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Liquid chromatographic determination of amino acid enantiomers by derivatization with *o*-phthaldialdehyde and chiral thiols
Applications with reference to food science

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Abstract

Using a fully automated liquid chromatograph, D- and L-α-amino acids (D- and L-AA) were determined in foods and beverages by precolumn derivatization with *o*-phthaldialdehyde (OPA) combined with the chiral thiol N-isobutyryl-L-cysteine (IBLC) or its enantiomer N-isobutyryl-D-cysteine (IBDC) with the structure $(CH_3)_2CHCONHCH(CH_2SH)COOH$. The resulting diastereomeric isoindole derivatives were resolved on an octadecylsilyl stationary phase using a linear gradient formed from sodium acetate buffer (pH 5.95) and methanol–acetonitrile. For the detection of the isoindoles, their fluorescence at 445 nm when excited at a wavelength of 230 nm was used. The suitability of the LC method was demonstrated by the detection of free D-AA as native constituents of grape juice, their determination in alcoholic fermented beverages (beer and wine), fermented dairy products (coumiss, dietary whey drink, hard cheese), lactic acid fermented cabbage juice, a commercial yeast extract and honey. D-AA were detected in all foodstuffs. The results provide further proof that D-AA are common in microbially fermented foods and that microorganisms (bacteria, yeasts) are major, but not exclusive, sources of free D-AA occurring in foodstuffs.

1. Introduction

For the precolumn derivatization of DL-α-amino acids (DL-AA) with chiral reagents followed by the liquid chromatographic (LC) separation of the diastereomeric derivatives formed, the use of various reactive chiral compounds has been reported. Examples are (+)- and (−)-1-(naphthyl)ethyl isocyanate [1], (*R*)-α-methylbenzyl isothiocyanate [2], acetylated β-D-glucopyranosyl isothiocyanates [3,4], dinitroaryl fluorides [5,6], monohalo-*s*-triazines [7], oxycarbonyl chlorides [8], L-α-amino acid carboxy anhydrides [9], and chiral thiols together with *o*-phthaldialdehyde (OPA). In the last instance, the diastereomeric isoindole derivatives formed from DL-AA and OPA together with various chiral thiols such as thio sugars [10,11], N-acetylpenicillamine [12], the chiral drug Captopril [13], D-3-mercapto-2-methylpropionic acid [14] and N-acetylcysteine [15,16] are resolvable by HPLC. In particular, the members of the homologous series N-acetyl- to N-octanoyl-L-cysteine [13,17–19] were systematically investi-

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gated for their suitability as reagents for the so-called indirect approach [20] of separating AA enantiomers as diastereomeric isoindole derivatives. We selected N-isobutyryl-L-cysteine (IBLC) and its enantiomer N-isobutyryl-D-cysteine (IBDC) as the most suitable reagents among the various thiols investigated. In combination with an appropriate ODS stationary phase and optimized gradient elution conditions, we completely and reproducibly separated a multicomponent standard consisting of all protein L-α-amino acids and their corresponding D-enantiomers, DL-cysteic acid, glycine, the non-protein α-aminoisobutyric acid (Aib) and DL-isovaline (Iva) and the internal standard L-*homo*-arginine [21]. The reagents, stationary phase and instrumental set-up are commercially available, and the method has been tested thoroughly with respect to its robustness and reliability in various analytical matrices [21,22–25]. This approach, therefore, is unique among the various HPLC methods proposed for the complete resolution of mixtures of D- and L-AA in realistic samples [26–30].

In continuation of work mainly concerned with the gas chromatographic determination of D- and L-AA in microorganisms [31,32], foodstuffs [23,33–37] and physiological fluids [38,39] by applying the chiral stationary phases Chirasil-Val [40], XE-60-L-Val-(*S*)-α-phenylethylamide and Lipodex E [41], we now show that the HPLC method is also very suitable for the determination of AA enantiomers in food and beverages.

2. Experimental

2.1. Instruments

For HPLC, an instrument that makes possible the fully automated pre-column derivatization of amino acid enantiomers with OPA and chiral thiols was used. It consisted of an HP 1090 Series L chromatograph constructed from a binary DR 5 solvent-delivery system, autoderivatizer and autoinjector (for details, see Ref. [22]), temperature-controlled column compartment and HP 1046 programmable fluorescence detector operated at an excitation wavelength of 230 nm and an emission wavelength of 445 nm (cut-off filter 280 nm). For data processing a Series HP 79994A ChemStation computer, Model 7440A ColorPro plotter and Model 2225B ThinkJet printer were used. All instruments were provided by Hewlett-Packard, Waldbronn Analytical Division (Waldbronn, Germany).

2.2. Chromatography

Columns (250 mm × 4 mm I.D.) and guard columns (20 mm × 2.1 mm I.D.) (Hewlett-Packard) packed with Hypersil ODS of particle size 5 μm (Shandon Scientific, Runcorn, UK) were used. The tested columns are available from Hewlett-Packard, Waldbronn Analytical Division, on request. The columns were kept at 25°C. Eluent A was prepared from 3.13 g (23 mmol) of sodium acetate trihydrate in 990 ml of doubly distilled water adjusted to pH 5.95 by addition of 10% (v/v) acetic acid and made up to 1.0 l. The eluent was filtered through a 0.45-μm filter (Sartorius, Göttingen, Germany). Eluent B consisted of 474 g of methanol and 39 g of acetonitrile (AN). Helium was continuously passed through the eluents. A linear gradient was applied for 75 min at a flow-rate of 1 ml min^{-1} from 0 to 53.5% B and then equilibrated with 100% A for 10 min.

2.3. Automated derivatization procedure

Derivatization of standards and samples was carried out as in previous investigations [21–25]: 260 m*M* IBLC (or IBDC) and 170 m*M* OPA in 1 *M* potassium borate buffer (pH 10.4) (Pierce, Rockford, IL, USA) were used as derivatization reagents (designated OPA–IBLC or OPA–IBDC reagent). Using the unique derivatization device of the instrument [22], amounts of 5 μl of 0.4 *M* sodium borate buffer (pH 10.4) (Hewlett-Packard), 1 μl of OPA–IBLC (or OPA–IBDC) reagent and 2-μl aliquots of the analyte solutions were consecutively drawn up by the autosampler and mixed by the autoderivatizer, programmed for five mixing cycles in the 8-μl mode. The

mixing procedure was completed in ca. 2 min, then the mixture was immediately and automatically injected on to the column. For quantification an external standard consisting of 100 pmol of L-AA and 5 pmol of D-AA in 2 μl of 0.1 *M* HCl was used [25]. The acid-sensitive AA (Gln, Asn and Trp) were dissolved in water and suitable amounts were added to the stock solution between analysis.

2.4. Solvents and chemicals

Solvents and chemicals were of chromatographic or analytical-reagent grade from Merck (Darmstadt, Germany). Dowex 50W-X8 cation exchanger (H^+ form, particle size 0.037–0.075 mm, analytical-reagent grade) and polyamide-6 powder (research grade) were obtained from Serva (Heidelberg, Germany) and were discarded after use. A saturated solution of picric acid (Fluka, Buchs, Switzerland) in water used for the precipitation of proteins is referred to as "picric acid" in the text. L- and D-AA were of the highest available purity and purchased from Sigma (St. Louis, MO, USA), Fluka or Serva. The internal standard L-*homo*-Arg was obtained from Serva and N-isobutyryl-D-cysteine (IBLC) was from Calbiochem–Novabiochem (Läufelfingen, Switzerland). N-Isobutyryl-L-cysteine (IBLC) was synthesized in our laboratory [22] and is also available from Calbiochem–Novabiochem or Calbiochem (La Jolla, CA, USA); IBLC and IBDC are now also available as ChiraSelect reagents from Fluka. The reagents used were of 99.91 ± 0.01% (IBLC) and 99.78 ± 0.02% (IBDC) optical purity.

