Amino Acids (1992) 2: 271–284

Amino
Acids

Liquid chromatographic determination of D-amino acids in cheese and cow milk. Implication of starter cultures, amino acid racemases, and rumen microorganisms on formation, and nutritional considerations*, **

H. Brückner[1], **P. Jaek**[1], **M. Langer**[1,3], and **H. Godel**[2]

[1] Institute of Food Technology, University of Hohenheim, Stuttgart
[2] Hewlett-Packard GmbH, Waldbronn Analytical Division, Waldbronn
[3] Untersuchungsinstitut des Sanitätsdienstes der Bundeswehr, Stuttgart
Federal Republic of Germany

Accepted September 12, 1991

Summary. Free L- and D-amino acids (L-AA, D-AA) were isolated from an Appenzeller cheese, from raw milk, and from an ethanolic extract as well as a total hydrolysate of cow's rumen microorganisms, and their relative amounts were determined by reversed-phase high-performance liquid chromatography after derivatization with *o*-phthaldialdehyde together with *N*-isobutyryl-L-(or D)-cysteine. D-Ala, D-Asp and D-Glu were found, among other D-AA in all cases and a microbial origin of free D-AA found in cheese and milk was rationalized. From the results, and taking other findings of the occurrence of D-AA in food and beverages into account, the highest intake of D-AA is to be expected from the consumption of ripened cheeses. From the presence of D-amino acid oxidases in human kidney, liver, and brain and from reports on the intravenous administration of racemic AA to humans and their metabolisation it is concluded that intake of free D-AA found in food is no threat for human beings.

Keywords: Amino acids – High-performance liquid chromatography – *o*-Phthaldialdehyde – *N*-Isobutyryl-cysteine – D-Amino acids – Food – Toxicology – Nutrition – D-Amino acid oxidase – Microorganisms

---

* Presented in part at the "2nd International Congress on Amino Acids and Analogues", Vienna, August 5–9, 1991, and at "Euro Food Chem VI", September 22–26, 1991, Hamburg.

** Dedicated to Prof. Ernst Bayer, University of Tübingen, on the occasion of his 65th anniversary.

Introduction

Relatively few liquid chromatographic methods have been reported attempting the separation of all protein L-amino acids (AA) from their corresponding D-enantiomers. Using a dedicated amino acid analyzer based on the ion-exchange principle, Manning and Moore (1968) separated D- and L-AA as diasteromeric dipeptides obtained by derivatization of the respective AA enantiomers with N-carboxyanhydrides of L-AA. Using such an instrument and the addition of L- or D-Pro and copper(II)sulfate to the elution buffer, Hare and Gil-Av (1979) separated DL-AA as diastereomeric copper complexes. Gübitz et al. (1982) used ligand-exchange chromatography with silica-bonded L-pipecolic acid as well as other L-AA as stationary phases for the separation of mixtures of DL-AA by high-performance liquid chromatography (HPLC), and Armani et al. (1988) separated Dansyl-DL-AA by reversed-phase HPLC using L-AA amides as chiral additives together with copper(II)acetate.

Among other approaches for the separation of mixtures of DL-AA (for a review see Lindner, 1988), which are more or less suitable for the separation of complex mixtures of DL-AA, precolumn derivatization and HPLC separation of a standard of DL-AA with (+)-9-fluorenylethyl chloroformate (Einarsson et al., 1987), is noteworthy, although chromatographic conditions are obviously not easily reproducible (Hofsommer et al., 1989). The use of *o*-phthaldialdehyde (OPA) together with mercaptoethanol or mercaptopropionic acid for the separation of AA was also extended to the resolution of DL-AA by derivatization with OPA together with chiral thiols. It was very recently shown that reversed-phase HPLC of diastereomeric isoindol derivatives of DL-AA formed by reaction of OPA together with *N*-isobutyryl-L-cysteine (IBLC) or *N*-isobutyryl-D-cysteine (IBDC) makes possible the complete separation of a 36-component AA standard with the exception of DL-Pro (Brückner et al., 1991, and references cited therein).

Chromatographic methods for the determination of D-AA are of interest as these AA have been found in microorganisms (Schleifer and Kandler, 1972), animals (Corrigan, 1969), plants (Robinson, 1976), crustaceae (D'Aniello and Giuditta, 1979), bivalve molluscs (Matsushima et al., 1984; Felbeck and Wiley, 1987), marine invertebrates (Preston, 1987), and in food and beverages (Brückner and Hausch, 1989; 1989a; 1990; 1990a; 1991; Brückner and Lüpke, 1991; Palla et al., 1989).

We demonstrate that the fully automated pre-column derivatization of DL-AA with OPA/IBLC or IBDC, separation by HPLC and fluorescence detection of derivatives are highly sensitive and suitable methods for the detection and resolution of DL-AA found in foodstuffs as well as microorganisms. Since highest amounts of free D-AA among foodstuffs are found in ripened cheeses, and low, but significant amounts are present in cow's milk, we also discuss the implication of microorganisms in their formation and some nutritional aspects of D-AA.

Materials and methods

Instruments and column

For chromatography a HP 1090 Series L HPLC system was employed, equipped with a binary DR5 solvent-delivery system, an auto injector and autosampler, a heated column compartment, and an HP 1046A programmable fluorescence detector. The excitation wavelength was set at 230 nm and the emission wavelength at 445 nm. For the printout of the chromatograms a plotter ColorPro model 7440A and ThinkJet printer model 2225A were used (all instruments from Hewlett-Packard GmbH, Waldbronn, Germany). The column (250 mm * 4 mm) and guard column (20 * 4 mm) used was filled with Hypersil ODS 5 μm (Shandon Scientific Ltd, Astmoor, Runcorn, Cheshire, England).

