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Determination of L- and D-amino acids in foodstuffs by coupling of high-performance liquid chromatography with enzyme reactors

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Summary. A technique is described for the enantiomeric determination of L- and D-amino acids. It works on the principle that the separation efficiency of high-performance liquid chromatography is coupled with the specificity of enzymes and the sensitivity of electrochemical detection. After separation on a lithium cation-exchange column the amino acids are converted into keto acids and hydrogen peroxide under catalyzation of L- or D-amino acid oxidase. Hydrogen peroxide is detected amperometrically. The method has been tested by the analysis of beer, port, sherry, wine and fruit juice.
A main emphasis was put onto the determination of D-alanine which can serve as an indicator for bacterial contamination. It is shown that a coupling of HPLC with enzyme reactors is a suitable technique for the rapid detection of this marker.

Keywords: Amino acids – Enzyme reactor – HPLC – D-Alanine – Microorganisms – Biosensor – Amino acid oxidase

Introduction

Quality control becomes more and more important both for industry as a cost factor and a question of image-building and as a part of consumer protection. Therefore, especially in the field of food analysis, the demand for specific, sensitive and also rapid analytical techniques increases. There have been several chromatographic methods reported which aim at the chiral separation of L- and D-amino acids (AA). In many attempts using chiral phases or after suitable derivatization it has been tried to separate the enantiomers by means of gas or liquid chromatography (for a review see Hare et al., 1985; Perrett, 1985; Engel and Hare, 1985; Brückner et al., 1991 and references therein).

Another different determination possibility arises from the combination of biosensors with HPLC followed by electrochemical detection. In this technique the separation efficiencies of HPLC are combined with the

specificity of enzymes and the sensitivity of an electrochemical detector, so that an extremely specific "filtration" and determination of certain substances even in complex matrices like there are in food analysis can be obtained. Generally, only a simple sample pretreatment is required, isolation and derivatization steps can be omitted. Further information on coupling of enzyme reactors with HPLC can be found in former publications (Galensa et al., 1988; Schwedt and Stein, 1994; Wagner and Guilbault, 1994). For the determination of the AA enantiomers commercially available L- and D-amino acid oxidases (AAOD) are immobilized and integrated into a HPLC system behind the column (Marko-Varga et al., 1991; Mögele et al., 1992; Voß and Galensa, 1999). The oxidative deamination of amino acids by AAOD leads to the formation of hydrogen peroxide which is detected electrochemically:

$$H_2N - CH(R) - COOH + H_2O + O_2 \xrightarrow{AAOD} O = CR - COOH + H_2O_2 + NH_3$$

Above all the determination of D-AA is of interest as these AA play an important role in the metabolism of microorganisms, although they have been found in higher developed organisms as well. Furthermore, they are components of the peptidoglycan moiety of the bacterial cell wall and can therefore be brought into foodstuffs by active bacterial cultures. Especially D-alanine is of high interest because besides D-glutamic acid it is common in bacteria (Brückner et al., 1994, 1995; Bugg and Walsh, 1992; Gandolfi et al., 1994; Man and Bada, 1987; Venturi et al., 1996). In addition D-AA may serve as indicators for adulteration e.g. additive of racemic AA to fruit juices (Hofsommer et al., 1989).

In this context the following paper presents the automated HPLC-enzyme reactor coupling as a technique which is suitable for the determination of L- and D-AA in foodstuffs. Furthermore, the method optimized for the determination of D-alanine allows the very sensitive detection of bacterial contaminations. The efficiency of the method is demonstrated with the investigation of several fermented and non-fermented beverages and beverage starter materials.

Materials and methods

Instruments and column

For chromatography a Beckman System Gold High Performance Liquid Chromatograph was employed equipped with two 116 Programmable Solvent Modules, a 502 Autosampler and an Analog Interface Module 406A (Beckman Coulter, Unterschleißheim, Germany). One of the pumps was controlled by a NEC PC-8300 (NEC Electronics, Düsseldorf, Germany). For the degassing of the eluents a gas expeller Degasys DG-1210 from uniflows (Tokyo, Japan) was used. The detection unit consisted of an electrochemical detector EP30 from Biometra (Göttingen, Germany), combined with a flowcell with a silane modified platinum sensorchip from Trace (Braunschweig, Germany). Besides a Valve Actuator (Sykam, Gilching, Germany), a column oven (Techlab, Erkerode, Germany), a mixing chamber (YMC Europe, Schermbeck,

Germany) and an additional pulse-damper (ESA, Chelmsford, Massachusetts, USA) were utilized. For the analysis of the chromatograms we used an IBM Personal System/1, System Gold-Software, Version 7.11 U, for the printout a Canon bubble jet printer 200ex. The analytical column was a lithium cation-exchange column (100 mm × 4 mm i.d.) from Pickering Laboratories (Mountain View, California, USA) with either a guard column (20 mm × 4 mm i.d.) from Pickering or a cation-exchange guard cartridge PRP-X200 (20 mm × 4 mm i.d.) from CS (Langerwehe, Germany).