2.5. Sources of food and beverage samples and treatment for analyses

Grape juice

Freshly harvested and selected ripe grapes *Vitis vinifera* L. (cultivar Trollinger) from a vineyard in the Stuttgart area were carefully washed with 70% ethanol. Grapes (ca. 200 g) were squeezed and the juice was collected using an automated juicer based on the centrifugal principle (Multipress MP 50, Type 4154, from Braun, Frankfurt, Germany). The juice was filtered using a fluted paper filter and centrifuged at 1650 *g*. To 1-ml aliquots of the juice, 31.3 μl of 1.6 m*M* L-*homo*-Arg in 0.1 *M* HCl were added, the pH was adjusted to 2 by addition of 2 *M* HCl and the mixture was transferred to the top of a column containing Dowex 50W-X8 cation exchanger (bed size 5 cm × 1 cm). Amino acids were eluted with 4 *M* aqueous ammonia (30 ml), the eluent was evaporated to dryness using a vacuum evaporator, the residue was dissolved in 0.1 *M* HCl (1 ml) and 2-μl aliquots were analysed.

Wines

Bottled wines [French rosé wine (Rosé d'Anjou, 4 years old) and Madeira wine (Leacocks, 10 years old)] were purchased in a local wine shop. To an aliquot of the rosé wine (10 ml), picric acid (10 ml) and 0.1 *M* HCl (10 ml) were added, the mixture was stirred for 15 min and then centrifuged at 1650 *g*. The clear solution was passed through a column packed with polyamide-6 powder (bed volume 5 cm × 1 cm) and then subjected to Dowex 50W-X8 treatment and analysed as described above. For the Madeira wine, an aliquot (25 ml) was adjusted to pH 2 by addition of 6 *M* HCl and the mixture was subjected to Dowex 50W-X8 treatment and elution of AA as described above. After evaporation to dryness, the residues were dissolved in 0.1 *M* HCl (2 ml) and 2-μl aliquots were used for analysis by HPLC.

Vinegar

Vinegar made from Sherry wine (Old Sherry wine vinegar, from Jerez de la Frontera) was investigated. The vinegar was matured for several years in oak barrels, bottled in Jerez, Spain, and imported and distributed in Germany by Probare, Skandinavien- und Süd-Import, Maisach, Germany. Aliquots of 2 μl were directly analysed after filtration.

Beer

A bottled German Export beer (Das echte Schwabenbräu, Stuttgart), produced with the aid of the bottom-fermenting yeast *Saccharomyces*

carlsbergensis, was investigated. The beer contained 6% (v/v) ethanol and had an original gravity of 13%. To an aliquot (10 ml) picric acid (10 ml) was added and the mixture was stirred for 10 min and centrifuged at 1630 *g* for 10 min. The supernatant was filtered using a fluted paper filter and the filtrate was extracted three times with 20-ml portions of light petroleum (b.p. 50–70°C)–diethyl ether (1:1, v/v). The aqueous phase was evaporated to dryness and the residue was dissolved in 0.01 *M* HCl (20 ml), filtered and subjected to cation exchanger treatment and ammonia elution of AA as described above. The dry residue was dissolved in 0.1 *M* HCl (3 ml) and filtered using a 0.45-μm disposable filter and aliquots of 2 μl were analysed.

Lactic fermented cabbage juice

Bottled and pasteurized lactic fermented (pickled) cabbage juice (Eden Sauerkrautsaft; Eden, Bad Soden, Germany) was purchased in a shop specializing in health food (Reformhaus). An aliquot of the juice was filtered using a Millex GV 13 filter (Millipore, Monheim, France). A 1-ml aliquot was diluted with 4 ml of doubly distilled water and 2-μl aliquots were analysed.

Coumiss

This commercially available product (Kumylac) was provided by Kurgestüt Hoher Odenwald (Waldbrunn, Germany). According to the manufacturer's declaration, this product is produced from the milk of mares by controlled addition of lactobacilli and yeast. To an aliquot (40 ml), acetonitrile (80 ml) was added and the mixture was stirred for 15 min and centrifuged at 1650 *g*. The supernatant was concentrated in vacuo to a volume of ca. 5 ml, then picric acid (5 ml) and 0.1 *M* HCl (10 ml) were added. The mixture was centrifuged at 1650 *g* and the supernatant was extracted with light petroleum (b.p. 50–70°C)–diethyl ether (1:1, v/v) (3 × 20 ml). The supernatant was passed through a Dowex 50W-X8 column and amino acids were eluted as described above. The effluent was evaporated to dryness, the residue was dissolved in 0.1 *M* HCl (2 ml) and 2-μl aliquots were analysed. Analogously, an unfermented mares milk (Equilac) sample, serving as starting material for coumiss, was analysed.

Dietetic whey drink

A 1-l package of heat-treated whey (Heirler Diät Kurmolke; Heirler, Gauting bei München, Germany) was purchased in a shop specializing in dietetic and health food (Reformhaus). According to the manufacturer's declaration, the dietetic whey was made from acidic whey with addition of Sanoghurt starter culture (*Streptococcus lactis* ssp. *lactis* or ssp. *cremoris*). In addition, whey proteins and pectin were added by the manufacturer. To whey (1 ml), solid 5-sulfosalicylic acid (80 mg) was added and the mixture was shaken for several minutes and centrifuged at 4000 *g*. The supernatant was filtered using a Millex GV 13 filter (see above) and 2-μl aliquots were analysed.

Cheese

A French pressed cheese, purchased in a cheese shop in France, of the protected origin (d'origin controllé) Cantal, was investigated. This popular traditional cheese from the Central Massif area of France is made from cow milk with the addition of mixed starter cultures, usually *Lactobacillus lactis* and *Leuconostoc citrovorum*. The cheese had 45% fat in dry matter and 58% dry matter. From the interior an aliquot (1.0 g) was removed, 96% ethanol (30 ml) was added and the mixture was stirred vigorously at 50°C for 45 min. Water (35 ml) was added and stirring was continued for another 15 min. The mixture was centrifuged (1600 *g*, 10 min) and the supernatant was evaporated to a volume of ca. 10 ml in vacuo. The pH was adjusted to 2 by addition of 0.1 *M* HCl and the solution was extracted with light petroleum (b.p. 50–70°C)–diethyl ether (1:1, v/v) and further treated as described for coumiss.

Yeast extract

A dietary yeast extract (Marmite, Burton on Trent, UK), used as a spread or seasoning, was purchased in the UK. According to the declaration, the ingredients were yeast extract, salt, vegetable extract, spices and vitamins. An

aliquot (1 g) was dissolved in doubly distilled water (10 ml). To a 1-ml aliquot, 5-sulfosalicylic acid (50 mg) was added, the mixture was centrifuged and the supernatant was diluted to a final volume of 10 ml. Aliquots of 2 μl were analysed.

Honey

White fir honey (collected in spring 1993 in the Stuttgart area by honey bees, *Apis mellifera carnica*) was kindly provided by Professor Dr. G. Vorwohl, Landesanstalt für Bienenkunde, Universität Hohenheim (Hohenheim, Germany). Samples (1 g) were dissolved in 0.01 *M* HCl (10 ml), evaporated to a volume of ca. 5 ml in vacuo and then subjected to Dowex 50W-X8 treatment as described above. The dry residue was dissolved in 0.1 *M* HCl (1 ml) and 2-μl aliquots were analysed.

3. Results and discussion

The elution profiles of AA (referred to as aminograms) from foodstuffs and derivatized with OPA–IBLC or OPA–IBDC are shown in Figs. 1–11 (for chromatograms of standards we refer to previous publications [21,25]; as IBLC or IBDC has to be used together with the dialdehyde, we omit OPA in the following text). The reversal of the elution order of AA enantiomers is extremely helpful for the certain assignment and quantification of D-AA, in particular when complex matrices are analysed. In order to demonstrate this, aminograms resulting from the use of both reagents are shown, with the exception of beer. The relative percentage values given in parentheses in the text are those determined with IBDC. In cases where no data are given, quantification or reliable assignment of the respective enantiomer was not possible. In the following, the relative amounts of D-AA that have been determined are presented and their relevance is discussed. The absolute amounts of D-AA determined in native, unprocessed grape juice, rosé wine, vinegar made from Sherry wine, juice of pickled cabbage, Cantal cheese, dietary whey drink, honey and edible yeast extract are displayed in Table 1.