Composition of amino acid standard and derivatizing reagent and sources of chemicals

The AA standard contained L-AA (100 pmol), Gly (100 pmol), and D-AA (50 pmol) in 2 μl 0.1 N HCl; AA were of analytical grade and purchased from either Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO, USA). The derivatizing reagent consisted of 260 mM IBLC (or IBDC) and 170 mM OPA in 1 M potassium borate buffer of pH 10.4 (fluoraldehyde™ dilutant; Pierce, Rockford, Ill., USA, product no. 27035). IBLC and IBDC were prepared in our laboratory (Brückner et al., 1991) and are also obtainable from Novabiochem, Läufelfingen, Switzerland, or Fluka, Buchs, Switzerland, on request. Sodium acetate (Merck) and picric acid (Merck) were of analytical grade, methanol (Merck) was of LichroSolv® quality and acetonitrile (Baker, Deventer, The Netherlands) was of HPLC grade.

Derivatization of standard and samples and gradient elution conditions

A 5 μl amount of 0.4 *N* sodium borate buffer of pH 10.4 (part no. 5061-3330, Hewlett-Packard), 1 μl OPA/IBLC (or IBDC) reagent, and 2 μl of AA standard solution (or 2 μl aliquots of food samples after work-up, see below) were mixed automatically by the derivatization device of the instrument (Brückner et al., 1991). The injector program special commands were 4 mixing cycles, 7 μl-mode; this corresponds to a reaction time of 2 minutes. For gradient elution two mobile phase systems were used: eluent A, 23 mM sodium acetate, adjusted to pH 6.0; eluent B, methanol/acetonitrile (600:50, v/v); linear gradient from 0% B to 53.5% B in 75 min at a flow rate at 1 ml/min.

Sources of milk, cheese, and rumen juice

Milk was from a German "Schwarzbunte" cow being at the end of its first lactation period and was analyzed immediately after milking manually. It showed a bacterial count of 24,000 microorganisms/ml and a cell count of 52,000 cells/ml (determined with a Bactoscan 8000 instrument and Combi-Foss 360 instrument, respectively; Foss Electric A/S GmbH, Hamburg). The cheese was an Appenzeller cheese of Swiss origin and purchased at a local retail outlet. Rumen juice was sucked out from the rumen of a cow (7 year old German "Braunvieh") which had implantated a rumen fistula.

Sample treatment

Appenzeller cheese (0.5 g) and 96% ethanol (30 ml), were heated under stirring at 50°C for 45 min; water (35 ml) was added and heating was continued for a further 15 min. The mixture was centrifuged at 1600 × *g*, the supernatant was concentrated *in vacuo* to a volume of approx. 15 ml, a saturated solution of picric acid in water (10 ml) and 0.1 *N* HCl (10 ml) were added to the supernatant. Proteins precipitated were removed by centrifugation (1600 × *g*), the supernatant was transferred into a separation funnel and extracted with diethyl ether/light petroleum, b.p. 40–60°C (3 × 20 ml), in order to remove lipids. The aqueous phase was passed through a column (bed size 1 cm * 5 cm) packed with Dowex®

50 WX8 cation exchanger (practical grade, 200–400 mesh, from Serva, Heidelberg). The column was washed with water (ca. 60 ml) and the AA adsorbed were desorbed with 2 *N* aqueous ammonia (30 ml). The effluent was evaporated to dryness *in vacuo* (bath temperature 40°C) and the residue was dissolved in 0.1 *N* HCl (2 ml). Aliquots of 2 μl were analyzed by HPLC.

Raw milk (40 ml) and 96% ethanol (80 ml) were stirred for 15 min, centrifuged at 1620 × *g* for 10 min, the supernatant was evaporated to a volume of approx. 10 ml *in vacuo* at 40°C and a saturated solution of picric acid in water (10 ml) and 0.1 *N* HCl (10 ml) were added. The precipitate formed was removed by centrifugation at 1620 × *g* and the supernatant was subjected to Dowex WX8 treatment as described for cheese. The mixture of free AA obtained therefrom was dissolved in 2 ml 0.1 *N* HCl and aliquots of 2 μl were analyzed by HPLC.

Rumen juice (1 l) was sucked from the cow, filtered immediately through several layers of a fine cheese cloth, and centrifuged at 5,000 × *g* for 30 min. The sediment formed was washed with 0.85% aqueous NaCl (3 × 100 ml) with repeated centrifugation and a 1 g amount of the sediment was extracted with 70% aqueous ethanol (3 × 50 ml). An aliquot (5 ml) of the combined supernatants was diluted with 0.1 *N* HCl (5 ml), and filtered by use of a PTFE membrane filter (pore size 0.5 μm, Millex®-SR, from Millipore, Monsheim, France). Free AA were isolated by Dowex® 50 WX 8 treatment and analyzed by HPLC as described for cheese. For a total hydrolysate of rumen microorganisms to an aliquot of the sediment (approx. 50 mg) in a 5 ml "reacti vial" (Wheaton, Millville, NJ, USA), 6 *N* HCl (4 ml) was added and the mixture was hydrolyzed under nitrogen at 110°C for 48 h. After evaporation to dryness *in vacuo* 0.1 *N* HCl (2 ml) was added, the mixture was stirred for 10 min at room temperature, and undissolved material was removed by centrifugation at 1620 × *g*. From the clear supernatant aliquots of 2 μl were used for HPLC.

Results

The HPLC elution profile of a standard, derivatized with OPA/IBLC and composed of 17 L-AA and their corresponding D-enantiomers, achiral Gly and the internal standard L-*homo*-Arg is shown in Fig. la (ratio D:L-AA 1:2 by weight). The reversal of the elution order of the isoindol derivatives by derivatization of AA with OPA/IBDC is shown in Fig. 1b. Derivatization of AA in a sample with IBLC as well as IBDC is therefore valuable for establishing the presence of AA enantiomers through peak reversal of the respective diastereomers (*cf.* Figs. 2 and 3). It is also noteworthy that all diastereomeric isoindol derivatives are satisfactorily separated from each other. The linearity of the method (peak area *versus* concentration) over a range of 1 pmol to 1 nmol was tested for a standard containing 1, 5, 25, 100, 250, or 1000 pmol of each AA (correlation coefficients ≥ 0.998. Different factors for the relative fluorescence yields of diastereomeric pairs of AA enantiomers have been determined (Brückner et al., 1991). The percent relative standard deviation values for all of the retention times were less than 0.6% and for the peak areas less than 1.4% except for His and Lys with 2.5%. The chromatograms of free L-AA and D-AA isolated from Appenzeller cheese and derivatized with OPA/IBLC and OPA/IBDC are shown in Fig. 2a and Fig. 2b, respectively. As can be seen D-Asp (24.8%), D-Glu (6.9%), D-Ala (44.2%), and D-Leu (1.6%) were found by derivatization with OPA/IBLC. By derivatization with OPA/IBDC reversal of the elution order of AA occurred and D-Asp (28.9%), D-Glu (5.7%), D-Ala (45.7%) and D-Leu (1.5%) were found. The relative amount of D-Pro (29.8%) which is also present in Appenzeller cheese was determined by chiral phase gas chromatography according to procedures

