Chromatographic determination of amino acid enantiomers in beers and raw materials used for their manufacture

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Abstract

Using gas chromatography (GC) on a chiral stationary phase, accompanied by high-performance liquid chromatography, beers and raw materials used for manufacturing (hops, barley grains, malts) were investigated for the pattern and quantities of amino acid enantiomers. Although L-amino acids were most abundant, certain D-amino acids were detected in all beers and most of the raw materials. Highest amounts of D-amino acids were detected in special beers such as Berliner Weisse that underwent bottle-conditioning with lactic cultures, and Belgian fruit beers produced by spontaneous fermentation. It is demonstrated that GC on chiral stationary phases is highly suitable for the quantitative determination of amino acid enantiomers in beers and raw materials used for their manufacture. Quantities, relative amounts and pattern of amino acid enantiomers can serve in particular as chiral markers for the authenticity of special beers. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Beer is defined as an alcoholic beverage from starch-containing raw materials serving as sources for maltose and glucose which are fermented by brewers yeast. In Germany, according to the famous law of purity from 1516, exclusively germinated and kilned barley (malt), hops, water and yeast are allowed to be used as ingredients. An exception is wheat beer which has to be defined as such and where a mixture of wheat and barley malt is allowed to be used [1].

Although barley malt is the most important cereal, wheat, wheat malt, corn, rice and millet are also used as starch-containing adjuncts or extenders and sources for fermentable sugars. Many breeds of barley, and an abundance of malts produced therefrom, are used to influence taste, color and body of the final product. For example, such malts are designated as Munich malt, Vienna malt, British pale ale malt and roasted black malt [1,2].

Over the past centuries many countries have developed their own local beer specialties. Germany is famous for its Pilsener, Düsseldorfer Altbier and Bavarian Weissbier, Great Britain for ales and stouts. In Belgium fresh fruit like cherries and raspberries are added in amounts of up to 15% to the finished beer followed by a further fermentation to produce a special fruit beer [1,3–5].

In beer production alcoholic fermentation takes place by the action of selected strains of the yeast

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Saccharomyces cerevisiae Hansen (top fermenting) or *Saccharomyces carlsbergensis* Hansen (bottom fermenting). Besides *Saccharomyces*, various wild yeasts together with lactic acid bacteria are involved in the brewing process of special local beers. For example, the Belgian lambic and fruit beers are produced by fermentation with a very complex mixture of wild yeasts, including *Brettanomyces lambicus* and *Brettanomyces bruxellensis*, combined with lactic cultures mainly consisting of species of *Pedicoccus* [4,5]. The characteristic tart taste of German Berliner Weisse is due to the lactic fermentation of the mash with *Lactobacillus delbrueckii*. Some manufacturers also add lactic cultures to the beer right before bottling for secondary fermentation named conditioning [1].

Among the constituents of beers, amino acids are of particular interest. The amounts and pattern of free L-amino acids in beers, and their enantiomers, the D-amino acids, are dependent on many factors. They comprise quantities of proteins in the raw materials used for production, activity of cereal proteolytic enzymes in the course of various mashing procedures, and microorganisms involved in the fermentation process.

Identification of sources of D-amino acids and estimation of their contribution to the D-amino acid content of finished beer is a difficult task. It has to be taken into account that the raw materials used for beer production might contain already free D-amino acids [6]. Thermal treatment of malt during kiln-drying and/or roasting might also induce conversion of L- into D-enantiomers [7]. This process is usually, but not correctly, referred to as racemization of amino acids. In the strict sense racemized amino acids consist of exactly equal amounts of the D- and the L-enantiomers. Further, raw materials are inevitably subject of microbial colonization and contamination [8] and might contribute to amounts of D-amino acids in beers.

Action of special microbial enzymes, so-called racemases and epimerases, on proteinogenic L-amino acids is known to cause formation of their optical antipodes i.e., D-amino acids [9]. Autolysis of bacterial peptidoglycan [10] in the course of the fermentation process additionally leads to the release of bonded D-amino acids of the cell walls and of free D-amino acids occurring in the cytoplasm [11,12].

D-Amino acids have also been detected in various yeasts and yeast autolysates [13,14]. Consequently, D-amino acids were detected in different beers [14–16] as well as in other microbially fermented foods [14,17,18].