Enzymes and immobilization

The specific reaction of the enantiomers was achieved by the use of L-amino acid oxidase (L-AAOD, E.C. 1.4.3.2., isolated from *Crotalus durissus* venom) and D-amino acid oxidase (D-AAOD, E.C. 1.4.3.3., isolated from porcine kidney) respectively. The enzymes were purchased either from Sigma, Fluka (Deisenhofen, Germany) or Boehringer Mannheim (Mannheim, Germany). The enzyme amounts used for immobilization were about 3 units (L-AAOD) and 70 units (D-AAOD). One unit L-AAOD was defined to oxidatively deaminate 1 μmole of L-phenylalanine per min at pH 6.5 and 37°C. One unit D-AAOD was defined to oxidatively deaminate 1 μmole D-alanine to pyruvate per min at pH 8.3 and 25°C in the presence of catalase. The oxidases were immobilized on controlled pore glass (Trisoperl amino 50–100 μm, Schuller, Wertheim, Germany) which was activated with glutardialdehyde. Therefore, 70 mg Trisoperl per reactor were suspended in 10 mL 2.5% glutardialdehyde solution in 0.1 M phosphate buffer (pH 7.5). The reaction was carried out under reduced pressure (water jet pump) for 30 minutes, afterwards for another 2 hours at atmospheric pressure. The glass pearls were washed with 5 × 10 mL buffer. The activated carrier was suspended with the respective enzyme in 4 mL 1 M phosphate buffer, pH 7.5. The reaction time was about 24 hours. Afterwards the composition was washed with 0.1 M phosphate buffer (pH 7.5) and was filled into plastomer reactor cartridges (8 mm × 6 mm i.d., Trace). These could be screwed directly onto the flowcell with the aid of special reactor holders (Trace). When not in use, the reactors were stored in 0.1 M phosphate buffer, pH 7.5, including 0.005% sodium azide at 4°C.

Composition of standards, eluents and sources of other chemicals

The L- and D-AA standards contained between 2 and 20 mg/L of each chiral AA in 0.1 M HCl dependent on the substrate-affinity of the oxidases (cf. Fig. 2, no Arg, Cys, Glu, His, Lys or Trp in standards). The AA of analytical grade were purchased in the form of a standard kit from Serva (Heidelberg, Germany) or as single standards from Sigma or Fluka respectively. As eluent a 0.1 M lithium buffer was used, consisting of 14.78 g trilithium citrate tetrahydrate (p.a., Fluka) and 2.0 g lithium chloride (p.a., Merck, Darmstadt, Germany) per liter. The pH was adjusted to 2.9 (Li 2.9) or 3.4 (Li 3.4) by adding 15% HCl. The post-column reaction buffer was either a 0.27 M phosphate buffer of pH 10.6 (method A) or a 0.41 M TRIS buffer of pH 9.0 (method B). The chemicals needed were sodium dihydrogen phosphate monohydrate, potassium dihydrogen phosphate and tris(hydroxymethyl)-aminomethane (TRIS), all of analytical grade and purchased from Merck. For the production of the regenerant we sued 6.0 g lithium chloride and 10.5 g lithium hydroxide monohydrate (p.a., Merck) per liter. As solid phase extraction (SPE)-cartridges we used Bakerbond SPE aromatic sulfonic acid-columns (3 mL, 500 mg, ion exchange capacity 0.8 meq/g, J. T. Baker, Phillipsburg, New Jersey, USA, product no. 7090-03). Methanol (HPLC grade) was from Baker as well, ammonium hydroxide solution (20–25%, purum) from Fluka, glutardialdehyde solution (25% in water) and sodium azide we obtained from Merck.

Sample sources and treatment

Beer, sherry, port, wine and fruit juice were purchased at retail shops. Authentic fruit juice concentrates and purees were put at our disposal from an industrial company.

A.) Sample treatment for the general determination of several AA (beer, sherry, port, wine, fruit juice): method A

For the determination of most of the L-AA and, concerning fermented food, in several cases of the D-AA as well, the degassing and filtration of the samples were sufficient as pretreatment. Otherwise, in order to accumulate the AA, the samples were acidified to pH 1 to 2 by the use of concentrated HCl. Samples of 10 mL were passed at a rate of 1 drop per sec through two SPE-columns which were put on top of each other and which had been conditioned before with methanol, methanol/0.1 M HCl and finally with 0.1 M HCl. The columns were washed with 2 mL water and the AA adsorbed were desorbed with 30 mL 1 M NH_3. The effluent was evaporated to dryness in vacuo (bath temperature 40°C) and the residue was dissolved in 2 mL water. Aliquots of 50 μL were injected into the HPLC after having been filtrated by the use of 13 mm CA membrane filters (pore size 0.45 μm, from Lida, Kenosha, Wisconsin, USA).

B.) Sample treatment for the determination of D-alanine (fruit juice concentrates and purees): method B

The fruit purees were centrifuged at 20.000 × g for 10 minutes and the supernatants were diluted with the same amount of water. The fruit concentrates were diluted using equal amounts of water. The solutions were acidified, put on the SPE-columns and the columns were washed analogous to procedure A (see above). As eluent we took the same TRIS buffer as the post-column reaction buffer. We eluted in two steps: 1. 700 μL TRIS, 2.