3.1. Grape juice

The elution profiles of D- and L-AA determined in a freshly pressed grape juice are shown in Fig. 1a and b. Relative amounts of 0.9% (1.2%) D-Asp, 0.7% (0.5%) D-Glu, 1.2% (1.4%) D-Ser, 1.2% (1.5%) D-Arg and 1.0% (1.2%) D-Ala were determined. As microbial contamination or spoilage is excluded, this result further corraborates that certain D-AA are native constituents of fruit juices [21,25]. Their amount and diversity increase as soon as bacteria

Table 1
Quantification of D-amino acids (D-AA) in foodstuffs

D-AA	Grape juice (μmol l^{-1})	Rosé wine (μmol l^{-1})	Vinegar (μmol l^{-1})	Pickled cabbage juice (μmol l^{-1})	Cheese[a] (μmol kg^{-1})	Dietary whey (μmol l^{-1})	Honey (μmol kg^{-1})	Yeast extract	
								μmol kg^{-1}	mg kg^{-1}
D-Asp	1.6	3.3	10.9	181	337	24	9.8	2153	287
D-Asn	–	–	–	–	–	–	–	2981	394
D-Glu	3.9	6.0	9.1	20	998	20	10.1	1866	275
D-Ser	1.9	–	1.4	49	n.d.	2.3	3.1	1283	135
D-Ala	5.4	6.2	2.3	718	987	29	8.1	3984	355
D-Arg	8.2	–	–	–	–	–	–	–	–
D-Tyr	–	–	–	–	–	–	–	548	99
D-Phe	–	–	–	–	n.d.	–	–	1026	135
D-Leu	–	–	3.5	59	104	–	3.1	–	–
D-Lys	–	–	–	2.4	–	1.0	1.5	–	–

For origin of foodstuffs see Experimental; – = not detected; n.d. = not determined.
[a] D-Pro (122 μmol kg^{-1}), D-*allo*-Ile (32 μmol kg^{-1}) and D-Phe (182 μmol kg^{-1}) determined by GC.

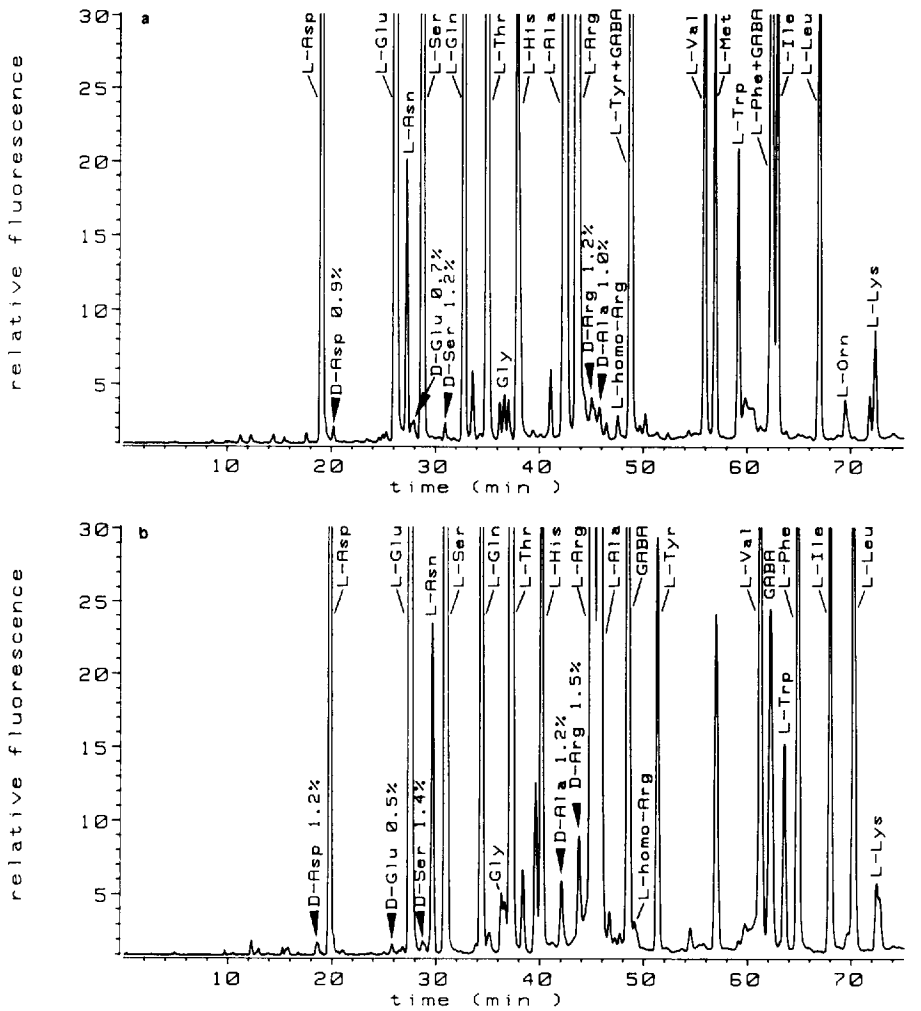

Fig. 1. Aminogram of freshly pressed grape juice derivatized with (a) IBLC and (b) IBDC. Arrows mark positions of D-AA in all aminograms.

are allowed to grow in plant juices (cf. Table 1 and Fig. 6). This might cause spoilage [36] or, if grown under controlled conditions, is used for manufacturing fermented foods or drinks [31] (see also below).

3.2. Wines

The aminograms of a French rosé wine are shown in Fig. 2a and b. Relative amounts of 3.4% (5.8%) D-Asp, 2.4% D-Glu, 2.9% D-Ala and 3.2% D-Tyr were determined. Using IBDC (Fig. 2b), D-Glu, D-Ala and D-Tyr elute together with unknown components, and the assignment of D-Ser and D-Leu is not confirmed by derivatization with IBLC. The total amounts of AA (D + L) in this wine was 901 mg l^{-1} and the sum of D-AA was 2.5 mg l^{-1}.

The chromatograms of a Madeira wine are shown in Fig. 3a and b. Relative amounts of 20.5% (20.5%) D-Asp, 22.5% (20.4%) D-Glu, 17.8% D-Asn, 4.4% (5.3%) D-Arg, 16.9% (17.8%) D-Ala, presence of D-Tyr, 3.2% D-Val and 7.7% (6.7%) D-Leu and, by derivatization with IBDC, 11.4% D-Ser were determined. D-Tyr and D-Phe are detectable using IBLC, but are not quantifiable relative to their L-forms as L-Tyr and L-Phe elute together with derivatives