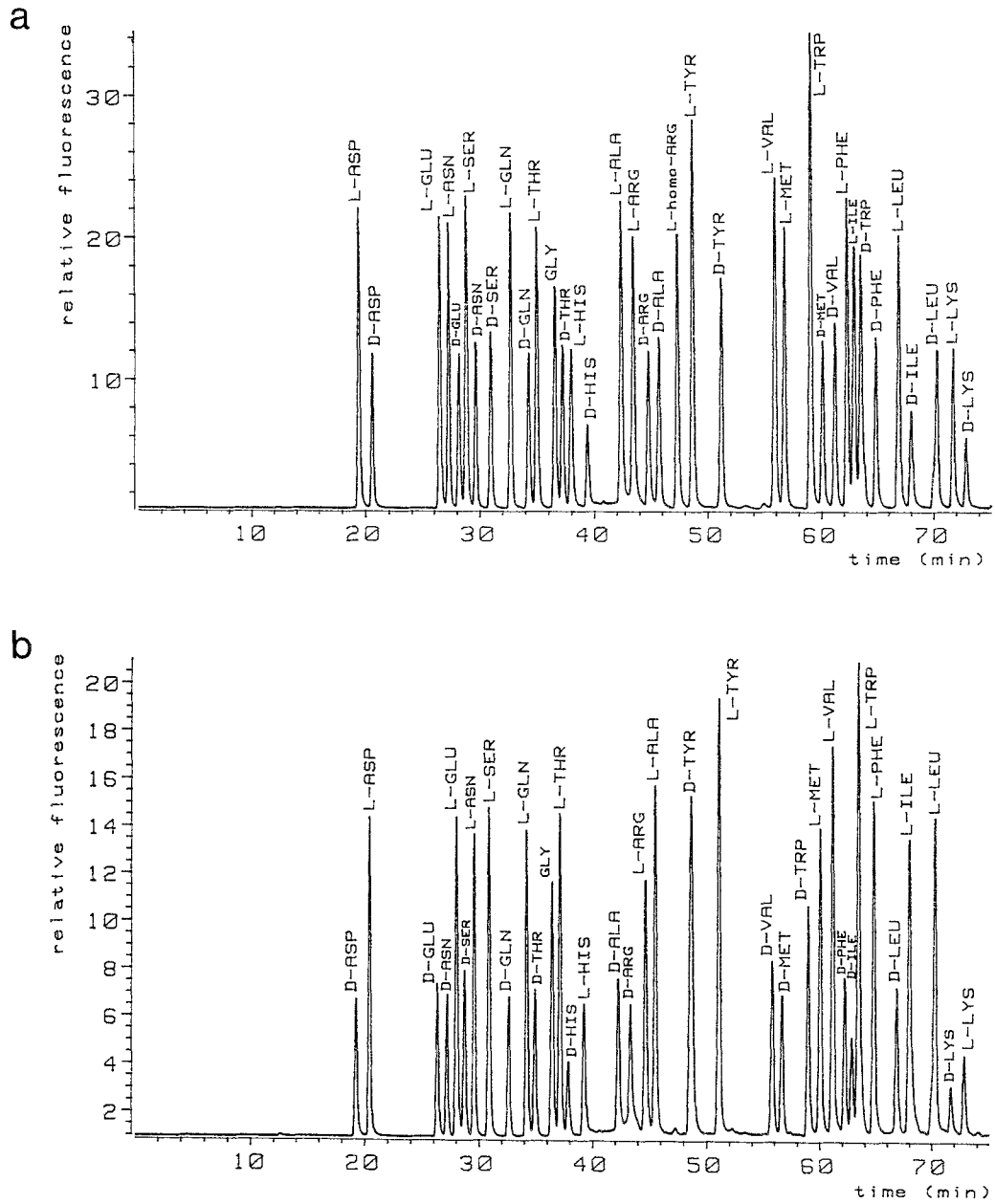

Fig. 1a, b. HPLC elution profiles of a standard of DL-amino acids (molar ratio L:D = 2:1), Gly, and L-*homo*-arginine (internal standard) derivatized with (**a**) *o*-phthaldialdeheyde/*N*-isobutyryl-L-cysteine (OPA/IBLC) and (**b**) OPA/*N*-isobutyryl-D-cysteine (OPA/IBDC). Note the reversal of the elution order of isoindol derivatives formed by derivatization. For chromatographic conditions of Figs. 1–4 see Materials and methods

described (Brückner and Hausch, 1990a), as imino acids, such as Pro and Hyp, fail to react with OPA/IBLC or OPA/IBDC. In this sample of Appenzeller cheese a total amount of 1770 mg AA were found in 100 g cheese, the combined amount of D-Ala, D-Pro, D-Asp, and D-Glu was 89 mg in 100 g cheese. This corresponds to totally 5832 mg AA in 100 g fat free dry matter (f.f.d.m.) and 295 mg