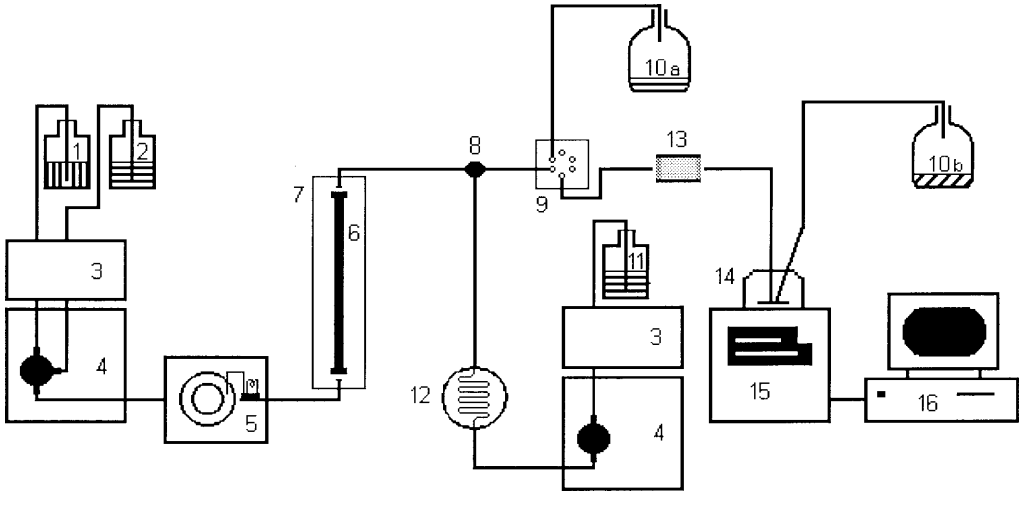

Fig. 1. Scheme of the instrument. pH values of the respective solution: *1* acidic; *2*, *10a*, *11* alkaline; *10b* neutral 1 Eluent (Lithium buffer); *2* Regenerant; *3* Degasser; *4* Solvent module; *5* Autosampler; *6* Column (Lithium cation-exchange column); *7* Column oven; *8* Mixing chamber; *9* Reversing valve; *10* Waste; *11* Post-column reaction buffer (phosphate or TRIS buffer); *12* Pulse damper; *13* Enzyme reactor; *14* Flowcell with platinum electrode (wall-jet); *15* Electrochemical detector; *16* Evaluation unit

1,800 μL TRIS into a 2 mL measuring flask. The first fraction was discarded, to the second fraction 120 μL 15% HCl were added, then the flask was filled up to the graduation mark with TRIS buffer. From the second fraction 50 μL aliquots were taken for analysis.

HPLC conditions

A.) General determination of several AA

Figure 1 shows a scheme of the instrumental configuration. Two different lithium buffers were used as mobile phase (pH 2.9 or 3.4), by the use of which the AA were separated in an isocratic way (flow rate at 0.36 mL/min, column oven temperature at 55°C). The pH value of 7.3 which was optimal for the enzymatic reaction was achieved by adding an alkaline phosphate buffer (flow rate at 0.54 mL/min (Li 2.9) or 0.52 mL/min (Li 3.4)) by means of a mixing chamber behind the column. After the enzymatic deamination of the AA the resulting hydrogen peroxide was detected electrochemically at an oxidative potential of 300 mV and a range of 50 nA. After each run the column was flushed for 2 minutes with a regenerant which was led into a waste receptacle with the aid of a reversing valve before coming into contact with the enzyme. The reversing valve was controlled by the autosampler equipped with a 50 μL-sample loop. The complete chromatographic part of the method could therefore become automated.

B.) Determination of D-alanine

As eluent we used the lithium buffer pH 3.4 (flow rate at 0.24 mL/min). In contrast to method A instead of the phosphate buffer a TRIS buffer was added at a flow rate of 0.36 mL/min behind the column in order to adjust the pH value to 8.5.

Reference analysis

The reference analysis was carried out analogously to a method reported by Brückner et al. (1991).

Instrument and column:

A Waters chromatographic system was employed, equipped with 2 pumps model 510, an automated gradient controller model 680 and a data module 740 (Waters, Eschborn, Germany). As fluorescence detector we used a Shimadzu RF-535 Fluorescence HPLC Monitor (Shimadzu Europe, Duisburg, Germany). The excitation wavelength was set at 330 nm and the emission wavelength at 445 nm. The eluents were degassed with helium. The column (250 mm × 4.6 mm i.d.) used was filled with Nucleosil 100 C18, 5 μm material (Macherey-Nagel, Düren, Germany).

Eluents and derivatizing reagent:

Eluent A was a 23 mM sodium acetate buffer (sodium acetate p.a., Fluka) adjusted to pH 5.95 with 10% acetic acid (acetic acid 100%, purum, Merck). Eluent B consisted of methanol/acetonitrile (600:50, v:v, both HPLC grade, J. T. Baker). The derivatizing reagent consisted to 170 mM *o*-phthaldialdehyde (OPA, for fluorescence applications, Fluka) and 260 mM *N*-isobutyryl-L-cysteine (IBLC, ChiraSelect, Fluka) in 1 M potassium borate buffer pH 10.4 (KOH purum, Riedel-deHaën, Seelze, Germany; boric acid p.a., Merck).

