors, nine amino acids were tested (Bassoli et al. 2014). This study reported that TAS1R2/TAS1R3 sweet receptors show a stereoselectivity with a preference for binding the D-AAs. Concerning the TASR2 bitter receptors, the TAS2R4 and TAS2R39 variants are activated by both enantiomers of tryptophan while the TAS2R43 and TAS2R49 receptors are activated by the L-enantiomer only. Indeed, a stereoselectivity was apparent for TAS3R8 and TAS2R4 receptors and phenylalanine.

Recently, it was reported that the clinically relevant respiratory Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* strains produce D-AAs that activate TAS1R/2 sweet taste receptors in solitary chemosensory cells and inhibit antimicrobial peptide secretion (Lee et al. 2017). This information is relevant because these nonpathogenic bacteria, which play a role in chronic rhinosinusitis, suppress *P. aeruginosa* virulence.

Are D-amino acids beneficial for human health?

The metabolic fate of D-AAs in humans is still controversial. In mammals, the flavoenzymes DAAO and DASPO convert the D-AAs (via oxidative deamination) to α-keto acids. DAAO is active on a number of D-AAs and it is largely expressed in liver and kidneys. Then, α-keto acids are catabolyzed or transaminated to L-AAs. In the human organism, racemization is restricted to few amino acids (D-Ser and, probably, D-Asp) and selected tissues. D-AA metabolism in mammals is largely due to gut microbiota.

Mice fed a synthetic all-amino acid diet were used to evaluate the nutritional value of D-enantiomers of amino acids (Friedman 2007; Friedman and Levin 2012). The nutritional

power of essential D-AAs depends on the form of administration (as free amino acids or as protein components), on the amino acid composition (the use of any D-AA may be affected by other D-AAs present in the diet), the digestibility, and utilization of protein-released amino acids. Actually, peptide bonds with D-L, L-D, or D-D configurations are resistant to proteolytic enzymes, thus inducing a reduced digestibility and eventually the formation of oligopeptides with unknown biological activity. No toxic effects were found in trials with parenteral nutrition of adults and children with free D,L-amino acids (Marchelli et al. 2007). Very recently, an investigation reported a decrease in metabolic activity of 3T3-L1 preadipocytes when the cell medium contained specific D-AAs in place of the L-enantiomer (Chen et al. 2019). While D-Lys showed a stronger inhibitory effect, no depression by D-Ser was apparent. Treatment with 10–200 μM D-AAs of Caco-2 cells, a model of the cells lining the gastrointestinal tract, did not affect the cell growth.

Reduced digestibility of dietary proteins containing D-AAs may in certain cases prove advantageous for nutrition, promoting considerable weight loss when consumed for a few days. Food products formulated to induce weight loss contain 50% D-Ser, 37% D-Asp, and 26% D-Phe; these high D-AA quantities might cause a risk if consumed as the sole source of dietary proteins (Finley 1985). D-AAs have been also classified among the anti-nutritional factors (Gilani et al. 2005; Gilani et al. 2012). Considering the single D-AA, D-Met is poorly used by humans when consumed orally or in parenteral nutrition, and D-Tyr significantly decreases growth in mice, D-Met and D-Lys as well (Friedman and Levin 2012). Actually, in the presence of high levels of free D-AAs, the DAAO degradation system may become saturated and, indeed, the proteins containing D-AAs are hydrolyzed at a slower rate than proteins containing L-AAs only and may generate D-D, D-L, or L-D peptides that are poorly degraded by proteases and that can compete with normal peptides for binding to the active site of the proteolytic enzymes. The ensuing slower adsorption, compared to L-AAs, may contribute to the decrease in protein digestibility.

Concerning a potential therapeutic application of D-AAs, D-Ser was proposed for the therapy of post-traumatic stress disorder (Heresco-Levy et al. 2009) and D-Phe to induce analgesia (Balagot et al. 1981): oral administration of D-Phe (750–1000 mg daily) should result in carboxypeptidase inhibition, which is involved in breaking down opioid pentapeptide in the brain and spinal cord and related to an increase in brain encephalin levels. Some D-AAs inhibit tumor growth in rats: D-Val administration impaired the nutritional status and inhibited the tumor growth of hepatoma-bearing rats compared to control diets (Sasamura et al. 1998). Furthermore, D-Ser supplementation has being investigated in the treatment of schizophrenia (MacKay et al. 2019). Some D-AAs are present in selected antibiotic peptides: it is also conceivable that proteolytic cleavage of D-AAs containing dietary proteins generates peptides with antibiotic properties.

The presence of various D-AAs in the human stratum corneum was reported by the Shiseido company, which also demonstrated that as levels of D-Asp decline during aging, collagen production decreases. After a trial of 2 months of D-Asp ingestion, the thickness of the skin was clearly higher. Accordingly, the first functional food enriched with D-AAs called “kireinosusume” was produced (Mutaguchi et al. 2016). Based on the evidence that D-Ala improves laminin-5 production in the basement membrane of skin and levels are restored in aged people, the same company intends to commercialize cosmetics supplemented with D-Ala as an anti-aging factor. Regarding the aging process, long-term administration of D-AAs to mice was recently reported to improve spatial reference memory (a test for cognitive function) and to increase the levels in the cerebral cortex (but not in plasma) of 5-hydroxytryptamine, various L-AAs (Ala, Ser, Val and Ile), and D-Ser (Kawase and Furuse 2019). The latter molecule was suggested as being responsible for improving the spatial reference memory by acting on NMDA receptors: accordingly, bacteria-derived molecules might affect central nervous system function.

Altered levels of D-AAs were reported to induce serious injury, especially to rodent kidneys. D-Ser has been reported to enlarge rat kidney cells (cytomegaly): sodium benzoate (Williams and Lock 2004), protein-deficient diets (Levine and Saltzman 2003), and α-aminoisobutyric acid (Krug et al. 2007) attenuated D-Ser nephrotoxicity in rats.

Conclusions

The presence of the “wrong enantiomers” of amino acids (the D-AAs) in foods is now well established. However, standardized methods designed to systematically ascertain D-AAs level in foodstuffs and their role in nutrition still represent a priority.

In order to clarify the effect of D-AAs on human health, we identified some unresolved questions:

- Do free and protein-associated D-AAs bind and modulate the proteolytic enzymes in the digestive tract?
- What is/are the biological effect(s) of D-AAs, depending on whether they are consumed in the free state or as food protein components?
- Do poorly digestible food proteins containing D-AAs serve as dietary fiber?
- Do metabolic interactions among free and protein bound D-AAs occur in vivo?

Notwithstanding amazing recent novel findings in D-AA research, this field is expected to grow considerably in the near future and we greatly anticipate the new scientific findings.

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Compliance with ethical standards

Conflict of interest GLM declares that she has no conflict of interest. ER declares that she has no conflict of interest. EC declares that she has no conflict of interest. LP declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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