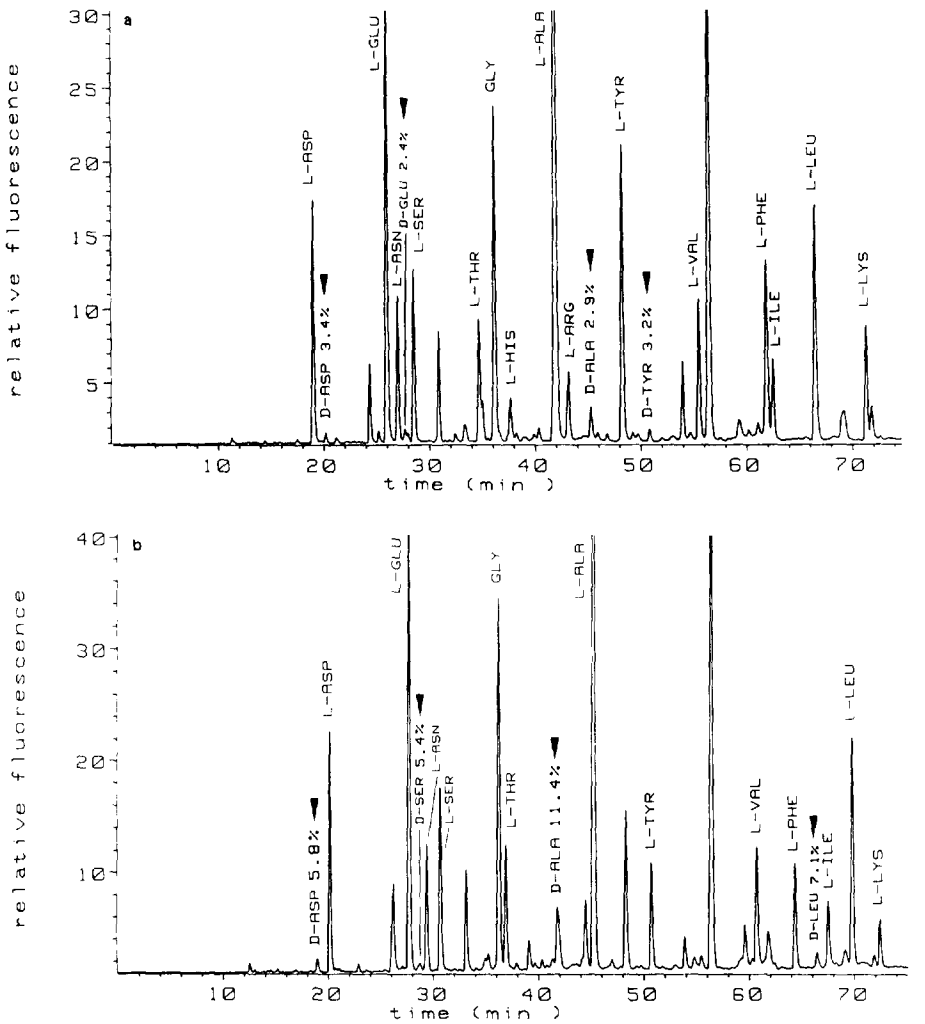

Fig. 2. Aminogram of a bottled rosé wine derivatized with (a) IBLC and (b) IBDC.

formed from GABA, named GABA(1) and GABA(2) (see Fig. 3b). In this wine, D-Ser is not quantifiable using IBLC as its derivative elutes close to an unknown component and D-Asn is not detectable using IBDC. The total amount of AA (D + L) in this wine was 517 mg l^{-1} and the sum of D-AA was 30 mg l^{-1}. In particular, the data show a characteristic difference for the relative amounts of D-AA between wines and fortified wines.

From the large number of white, red and rosé wines which we have investigated [33–35], the presence and the relative amounts of ca. 1–3% of D-Asp, D-Glu and D-Ala are typical (higher amounts of certain D-AA have been reported to occur in Portuguese elementary wines [42]). In fortified wines (Madeira, Sherry, Port), however, in general high amounts of D-Asp, D-Glu and D-Ala and a greater diversity of D-AA are found (see Fig. 3). This is attributed to the manufacturing procedures for fortified wines, where alcohol is added to the vigorously fermenting must in order to stop the fermentation process, followed by curing and maturing of these fortified wines under typical conditions for several years. The raw materials, manufacturing

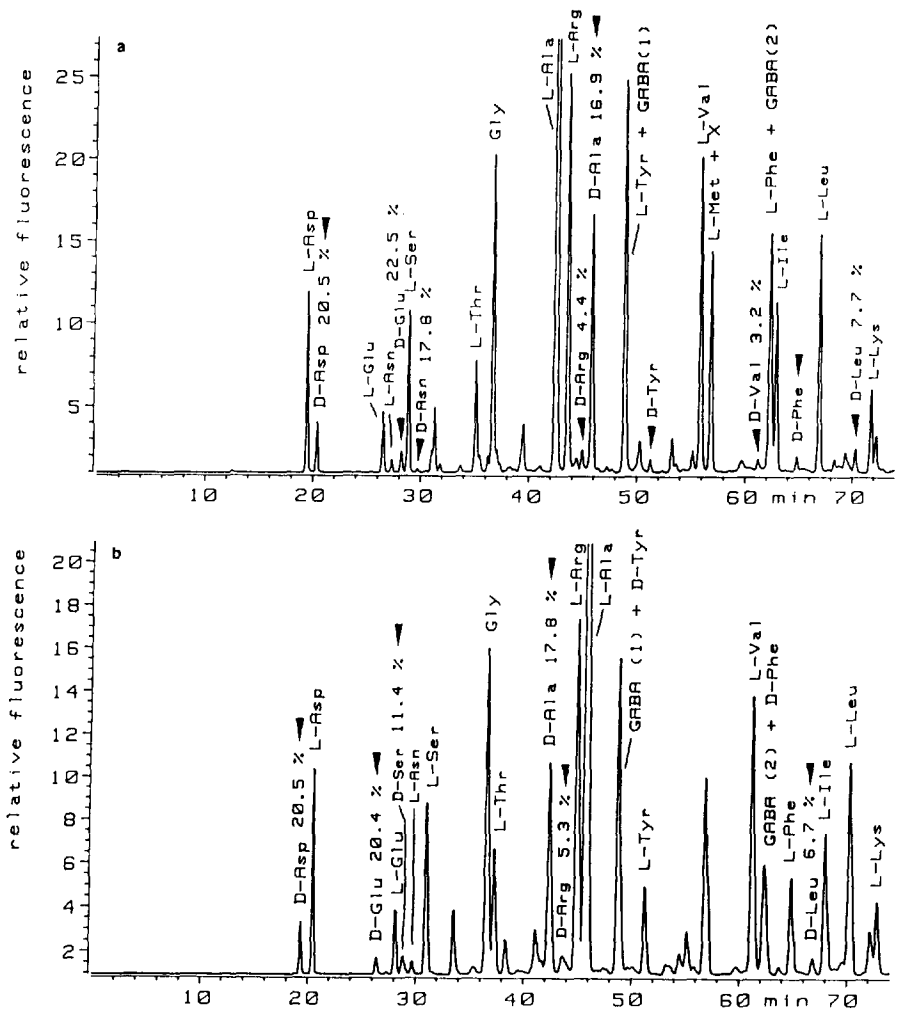

Fig. 3. Aminogram of a 10-year-old Madeira wine derivatized with (a) IBLC and (b) IBDC.

processes, fermentation procedures (including yeasts and bacteria) and maturing conditions of the various wines show a great variety and the blending of fortified wines is also common. In the context of this work, it should briefly be stated that the action of microbial and plant enzymes such as amino acid isomerases and transaminases is assumed to be responsible for the occurrence of D-AA in wines, rather than an acid-catalysed racemization of L-AA.

3.3. Vinegar

The chromatograms of vinegar made from Sherry wine are shown in Fig. 4a and b. Relative amounts of 22.1% (25.4%) D-Asp, 17.9% (13.4%) D-Glu, 6.0% (4.7%) D-Ser, 21.7% (24.4%) D-Ala and 10.0% (9.4%) D-Leu were found. Such high relative amounts of D-AA are also found in other high-quality vinegars such as Italien aceto balsamico [35], which is produced and stored in wooden barrels for several years. As with wine, the formation of D-AA is attributed to the action of microbial enzymes. At the beginning of the fermentation the yeast *Saccharomyces cerevisiae* converts sugars into ethanol, which is then fermented to acetic acid by species of *Acetobacter*. Consequently, D-AA