Derivatization and gradient elution conditions:

50 μL 0.4 N sodium borate buffer pH 10.4 (NaOH purum, Riedel-deHaën), 10 μL OPA/IBLC reagent and 20 μL standard or sample solution after treatment were mixed and a 20 μL aliquot was injected after 2 minutes reaction time. For elution a linear gradient from 0% B to 53% B in 75 minutes was used at a flow rate at 1 mL/min.

Standards and samples:
Each standard solution contained 50 μmol/L of the L-AA and 100 μmol/L of the D-enantiomer in 0.1 M HCl. The samples were treated as described above for method A.

Results and discussion

Characterization of the amino acid oxidases, consequences for the method

For more detailed characterization of the used enzymes the substrate spectrum and affinity to the respective enantiomers were examined. It ensued that the enantiomer-specific enzymes did not catalyze the deamination of all the different AA to the same degree. Some were not turned over at all. Concerning the protein AA the following AA reacted under the influence of the L- or D-AAOD employed: L-AAOD: L-Ala, L-Val, L-Leu, L-Ile, L-Asn, L-Gln, L-Glu, L-Lys, L-His, L-Arg, L-Phe, L-Tyr, L-Trp, L-Cys, L-Met; D-AAOD: D-Ala, D-Val, D-Pro, D-Leu, D-Ile, D-Ser, D-His, D-Phe, D-Tyr, D-Trp, D-Cys, D-Met. Additionally the affinity of the enzymes towards each AA differed considerably. Marko-Varga et al. (1991) found that 1 nmol L-Phe, for example, led to a signal hundred times higher than 1 nmol L-Ala. The exact conversion factors were not investigated in this study, but the work of Marko-Varga et al. could be confirmed qualitatively. The only deviation we found was an additional affinity of the L-AAOD towards L-Glu. The conversion factor, normalized with L-Gln as 100%, was merely about 2.2% corresponding to a detection limit of approximately 50 mg/L. Thus, the detection of L-Glu was about one decimal power less sensitive than that of the other AA (see statistics). The different conversion efficiencies necessitated standard mixtures containing all AA investigated. The enzymatic reaction yield was not quantitative in any case, but at any rate it was reproducible. The reaction rates and their proportion to each other were dependent on the pH value. For the determination of several L-AA as well as for the D-AA a pH value of 7.3 was suitable because as many AA as possible reacted to a relatively high degree. This pH value could be adjusted by adding a phosphate buffer after the separation. On the other hand for the determination of D-alanine a higher pH value of 8.5 turned out to be more suitable. Because this pH could not be adjusted with the phosphate buffer without baseline disturbances, we switched to a TRIS buffer. The careful selection of a suitable reaction buffer was of extreme importance because of the very sensitive method of detection. In order to minimize the unsteadiness of the baseline the eluent and the reaction buffer had to be mixed in a ratio of 1:1 to 1:2. Several buffer systems could not be used because of their insufficient buffering capacity. Since gas formation had to be avoided, carbonate buffers could not be used. The buffer mixture required an appropriately small chamber volume (3.1 μL) in order not to remove the preceding separation. After each run a regeneration step was necessary in order to flush the remaining matrix components from the column. As the enzymes would certainly be denaturated at such an alkaline pH the regenerant was diverted after the column into a waste receptacle. Typically the enzyme activity decreased during the first 24 h after

immobilization about 50–70%, then finding its level. The following slight continuous decrease lasted about half a year up to more than one year dependent on the use of the enzymes.

Determination of several AA

Since the used electrochemical detector was not suitable for gradient elution, an isocratic mode of operation was necessary. In addition to that we could not take organic solvents because of the biological component. The separation of all AA in one single run was therefore not possible. Experiments using reversed-phase- or diol-stationary phases were not successful. Finally we succeeded using a lithium cation-exchange column. In order to determine as many AA as possible two mobile phase systems were established. At pH 2.9 the first eluted AA were still separated satisfactorily, whereas at pH 3.4 those with longer retention times were determined. Nevertheless, it was not possible to investigate all amino acids in an appropriate period of time, e.g. Arg, Lys, His and Trp were not eluted over 50 minutes at the chosen conditions. In Fig. 2 standard chromatograms for the two systems are shown. As the employed AA concentrations differ the peak-heights do not give any information about the substrate affinity of the oxidases. The chromatogram of free L-AA in pils beer is shown in Fig. 3. The elution profile of the free D-AA isolated from a sherry is shown in Fig. 4. In all cases the quantitative determination was performed by multiple injection of the external standard.

The investigations included 11 kinds of pils beer and for comparison, several sherry, port, wine and fruit juice samples. The AA amounts found in the beer samples are summarized in Table 1. All in all there were strong variations concerning the total AA amounts in beer, but we found that