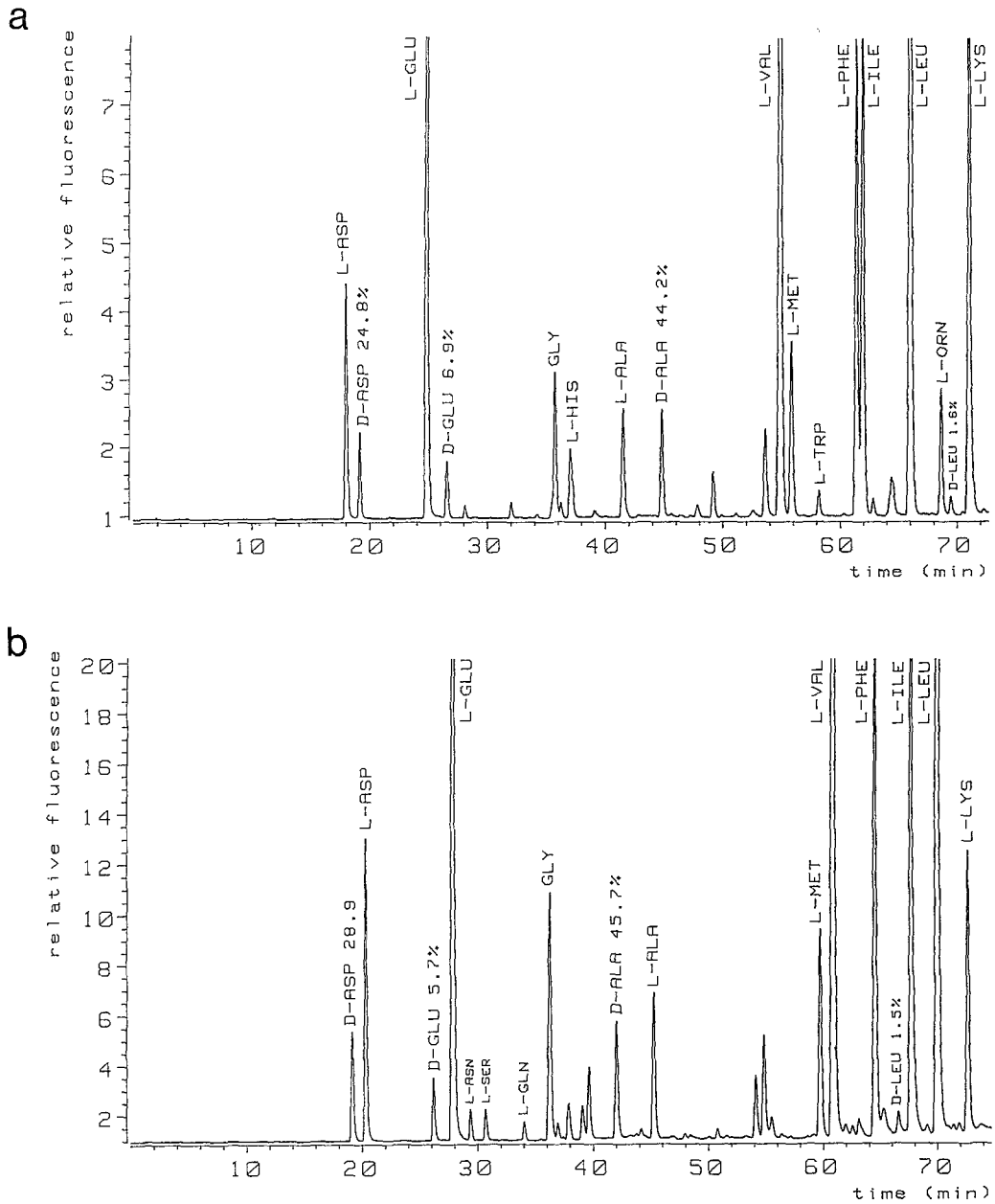

Fig. 2. HPLC elution profile of amino acids isolated from Appenzeller cheese; derivatization with (**a**) OPA/IBLC, and (**b**) OPA/IBDC. For origin and treatment of samples of Figs. 2–4 see Materials and methods

of the above D-AA in 100 g f.f.d.m. For comparison the relative amounts of D-AA determined by GC in an old Gouda cheese, ripened for > 1 year are given (chromatogram not shown): D-Ala (44.2%), D-Asx (19.8%), D-Thr (12.9%), D-Glx (4.0%), D-Ser (1.9%), D-Leu (0.14%). Totally 6373 mg free AA were found in this cheese; the combined amount of D-AA was 285 mg in 100 g Gouda or 537 mg D-AA in 100 g f.f.d.m. (for HPLC of another Gouda cheese cf. Langer et al., 1991).