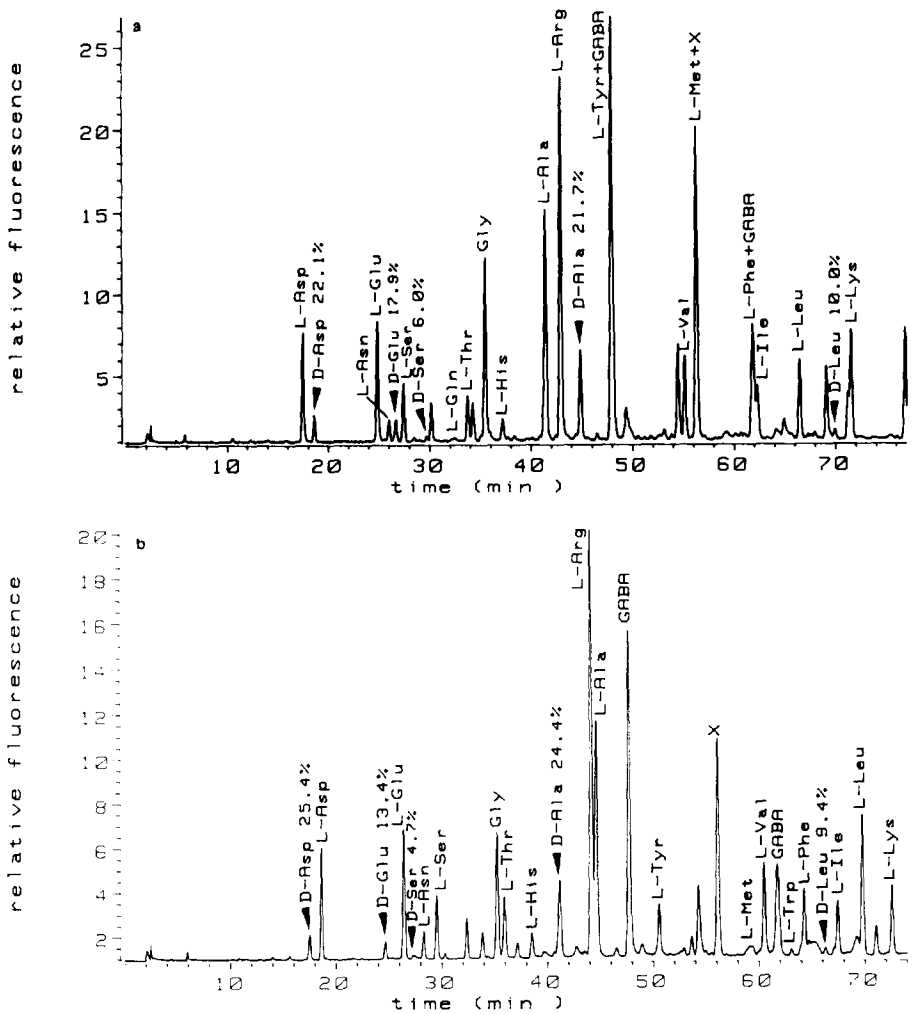

Fig. 4. Aminogram of a vinegar produced from Sherry wine derivatized with (a) IBLC and (b) IBDC.

have also been detected in the acetic acid bacterium *Acetobacter esbia*, which is used as a starter culture for the production of vinegar [31].

3.4. Beer

The elution profile of AA from a beer, derivatized with IBLC, is shown in Fig. 5. Relative amounts of 29.0% D-Asp, 5.9% D-Glu and 3.3% D-Ala were found. The total amount of AA (D + L) was 697 mg l^{-1} and the sum of D-AA was 11 mg l^{-1}. Like wine, beer belongs to the alcoholic fermented beverages and, consequently, D-AA have been detected by GC in all beers which were brewed under various conditions [33–35]. The most abundant D-AA is D-Asp, followed by D-Glu and D-Ala. Although varieties of the yeasts *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* play the major role in the brewing process, bacteria are also involved and the possible contribution of the processes of malting, liquating and torrefying on the formation of D-AA, as well as the contribution of plant D-AA [25], still has to be evaluated.

3.5. Lactic acid fermented cabbage

The chromatograms of AA isolated from fer-

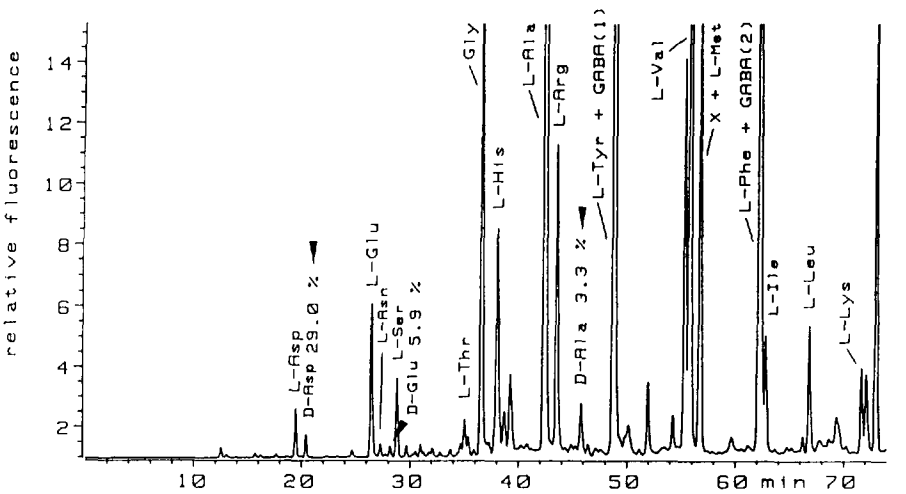

Fig. 5. Aminogram of bottom-fermented beer derivatized with IBLC.

mented cabbage are shown in Fig. 6a and b. Relative amounts of 5.2% (4.7%) D-Asp, 13.5% (12.3%) D-Glu, 3.0% (2.5%) D-Ser, 11.2% (8.2%) D-Ala 7.5% (7.2%) D-Leu and 10.2% (4.9%) D-Lys were found. Fermented (pickled) cabbage juice is used as a dietary drink. The bacterium *Leuconostoc mesenteroides* is involved in the beginning of fermentation, and later *Lactobacillus brevis* and *Lactobacillus plantarum* dominate. Lactobacilli show a high racemase activity [21,31,37] and are responsible for the relatively high amounts of D-AA determined.

3.6. Coumiss

Fig. 7a and b show the AA determined in the fermented milk of mares; 33.4% (34.5%) D-Asp, 9.5% (9.7%) D-Glu, 14.0% (15.3%) D-Ser and 76.1% (73.7%) D-Ala were found. These high amounts of free D-AA are the result of the microbial fermentation of the milk in which, again, amino acid isomerases and racemases are involved. In the fresh milk of mares, serving as a blank, only low amounts of D-Ala (0.9%), D-Glu (0.5%), D-Ser (0.3%) and traces of D-Asp (not quantifiable) were detectable using derivatization with OPA–IBLC.

3.7. Cheese

The AA determined in a ripened cheese are shown in Fig. 8a and b; 6.1% (7.1%) D-Asp, 14.3% (15.4%) D-Glu, 32.7% (32.4%) D-Ala and 0.8% (2.0%) D-Leu were found. With the exception of D-Leu, these values show good agreement, taking the very complex food matrix into account. The higher amount of D-Leu as determined by derivatization with OPA–IBDC is attributed to the co-elution of a background impurity. The total amount of AA (D + L) in the Cantal cheese was 7724 mg kg^{-1} and the sum of D-AA was 435 mg kg^{-1}. Quantitative data for D-AA determined in many cheeses have been reported [24,33–35,37,43] and their amounts and kinds have been rationalized as a result of the concerted action of high microbial protease and racemase activities [37,43].