Table 1. L- and D-amino acids in mg/L in pils beer samples

Sample no.	1	2	3	4	5	6	7	8	9	10	11
L-amino acids:											
L-Gln	n.d.	n.d.	n.d.	n.d.	n.d.	1.2	1.3	n.d.	0.8	n.d.	1.6
L-Ala	28.5	89.7	32.6	76.7	29.2	48.6	65.2	59.6	47.8	49.2	79.6
L-Val	20.1	75.0	28.9	65.4	12.1	28.2	62.2	33.5	25.5	42.9	59.0
L-Met	n.d.	5.8	0.7	2.5	n.d.	6.7	8.8	2.4	1.6	n.d.	6.1
L-Ile	7.6	29.2	17.9	35.3	4.9	41.8	35.7	22.5	12.2	15.0	21.4
L-Leu	8.7	65.2	25.5	49.8	2.6	66.6	91.7	45.4	38.8	33.8	55.5
L-Tyr	47.3	70.2	48.2	67.0	54.4	78.3	124.7	59.9	94.4	81.6	110.1
L-Phe	37.0	79.6	48.7	73.4	19.7	85.8	154.9	71.6	64.9	90.5	129.4
D-amino acids:											
D-Ala	1.3	1.5	1.1	2.4	1.8	4.9	3.8	1.7	1.3	1.3	2.3

D-Pro was detectable in all samples but not determinable because it coeluted with an unknown compound.

n.d. not detectable. L-AA chromatograms of sample 11 see Fig. 3.

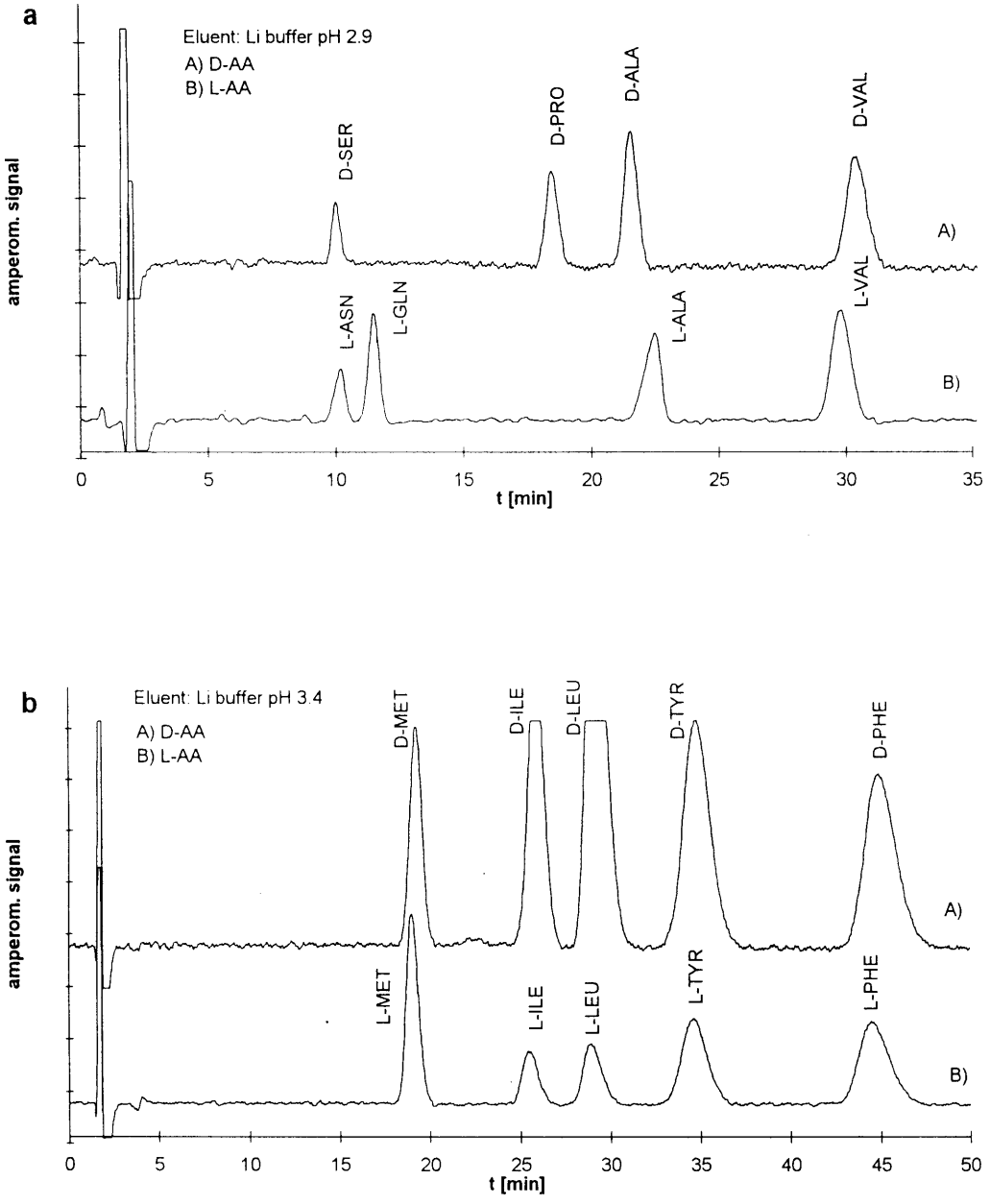

Fig. 2a,b. Chromatograms of L- and D-AA standard solutions. **a** Eluent: lithium buffer pH 2.9. Amounts [mg/L]: L-Asn 12.8, L-Gln 11.0, L-Ala 18.7, L-Val 9.2, D-Ser 8.2, D-Pro 3.6, D-Ala 10.4, D-Val 13.1. **b** Eluent: lithium buffer pH 3.4. Amounts [mg/L]: L-Met 11.0, L-Ile 18.8, L-Leu 15.0, L-Tyr 12.2, L-Phe 19.0, D-Met 10.5, D-Ile 9.5, D-Leu 15.1, D-Tyr 22.1, D-Phe 21.2. For chromatographic conditions see materials and methods (method A)

either all AA could be found in high amounts or in low amounts in one sample. D-proline was detected in all beer samples but could not be determined quantitatively because it was eluted at the same time as another electrochemically active disturbance substance. This could be