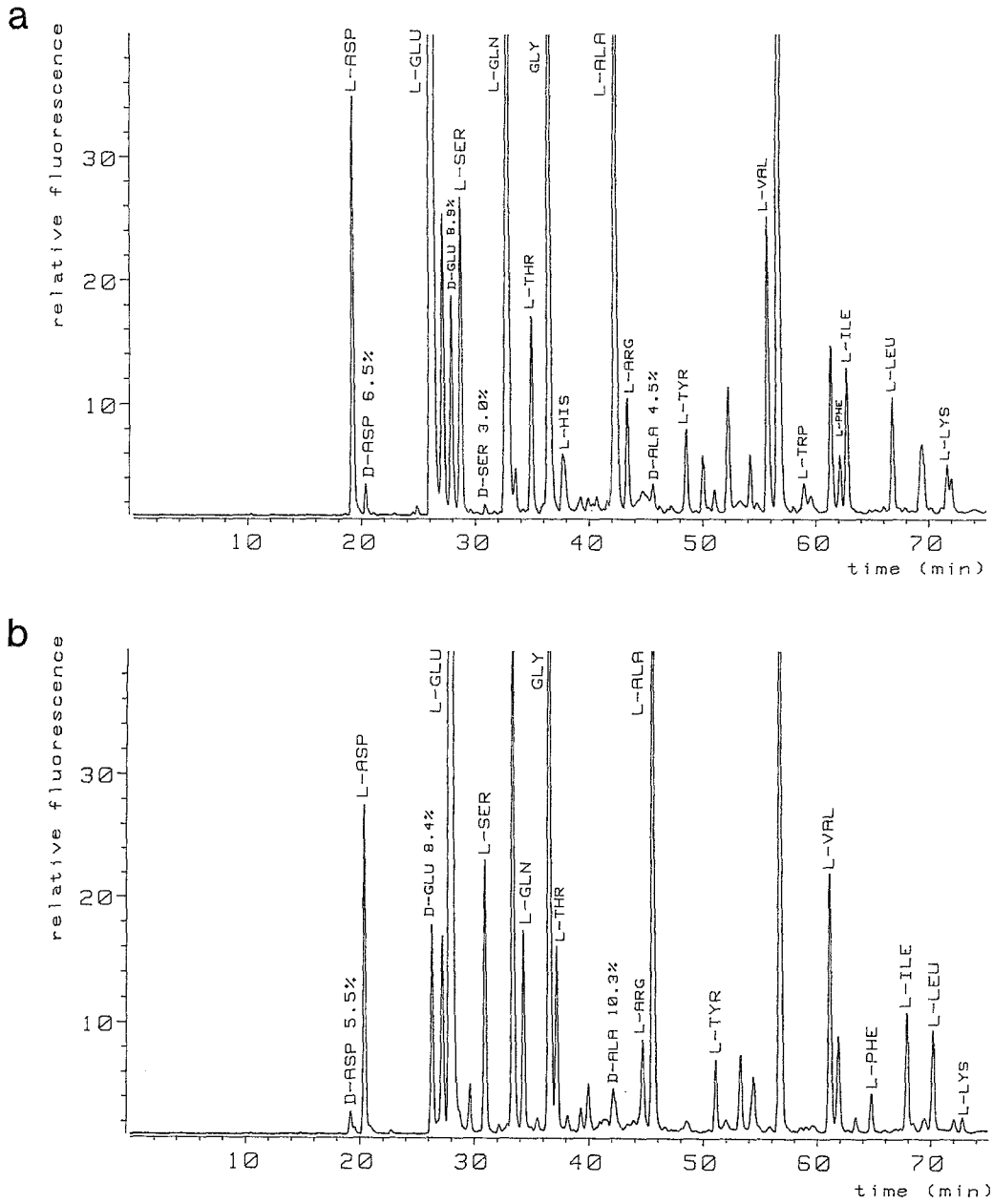

Fig. 3a, b. HPLC elution profile of amino acids isolated from cow's raw milk; derivatization with (**a**) OPA/IBLC, and (**b**) OPA/IBDC

The chromatogram of free AA isolated from a raw milk sample and derivatized with OPA/IBLC is shown in Fig. 3a. D-Asp (6.5%), D-Glu (8.9%), D-Ser (3.0%) and D-Ala (4.5%)) were found. The results are also confirmed by the opposite elution order of diastereomers of this sample obtained by derivatization with OPA/IBDC (Fig 3b). D-Asp (5.5%), D-Glu (8.4%), D-Ala (10.3%) were found; D-Ser elutes as a shoulder of L-Glu (Fig. 3b). These results confirm gas chromatographic data on the occurrence of free D-AA in cow milk reported earlier (Palla et al., 1989; Brückner and Hausch, 1989, 1990; Langer et al., 1991).

The AA elution profiles of an ethanol/water extract of AA from microorganisms from a cow's rumen, and of a total hydrolysate of these microorganisms, derivatized with OPA/IBLC are shown in Figures 4a and 4b, respectively. As can be seen (Fig. 4a) derivatization with OPA/IBLC revealed D-Asp (7.1%), D-Glu (6.7%), and D-Ala (29.5%) (D-Leu coeluted with an impurity and was not determinable). In the total hydrolysate of the rumen microorganisms relative amounts of D-Asp (9.8%), D-Glu (7.9%), D-His (38%), D-Ala (7.8%), D-Tyr