3.8. Whey

Fig. 9a and b show the chromatograms of AA determined in a dietary whey beverage. Amounts of 23.7% (26.1%) D-Asp, 6.0% (5.7%) D-Glu, 10.0% (10.5%) D-Ser, 22.2% (24.8%) D-Ala and 15.2% (16.5%) D-Lys were found. Whey is milk serum obtained when caseins of milk are precipitated for cheese production by the action of rennet and cheese starter cultures. Depending on the starter cultures used and the manufacturing procedures applied, a high bacterial racemase activity in cheese and whey is observed [37]. In the dietary

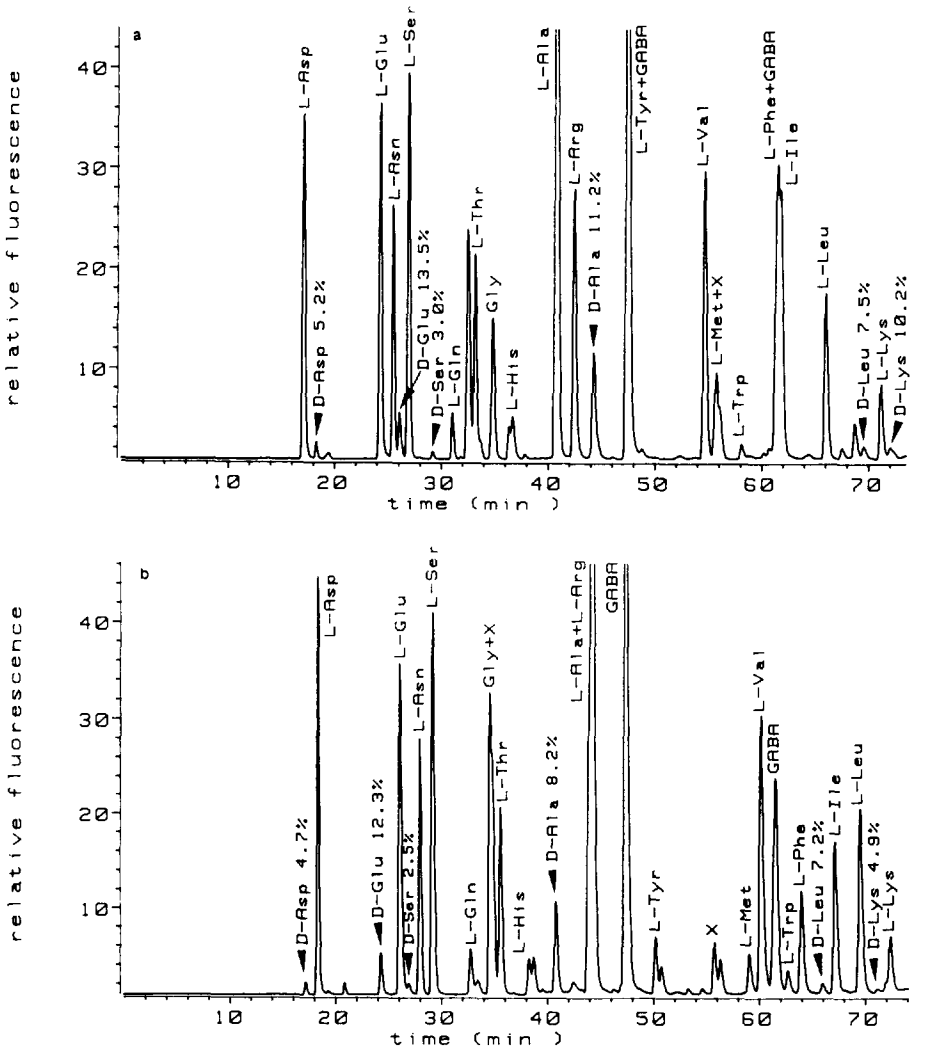

Fig. 6. Aminogram of lactic fermented cabbage juice derivatized with (a) IBLC and (b) IBDC.

whey drink investigated, additional species of *Streptococcus* that show a high racemase activity [31] were added to acid whey.

3.9. Honey

Fig. 10a and b show the aminograms of AA isolated from white fir honey; 5.2% (7.3%) D-Asp, 4.1% (4.2%) D-Glu, 1.8% (1.2%) D-Ser, 3.3% Ala, 1.8% D-Phe and 6.9% (5.7%) D-Leu were determined. D-Ala and D-Phe were not determinable using IBDC as a result of the co-elution with an unknown component (X) and GABA, respectively. Using chiral phase GC with another sample of a commercially available fir honey, 3.3% D-Asp, 5.7% Glx, 5.7% D-Ala and 3.8% D-Phe were detected. No D-Pro (which is not determinable using the method described) was found by GC, although L-Pro is the major AA in both honeys. Although the occurrence of AA in honey is well documented and the ratios between the concentrations of the individual AA have been used to determine the geographic source of honeys [44], the presence of D-AA in honey has not previously been reported. Honey, per se, is not considered to be a fermented food. Concerning the origin of D-AA in honey, clues might be that D-AA have been detected in

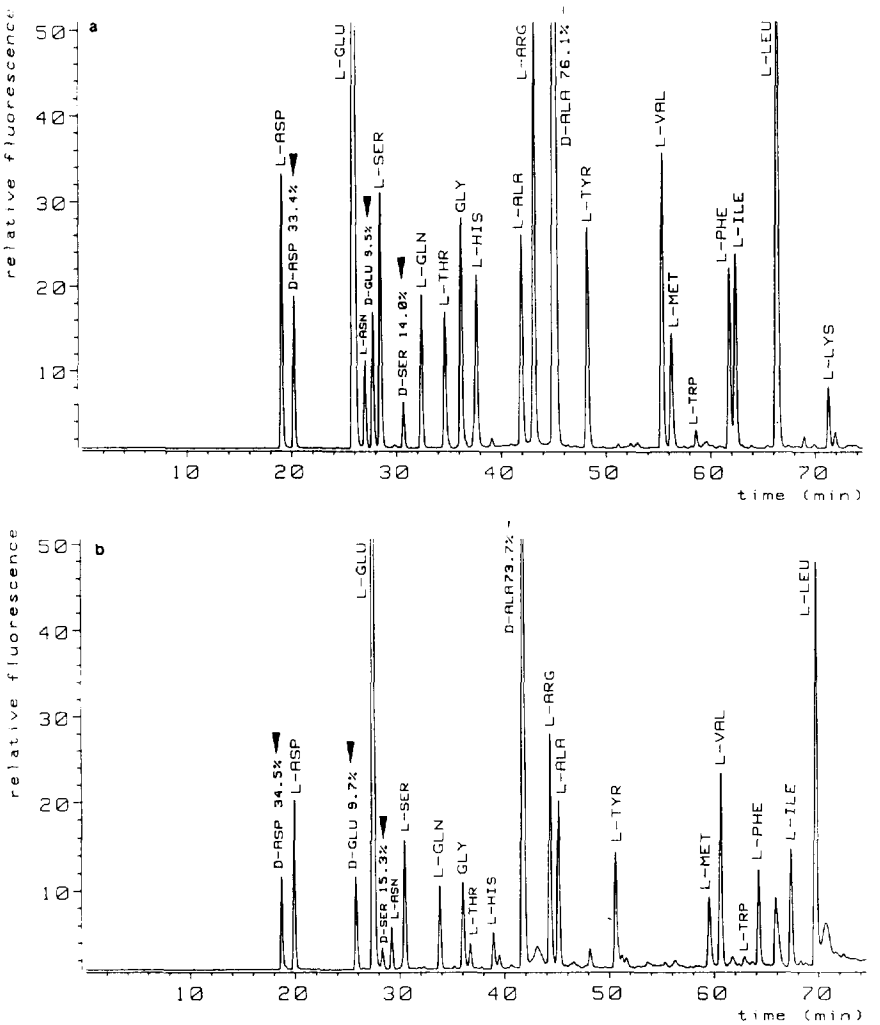

Fig. 7. Aminogram of coumiss derivatized with (a) IBLC and (b) IBDC.

insects [45], in nectar exudates of orchids [46] and in fruits and vegetables [25].