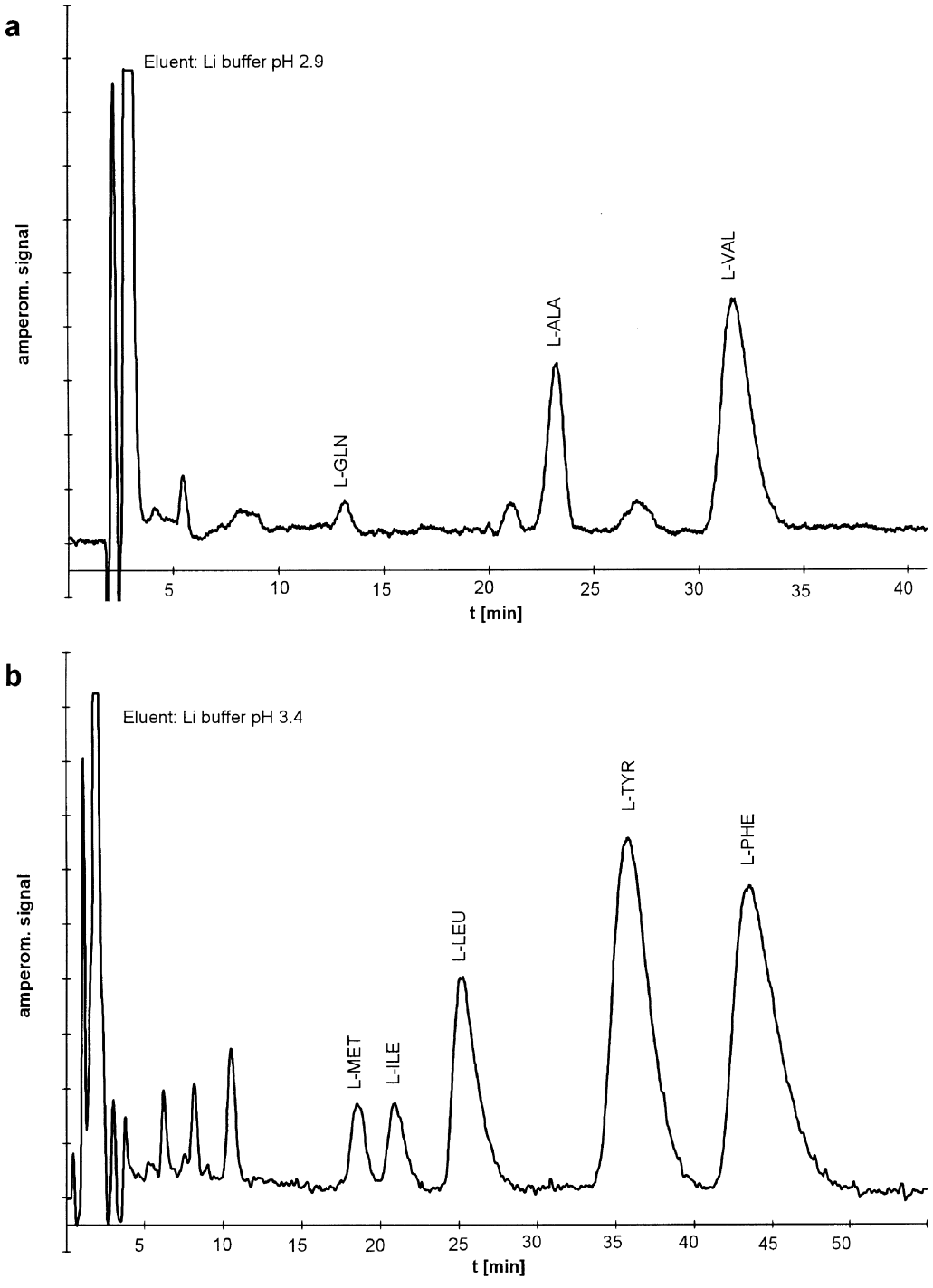

Fig. 3a,b. Elution profiles of free L-AA in pils beer, chromatograms of sample 11 (see Table 1). **a** Eluent: lithium buffer pH 2.9, **b** Eluent: lithium buffer pH 3.4, sample directly injected without pretreatment. Amounts [mg/L]: L-Gln 1.6, L-Ala 79.6, L-Val 59.0, L-Met 6.1, L-Ile 21.4, L-Leu 55.5, L-Tyr 110.1, L-Phe 129.4. For sample treatment and chromatographic conditions of Figs. 3 and 4 see Materials and methods (method A)