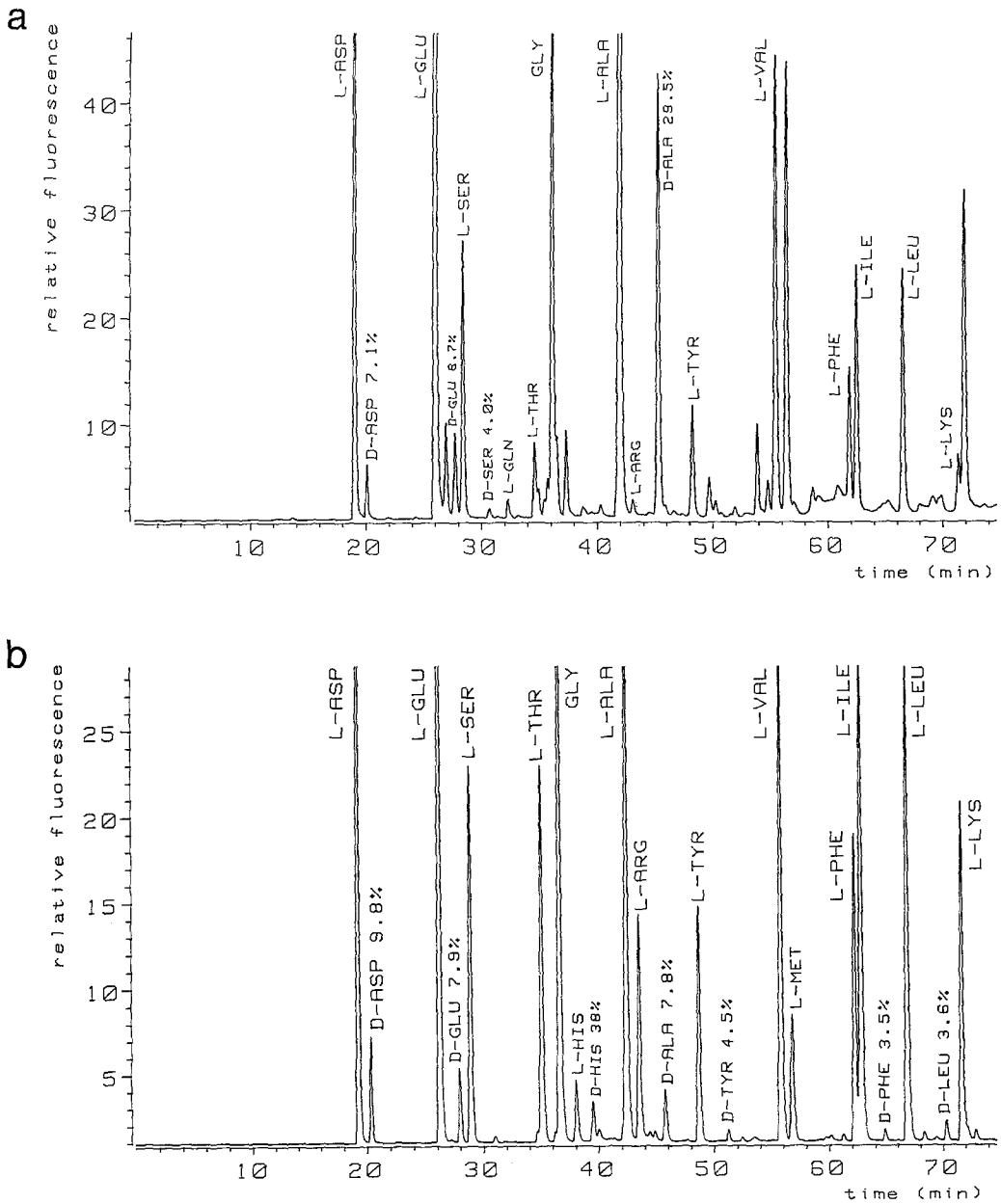

Fig. 4a, b. HPLC elution profile of amino acids from (**a**) an ethanolic extract of rumen microorganisms, and (**b**) a total hydrolysate of rumen microorganisms; derivatization with OPA/IBLC

(4.5%), D-Phe (3.5%) and D-Leu (3.6%) were found (Fig. 4b). These results were also confirmed by derivatization with OPA/IBDC (chromatograms not shown).

Discussion

It is demonstrated that the fully automated derivatization of L- and D-AA with OPA and IBLC or IBDC and separation of the resulting diastereomeric isoindol derivatives by reversed-phase high-performance liquid chromatography is a highly suitable method for the detection and quantification of free L-and D-AA isolated from complex matrices such as ripened cheeses, raw milk and in rumen microorganisms.

In comparison with gas chromatographic procedures using chiral stationary phases such as Chirasil-L-Val® or Chirasil-D-Val® (Brückner and Lüpke, 1990), the advantage of the described method is that acid-labile AA such as Asn, Gln, and Trp, and the basic AA His and Arg, are routinely determinable and do not require special care and that the derivatization procedure is fully automated. The disadvantages are that enantiomers of Pro and Hyp are not yet determinable and cysteine and cystine (which, however, were not present in the samples investigated) would give fluorescent derivatives with relative fluorescence yields of $<5\%$ of that of Ala. In the case of the Appenzeller cheese L- and D-Pro were determined by GC as described (Brückner and Hausch, 1990a).

Origin of D-amino acids in cheese

The analytical data also make it possible to draw conclusions on the origin of D-AA in cheese and raw milk. Ripened cheeses are manufactured from raw or pasteurized milk by the addition of rennet and bacterial starter cultures and receive their final texture and flavour through the action of an agency of enzyme systems produced by bacteria which are continuously changing both in numbers and species during cheese ripening. The most frequently used bacteria for cheese manufacturing (Scott, 1986), are species of *Lactobacilli*, *Streptococci*, *Leuconostoc* or *Propionibacteria*, but others originating from the environment also play an important role. In the case of Appenzeller cheese, which has been selected as an example of a ripened cheeese, mixed bacterial cultures composed of selected species such as *Lactobacillus delbrueckii spp. lactis*, *Lb. helveticus*, *Lb. brevis*, *Streptococcus cremoris*, *Sc. lactis*, *Sc. diacetylactis*, and *Leuconostoc cremoris* are usually used. Occasionally typical joghurt starters such as *Lactobacillus delbrueckii* spp. *bulgaricus* or *Streptococcus thermophilus* are also added together with *Propionibacterium shermanni*. Appenzeller cheese is usually ripened for 4–9 months by periodically smearing the surface with so-called "sulz", that is a mixture of yeast (*Saccharomyces cerevisiae*) salt, and spices in white wine or cider.

The cell walls of gram-positive and gram-negative bacteria are composed of polysaccharides crosslinked by oligopeptides which contain D-Ala, D-Asx (Asx = aspartic acid, asparagine or isoasparagine), and D-Glx (Glx = glutamic acid, glutamine or isoglutamine) as most frequent D-AA (Schleifer and Kandler,

1972; Tipper and Wright, 1979). These D-AA are also those found most abundantly in ripened cheeses, as well as in other fermented food and beverages. Other D-AA such as D-Lys, D-Orn, D-Ser, D-Pro have also been found in bacterial peptidoglycans (Schleifer and Kandler, 1972; Bottazzi, 1988). Further, the respective D-enantiomers of most protein L-AA have been found in the free state in the cell pool of certain bacteria (Bhattacharyya and Banerjee, 1974). The formation of D-AA in bacteria, therefore, is essential for their life cycles, and this explains that enzymes catalyzing either racemization or epimerisation of all AA occure in bacteria (Adams, 1972).

Using GC we found the following amounts of D-AA in a totally hydrolyzed strain of the frequently used starter *Lactobacillus casei*: D-Asx (20.4%), D-Glx (45.8%), D-Ala (63.5%), D-Leu (25.0%), D-*allo*-Ile (22.1%), D-Val (4.8%), and D-Ser (18.8%). In a total hydrolysate of *Lactobacillus acidophilus* the following relative amounts of major D-AA were found: D-Asx (14.6%), D-Glx (12.6%), and D-Ala (37.2%), Brückner and Hausch (1990); (amino acid amides are hydrolysed to their respective acids under acidic conditions).

Therefore it is certainly justified to assume that the relatively high amounts of D-AA found in ripened cheeses originate from the action of microorganisms implicated in the cheese manufacturing and ripening process. This is also in agreement with the findings that free D-AA occur in all fermented dairy products (Brückner and Hausch 1990, 1990a) as well as fermented food and beverages in general (Brückner and Hausch, 1989; 1989a; 1990b; Langer et al., 1991).

From the results reported here and elsewere (Brückner and Hausch, 1989; 1989a; 1990a) it is obvious that ripened cheeses are the food with highest amounts of free D-AA consumed by mankind (drastically alkali-treated food is not considered as suitable for human nutrition). Consumption of Appenzeller cheese, old Gouda cheese, or Parmesan cheese (Brückner and Hausch, 1990b), raclette cheese or cheese fondue might result in an intake of several hundred milligrams of D-AA in total (for discussion of physiological effects see section "Nutritional considerations").