3.10. Yeast spread

In the yeast extract (Fig. 11a and b), amounts of 3.0% (2.7%) D-Asp, 1.8% (2.3%) D-Glu, 5.2% (4.9%) D-Asn, 1.3% (1.2%) D-Ser, 2.3% (2.6%) D-Ala, 1.9% D-Tyr and 1.7% D-Phe were determined (D-Tyr and D-Phe were determinable using the IBLC reagent, but not with the IBDC reagent as a result of the co-elution of the derivatives with the two arising from GABA [21]. It is of interest that dietary yeast also contains D-AA. The high absolute amounts are worth noting: a total of 1.7 g of D-AA in 1 kg of the yeast spread (see Table 1 for amounts of individual D-AA). As yeast extracts are widely used as seasonings in the food industry, these extracts are potential sources of D-AA in foodstuffs. As we have also found ethanol-extractable, free D-AA in baker's yeast, brewer's yeast and wine yeast (relative amounts of 1–2% with respect to the L-enantiomers have been determined by GC for D-Ala, D-Val, D-Ser, D-Asx and

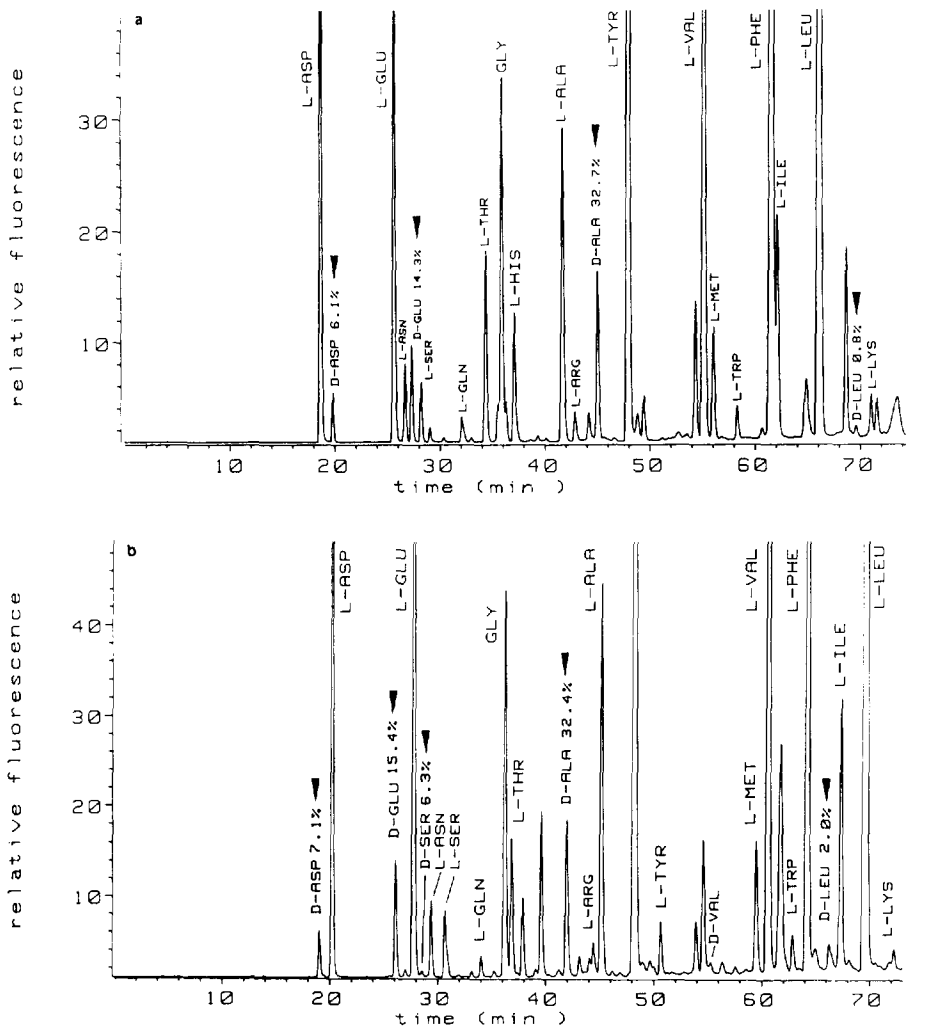

Fig. 8. Aminogram of a French Cantal cheese derivatized with (a) IBLC and (b) IBDC.

D-Glx), it is evident that D-AA also originate from the yeasts used for food production.

3.11. Practical aspects

As with biosamples [21], fully automated pre-column derivatization with OPA and the chiral thiols IBLC or IBDC has proved to be a rapid and reliable method for the indirect determination of the enantiomers of primary AA in foods and beverages. Although in several instances, such as with fruit juices, direct analysis is possible [25], the isolation of food AA using a cation exchanger is advantageous and gives cleaner aminograms and increases the lifetime of the column. Proteins have to be precipitated by addition of 5-sulfosalicylic acid, picric acid or organic additives and fat and other lipophilic compounds should be removed, e.g., by extraction with organic solvents. The suitability of polyamide-6 powder, which leads to partial decolorization of wines and other beverages, has to be further investigated. When low amounts of D-AA have to be determined, precautions with respect to microbial contamination have to be applied [21]. Samples subjected to cation-ex-

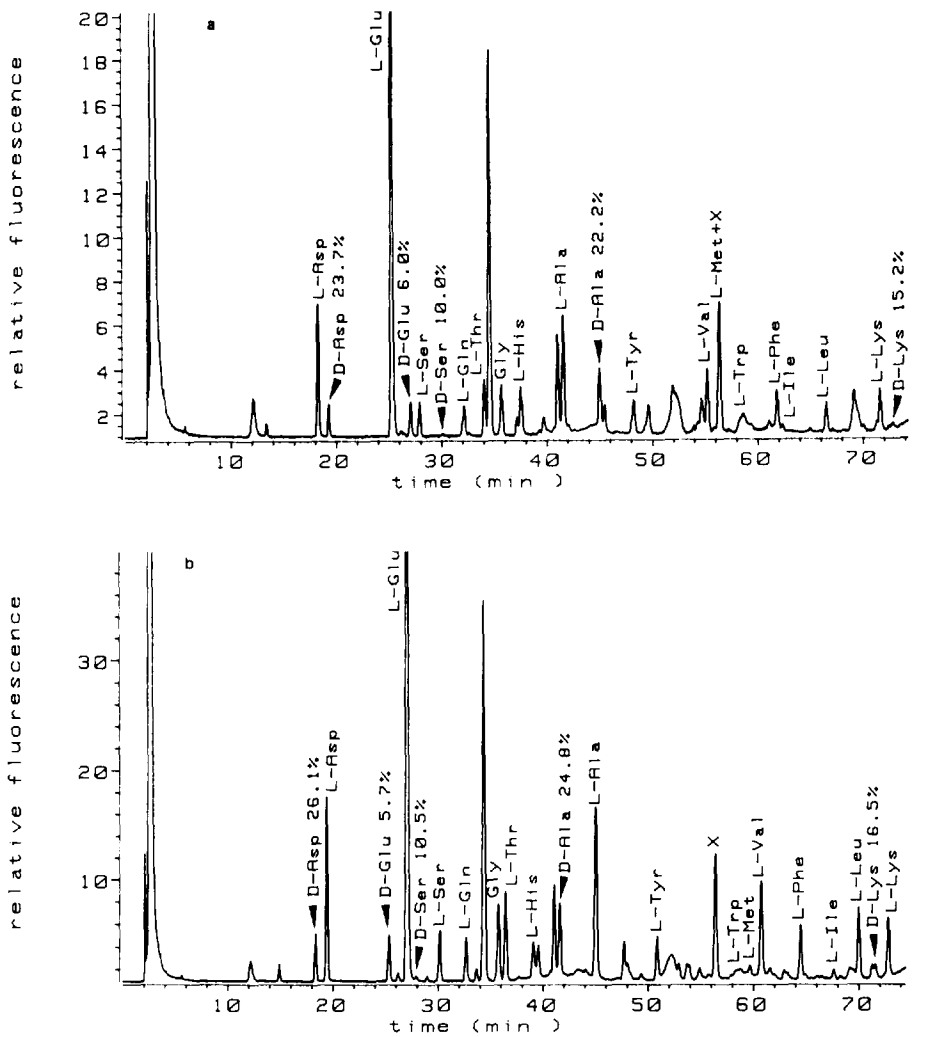

Fig. 9. Aminogram of dietetic whey drink derivatized with (a) IBLC and (b) IBDC.

change treatment should not have a pH value less than 2, as loss of acidic AA has been observed. In some instances low recoveries of the internal standard L-*homo*-Arg were recognized after ion-exchange treatment. This might depend on the food matrix and should therefore be tested. We discarded the ion exchanger after a single use as we have observed a rapid decrease in its capacity and quality, even when thoroughly regenerated, when food and biosamples were applied several times.