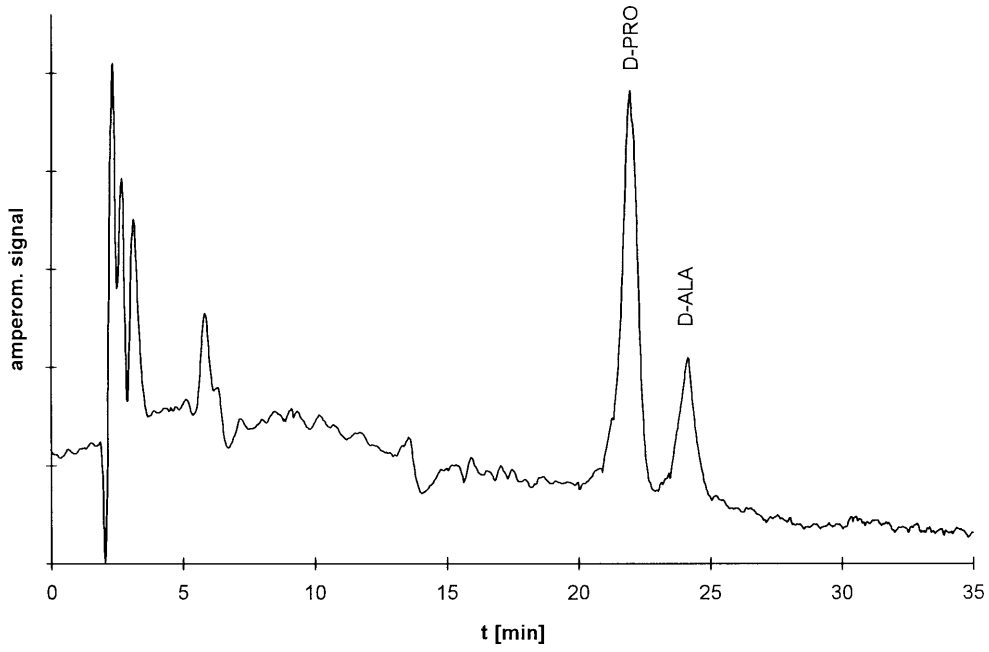

Fig. 4. Chromatogram of free D-AA in Sherry. Eluent: lithium buffer pH 2.9. Amounts [mg/L]: D-Pro 0.9, D-Ala 0.7

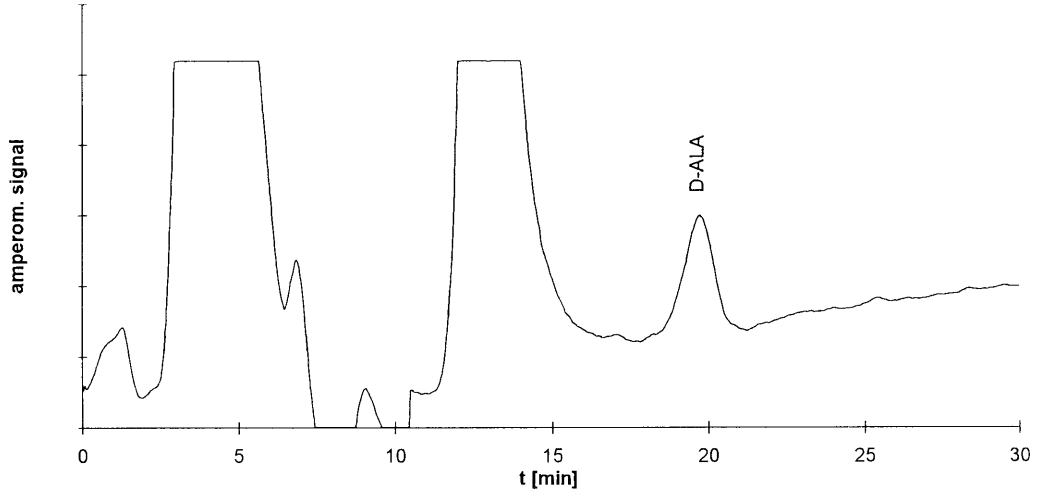

Fig. 5. Determination of D-alanine in mango puree. Eluent: lithium buffer pH 3.4. Amount: D-Ala 8.8 mg/L. For sample treatment and chromatographic conditions of Figs. 5 and 6 see Materials and methods (method B)

established by measuring a blank without using the enzyme reactor. Thus, the method offers an additional possibility of checking results other chromatographic techniques do not contain (see Fig. 6). Concerning the other samples examined, the highest L-AA amounts were found in a wine sample which contained 267.0 mg/L L-Ala and 270.3 mg/L L-Leu. D-AA could be