Origin of D-amino acids in raw milk

Raw milk is usually contaminated with a certain degree of environmental microorganisms as a result of the milking procedure, sampling, storage etc.. Further, udder infection (mastitis) might contribute to microorganisms found in raw milk. Therefore, in order to prevent microbial growth, bulk milk used for dairying is immediately cooled to approx. 4°C during sampling and storage. However, even in chilled milk psychrotrophic bacteria are able to grow (Cousins and Bramley, 1981).

If the microbial count is high enough the bacterial indicator amino acids, namely D-Ala, D-Asp, and D-Glu, will be detectable and, if bacteria are allowed to grow vigorously, souring of the milk and finally curdling will occure. In the case of the raw milk sample investigated (*cf.* Fig. 4) the low microbial count of 24×10^3 microorganims/ml is not assumed to be responsible for the D-AA found in this sample or for similar amounts of D-AA in a number of other raw milk samples investigated.

A reasonable explanation for the occurrence of a background level of certain D-AA is given by the special physiology of ruminants. Briefly, ruminants cover to a large extent their need for nitrogen and amino acids by digestion of rumen microorganisms in their intestinal tract, *viz.* mainly anaerobic bacteria of the genus *Bacterioides*, *Ruminococcus*, and *Butyrivibrio*, but also of protozoa (Hungate, 1966).

Chromatograms shown in Fig. 4a and 4b demonstrate that rumen microorganisms also contain extractable, free D-AA as well as those released by total hydrolysis. Those found most abundant in cow milk, i.e. D-Ala, D-Asp and D-Glu are also the most prominent found in the cell pool and in the total hydrolysate of rumen microorganisms.

It is reasonable to conclude that the low but significant background level of D-AA found in raw milk is the result of the digestion and autolysis of rumen microorganisms. They might enter the nortal blood stream via the mucosa of the small intestine and, finally, the alveoles of the mammary gland, despite the fact that in the liver of the cow (as well as human beings) D-amino acid oxidases are found (Neims et al., 1966; Barker and Hopkinson, 1977; Tokuhisa et al., 1981; Konno and Yasumura, 1981). Although the majority of D-AA are oxidased with D-AA oxidases it seems that minute amounts of certain D-AA were not affected by the liver enzymes. It is also not to exclude that very small amounts of D-AA could be transported by the lymph system following direct uptake of these acids by the mesemeric veins (Holmes and Wilson, 1987).

Nutritional considerations

From reports on the toxicity of certain D-AA administered to experimental animals (Ganote et al., 1974; Cherken et al., 1978; Kampel et al., 1991) the question of possible noxious effects of D-AA to mankind has to be addressed. Although the role of dietary D-AA has been reviewed (Man and Bada, 1987; Friedman and Gumbmann, 1989) we feel, however, that in the respective discussion the wealth of information available from the use of mixtures of racemic AA used for parenteral nutrition of human beings has not yet been sufficiently recognized. These findings, however, are of major importance as at the beginning of parenteral nutrition mainly DL-AA had been administered (Bansi et al., 1964). Racemic AA have still been used until very recently; as an example Alvesin® is mentioned (Heine et al., 1983). In parenteral nutrition much higher amounts of D-AA were administered intravenously than would be expected to be consumed by the intake of foodstuffs.

Using the ^{15}N-tracer technique (Heine and Drescher, 1975; Heine et al., 1983) it was shown that most D-AA administered parenterally are mainly excreted renally in unmodified form and, in part, as urea or are used as nonspecific nitrogen sources. That D-Ala, however, can be metabolized relatively well by humans is explained that it is subject to enzymic oxidative deamination yielding the respective α-keto acid which is, in part, reaminated to yield L-AA (Heine et al., 1983). Using a deuterium labelling technique Tokuhisa et al. (1981) showed that orally administered D-Phe was inverted to L-Phe at a reasonably rapid rate. D-amino acid oxidases have been found in human kidney and liver (Barker

and Hopkins, 1977) which are capable of oxidising all D-enantiomers of the respective protein L-AA, inclusive those for which toxicity in experimental animals has been reported. It is noteworthy that D-amino acid oxidase isolated from human liver shows highest oxidation rate among D-AA for D-Pro (Krebs, 1948; Konno and Yasumura, 1981), and a medium oxidation rate for D-Ser. It has been pointed out that no toxic effects have been found by intravenious administration of chemically pure, racemic AA to adults and infants (Bansi et al., 1964; Heine and Drescher, 1975; Heine et al., 1983). In conclusion as no toxic effects attributable to D-AA have been reported by intravenous administration of decagramm amounts of racemic D-AA to adults and infants this is not to be expected for free D-AA consumed by food intake.

From the results reported here and elsewhere (Brückner and Hausch, 1989; 1990a) we conclude that the highest intake of dietary D-AA is to be expected by consumption of certain ripened cheeses such as old Gouda cheese (see above), Appenzeller cheese, Parmesan cheese (Brückner and Hausch, 1990b), raclette cheese, or cheese fondue (usually a mixture of Emmental cheese – for D-AA *cf.* Brückner and Hausch, 1990a – together with Gruyere cheese or Appenzeller cheese). In particular in the latter cases 100–200g might be consumed during a meal which might contain several hundred milligrams of D-AA in total. From the discussion above and taking into account the presence of D-AA oxidases, including D-aspartic acid oxidase, in human liver, kidney, and brain (Krebs, 1948; Neims et al., 1966; Barker and Hopkinson, 1977), as well as the finding of free D-AA in human plasma (Nagata et al., 1987) the effects of free D-AA consumed in food are not expected to be harmful. Whereas the formation of protein-bonded D-AA by intensive technological treatment of food proteins (Masters and Friedman, 1980; Tovar and Schwass, 1983; Liardon and Hurrell, 1983) is avoidable in most cases by changing the technological process, formation of free D-AA in fermented foodstuffs, in contrast, is a result of the action of microorganisms implicated in the respective fermentation processes. D-AA, therefore, are omnipresent in fermented foods and beverages (Brückner and Hausch, 1989a, 1990b) and their formation is unavoidable.