An important aspect of this work is that it further supports the thesis that free D-AA are common [34] or ubiquitous [35] constituents of microbially fermented foods and beverages. D-Ala, D-Asx and D-Glx are the D-AA found most frequently in the peptidoglycan [47] of all Eubacteria (i.e., Gram-positive and Gram-negative bacteria) which play a role in food production [31]. These D-AA are the same as those found to be must abundant in foodstuffs. It should be pointed out, however, that the peptidoglycan of several bacteria contains further D-AA such as D-Ser (*Bifidobacterium bifidum*), D-Orn (*Acetobacter woodii*) or D-Lys (*Clostridium innocuum*); these D-AA have also been detected in

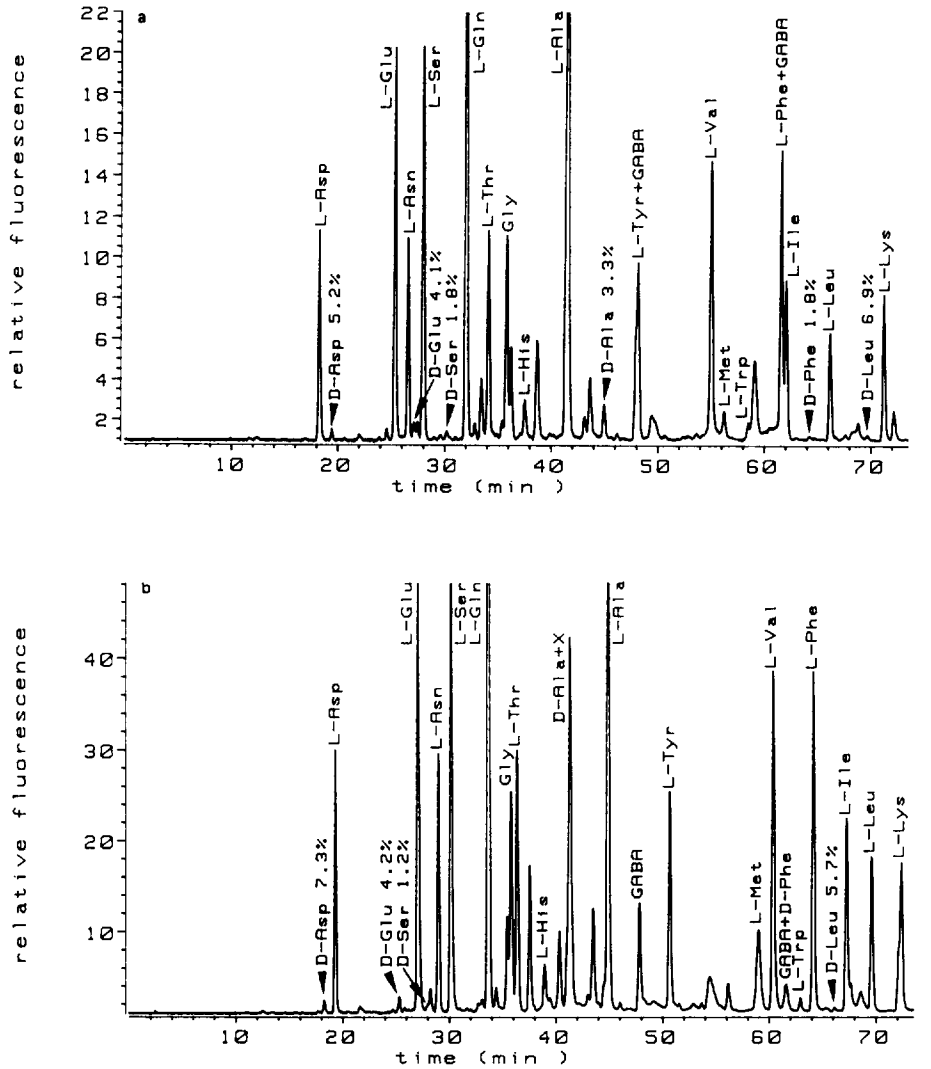

Fig. 10. Aminogram of fir honey derivatized with (a) IBLC and (b) IBDC.

certain foodstuffs. Moreover, a great diversity of D-AA, obviously not involved (or not known to be involved) in the formation of peptidoglycan, such as D-Pro, D-Val, D-Thr, D-Ile, D-*allo*-Ile, D-Leu, D-Phe, D-Tyr and D-Met, has been detected as free AA in the cytoplasm of bacteria used as starters for the production of fermented foods [31]. As shown above, yeasts are also potential sources of D-AA. Remarkably, free D-AA, not directly attributable to microbial activities, have been detected as native constituents of fruits and vegetables [21,25]. From a nutritional point of view, uptake of dietary D-AA is therefore the rule and not the exception as is the case with certain peptide-bonded D-AA that are formed in food proteins when processed severely enough [48–50]. Foods, together with intestinal microorganisms [31], are therefore assumed to be potential sources of the free D-AA that are regularly found in physiological fluids and certain tissues of man and animals (see Ref. [21] and references cited therein).

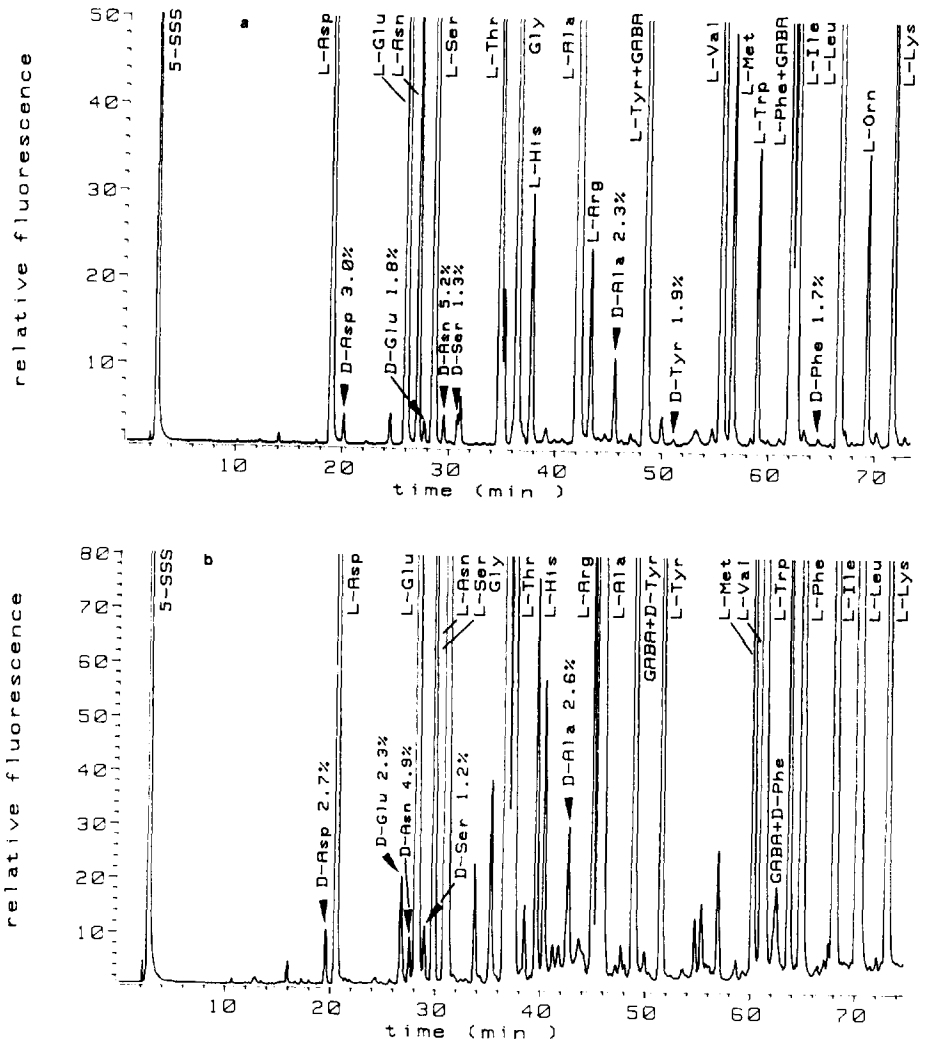

Fig. 11. Aminogram of a yeast spread derivatized with (a) IBLC and (b) IBDC.

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