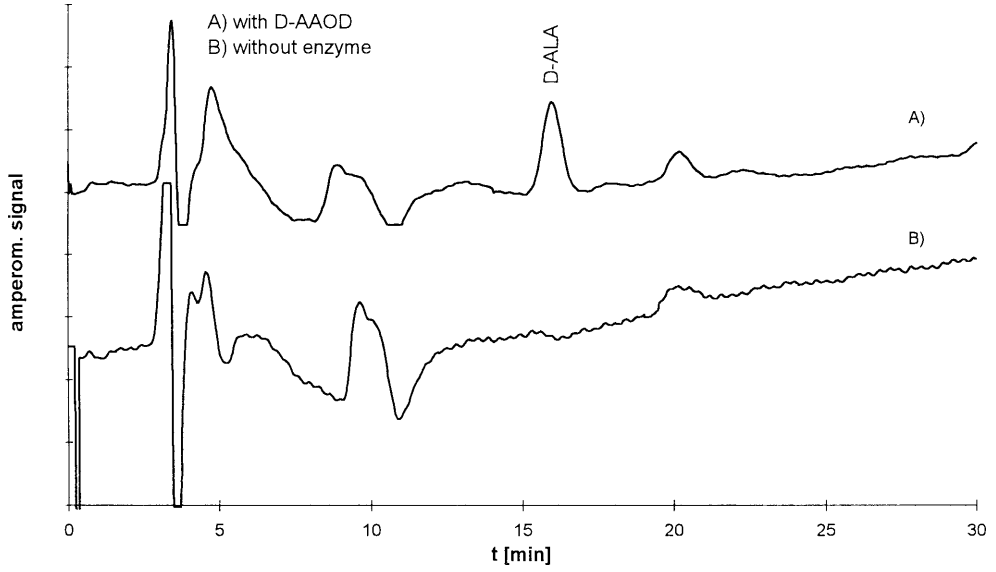

Fig. 6. Determination of D-alanine in apple juice concentrate. Eluent: lithium buffer pH 3.4. Chromatogram **A** with D-AAOD reactor, **B** without enzyme. Amount: D-Ala 2.8 mg/L. Note the missing peak in **B** as a means of identification

determined in every fermented beverage, whereas in fruit juice D-AA were not detectable.

Determination of D-alanine

In order to increase the sensitivity for the determination of D-alanine sample pretreatment as well as measuring parameters were modified. So after SPE less than 0.1 mg/L (1 μmol/L) D-alanine could be detected. We investigated 32 fruit concentrates or purees respectively, some of which had become apparent because of enhanced fermentation parameters (e.g. lactic acid). The chromatograms of a mango puree and an apple juice concentrate are shown in Fig. 5 and 6. The different elution volumes are due to different guard columns. In Fig. 5 a guard column from Pickering, in Fig. 6 a guard cartridge from CS was used. One analysis run took 55 minutes including the regeneration step. Analogous to the wide range of amounts of lactic acid, the D-alanine contents amounted from "not detectable" to 135.2 mg/L. A significant correlation between D-alanine and especially D-lactate could be determined. D-lactate is not found in fresh food but can serve as a diagnostic feature of the specific presence of bacteria (Dainty, 1996). More detailed results will be published soon (Flüssiges Obst, in press).

The results were confirmed by reference analyses using a method described by Brückner et al. (1991) (chromatograms not shown). Ten of the fruit concentrates containing different amounts of D-alanine were therefore worked up once again (see materials and methods). For these measurements D-alanine could not be directly eluted from the SPE-columns with TRIS

buffer because the buffer would have led to disturbances of the derivatization and the chromatograms with the ODS-column. After derivatization with OPA/IBLC the AA were determined by fluorescence detection. Including a regeneration step a run lasted 90 minutes. After elimination of one outlier (Grubbs' and Dixon's test) the reference results correlated to the results achieved with HPLC-enzyme reactor with a correlation factor of 0.999, the results obtained with fluorescence detection generally exceeding the biosensor results by 15%. The retention times of the other protein AA enantiomers differed sufficiently to that of D-alanine. Thus, a coelution of that type could be excluded, whereas it could not be settled what about interferences with other matrix components. Finally, the deviation of the results could not be explained satisfactorily, but the high correlation factor can be interpreted as a confirmation of the results determined with the biosensor technique.

Considering the total amount of samples investigated, a limit value of 1 mg/L D-alanine seems to be significant for a bacterial contamination. This corresponds to the limit value postulated by Gandolfi et al. (1994). The suitability of D-alanine as a microbial marker has been discussed elsewhere (Brückner and Lüpke, 1991). Aside from microbial spoilage D-AA might originate from adulteration of the respective concentrate by the addition of racemic AA or of vigorously treated protein hydrolysates. Besides a natural occurrence of D-AA in certain fruits cannot be excluded with certainty. However, the fact that in authentic fruit concentrates of high quality D-alanine was not detectable and the correlation between the D-alanine and D-lactate contents confirm the assumption that D-alanine was of bacterial origin.

Statistics

The repeated measurement of the same sample alternately with a standard solution for more than 24 hours resulted in a coefficient of variation of 8.50%. In order to investigate the precision of the complete method including the SPE the sample was pretreated 10 times. We found a slightly worse coefficient of variation of 8.64%. Within the limits of accuracy of measurement the amounts recovered were between 90 and 110%. The linearity of the detector response to the various amino acids was generally two orders of decimal power.

It is difficult to indicate general detection limits because they strongly depend on electrode sensitivity and enzyme activity. Using a 50 μL-sample loop and relatively "new" enzymes, i.e. the immobilization had been carried out less than 3 months ago, the detection limits ranged from 0.1 mg/L (D-Pro, D-Met) to 5 mg/L (L-Ala) dependent on the respective AA. As an exception the detection limit of L-Glu was about 50 mg/L. D-alanine could be detected in amounts of 0.5 mg/L.

Conclusions

In conclusion the rapidity of the determination of D-alanine without an evaporation or derivatization step, relatively short chromatographic runs and

in addition to the retention time the possibility of a confirmation of the results by measuring without an enzyme reactor makes the HPLC-enzyme reactor technique an interesting possibility to identify a probable bacterial contamination of food.

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