Acknowledgements

We thank Dr Steingaß, Institut für Tierernährung, for collecting rumen juice from a cow with fistula, Dr Birnkammer, Versuchsstation für Landwirtschaftliche Betriebswirtschaft und Agrartechnik for providing raw milk, the Milchprüfring Stuttgart for milk analyses for microbial and somatic cell count, and Prof. Dr. Fürst, Institut für Biologische Chemie und Ernährungswissenschaft for valuable discussion.

References

Adams E (1972) Racemases and epimerases. In: Boyer PD (ed) The enzymes, vol 6. Academic Press, New York, pp 479–507
Armani E, Barazzoni L, Dossena A, Marchelli R (1988) J Chromatogr 441: 287–298
Bansi HW, Jürgens P, Müller G, Rostin M (1964) Klin Wochenschr 42: 332–352
Barker RF, Hopkinson DA (1977) Ann Hum Genet Lond 41: 27–42
Bhattacharyya SR, Banerjee AB (1974) Folia Microbiol 19: 43–50
Bottazzi V (1988) Biochimie 70: 303–315

Brückner H, Hausch M (1989) J High Resol Chromatogr 12: 680–684
Brückner H, Hausch M (1989a) Chromatographia 28: 487–492
Brückner H, Hausch M (1990) Milchwissenschaft 45: 357–360
Brückner H, Hausch M (1990a) Milchwissenschaft 45: 421–425
Brückner H, Hausch M (1990b) D-Amino acids as ubiquitous constitutents in fermented food. In: Lubec G, Rosenthal GA (eds) Amino acids: chemistry, biology and medicine. Escom Science, Leiden, pp 1172–1182
Brückner H, Lüpke M (1991) Chromatographia 31: 123–128
Brückner H, Wittner R, Godel H (1991) Chromatographia 32: 383–388
Cherken A, Davis JL, Garman MW (1978) Parmacol Biochem Behav 8: 623–625
Corrigan JJ (1969) Science 164: 142–149
Cousins CM, Bramley AJ (1981) The microbiology of raw milk. In: Robinson RK (ed) Dairy microbiology, vol 1. Applied Science Publishers, London, pp 119–163
D'Aniello A, Giuditta A (1979) Comp Biochem Physiol 66B: 319–322
Einarsson S, Josefsson B, Möller P, Sanchez D (1987) Anal Chem 59: 1191–1195
Felbeck H, Wiley S (1987) Biol Bull 173: 252–259
Friedman M, Gumbmann R (1989) Dietary significance of D-amino acids. In: Friedman M (ed) Absorption and utilization of amino acids, vol 2. CRC Press, Boca Raton, FA, pp 173–190
Ganote CE, Peterson DR, Carone FA (1974) Am J Pathol 77: 269–276
Gübitz G, Juffmann F, Jellenz W (1982) Chromatographia 16: 103–106
Hare PE, Gil-Av E (1979) Science 204: 1226–1228
Heine W, Drescher U (1975) Dtsch Gesundheitswesen 30: 1563–1566
Heine W, Wutzke K, Drescher U (1983) Clin Nutr 2: 31–35
Hofsommer HJ, Klein I, Grüning J, Höpker HR (1989) Flüssig Obst 56: 646–651
Holmes CW, Wilson GF (1987) Milk production from pasture. Butterworths, Wellington, New Zealand, pp 88–106
Hungate R (1966) The Rumen and its microbes. Academic Press, New York
Kampel D, Kupferschmidt R, Lubec G (1991) Toxicity of D-proline. In: Lubec G, Rosenthal GA (eds) Amino acids: chemistry, biology and medicine. Escom Science, Leiden, pp 1164–1171
Konno R, Yasumura Y (1981) Zoological Magazine Jpn 90: 368–373
Krebs HA (1948) Biochem Soc Symp Camb 1: 2–19
Langer M, Wittner R, Jaek P, Godel H, Brückner H (1991) Determination of amino acid enantiomers in food and beverages by HPLC and GC. In: Baltes WJ, Eklund T, Fenwick R, Pfannhauser W, Ruiter A, Thier HP (eds) Strategies for food quality control and analytical methods in Europe. Proceedings of Europ Food Chem VI, Hamburg, September 22–26, 1991. Behr's Verlag, Hamburg, pp 385–390
Liardon R, Hurrell RF (1983) J Agric Food Chem 31: 432–437
Lindner W (1988) Indirect separation of enantiomers by liquid chromatography. In: Zief M, Crane LJ (eds) Chromatographic separations. Dekker, New York Basel, pp 91–129
Man EH, Bada JL (1987) Ann Rev Nutr 7: 209–225
Manning JM, Moore S (1968) J Biol Chem 243: 5591–5597
Masters PM, Friedman M (1980) Am Chem Soc Symp Ser 123: 165–194
Matsushima O, Katayama H, Yamada K, Kado Y (1984) Mar Biol Lett 5: 217–225
Nagata Y, Akino T, Ohno K, Kataoka Y, Ueda T, Sakurai T, Shiroshita K-I, Yasuda T (1987) Clin Sci 73: 105–108
Neims AH, Zieverink WD, Smilack JD (1966) J Neurochem 13: 163–168
Palla G, Marchelli R, Dossena A, Casnati G (1989) J Chromatogr 475: 45–53
Preston RL (1987) Comp Biochem Physiol 87B: 55–62
Robinson T (1976) Life Sci 19: 1097–1102
Schleifer KH, Kandler O (1972) Bact Rev 36: 407–477
Scott R (1986) Cheesemaking practice, 2nd edn., Elsevier, London
Tipper DJ, Wright A (1979) The structure and biosynthesis of bacterial cell walls. In: Sokatch JR, Ornston LN (eds) The bacteria, vol. 7. Academic Press, New York, pp 291–426

Tokuhisa S, Saisu K, Naruse K, Yoshikawa H, Baba S (1981) Chem Pharm Bull Jpn 29: 514–518
Tovar RL, Schwass DE (1983) Am Chem Soc Symp Ser 234: 169–185

Authors' address: Prof. Dr. H. Brückner, Institute of Food Technology, University of Hohenheim, D-W-7000 Stuttgart 70, Federal Republic of Germany.

Received August 5, 1991