So far, only a few systematic investigations of different groups of beers with the aim to quantify amino acid enantiomers have been carried out [15,16]. Therefore, the aim of this work was the characterization of beers by quantitative determination of the enantiomeric amino acid ratios using mainly gas chromatography (GC), complemented by high-performance liquid chromatography (HPLC). Samples of grains, malts and hops were also investigated in order to evaluate their contribution to the D-amino acid content of beer. Further, the results should be discussed with regard to aspects of beer quality and authenticity.

2. Experimental

2.1. Instrumental

2.1.1. Gas chromatography

A Shimadzu GC-14A gas chromatograph with a flame ionization detection (FID) system and a Shimadzu GC-17A coupled with a Shimadzu QP-5000 mass spectrometer (Shimadzu, Kyoto, Japan) were used.

The GC columns used in both instruments for the separation of amino acid enantiomers were fused-silica Chirasil-L-Val (*N-tert*.-butyl-L-valine-polysiloxane) [19] capillary columns (25 m×0.25 mm I.D.; Chrompack, Middelburg, The Netherlands). Carrier gas was helium (Messer Griesheim, Krefeld, Germany, 99.999% purity) at an inlet pressure of 50 kPa (GC-14A) and 80 kPa (GC-17A), respectively. The temperatures of the injector and interface were 250°C; samples were injected in split mode (split ratio 1:30).

Temperature program for GC-14A: initial temperature 85°C held for 7 min; heating rate A: 2.0°C/min to 90°C; rate B: 2.0°C/min to 110°C; rate C: 3.5°C/min to 160°C; rate D: 5.0°C/min to 175°C held for 1 min; rate E: 2.5°C/min to 190°C held for 15 min. Isobaric pressure was 50 kPa.

For temperature and pressure program of GC-17A see Ref. [17].

Data acquisition was carried out with a Cromatopac C-R3A integrator (Shimadzu) at the GC–FID system and with Class 5000 software and workstation (Shimadzu) at the GC–MS system.

2.1.2. High-performance liquid chromatography

For amino acid analysis by HPLC a HP 1090 Series L [Hewlett-Packard (HP), Waldbronn, Germany] equipped with a HP 1046A programmable fluorescence detector and a Hypersil ODS2 column were used as described in detail previously [14]. Data acquisition was carried out with the HP Chem-Station for LC software.

2.2. Chemicals

Dichloromethane (DCM), 2-propanol (2-PrOH), 1-PrOH, acetyl chloride (AcCl), 32% aqueous hydrochloric acid (HCl) (all from Merck, Darmstadt, Germany), methanol (MeOH), 25% aqueous ammonia (NH$_3$), *n*-hexane, acetic acid (100% analytical-reagent grade) (all from Roth, Karlsruhe, Germany), pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFAA), trifluoroacetic acid (TFA), 2,6-di-*tert*.-butyl-*p*-cresol (BHT), cation exchanger Dowex 50W-X8, 200–400 mesh, corresponding to a particle size of 0.037–0.075 mm, H$^+$-form (all from Fluka, Buchs, Switzerland); sodium hydroxide (NaOH, 99% analytical-reagent grade) and potassium hydroxide (KOH, 99% analytical-reagent grade) (from Roth); *N*-isobutyryl-D-cysteine (IBDC) and *N*-isobutyryl-L-cysteine (IBLC) (Novabiochem, Läufelfingen, Switzerland), *o*-phthaldialdehyde (OPA) (analytical-reagent grade, Merck); 0.13 *M* sodium borate buffer (HP).

An equimolar amino acid standard H (1-ml sealed ampoules from Pierce, Rockford, IL, USA) was used, consisting of Gly and the L-enantiomers of alanine (Ala), valine (Val), threonine (Thr), isoleucine (Ile), proline (Pro), serine (Ser), leucine (Leu), aspartic acid (Asp), methionine (Met), glutamic acid (Glu), phenylalanine (Phe), tyrosine (Tyr), lysine (Lys), arginine (Arg), histidine (His) (2.5 m*M* each) and L-cystine (1.25 m*M*). L-Ornithine (Orn) and δ-aminobutyric acid (GABA) were added in equimolar amounts. The enantiomeric purity of

the L-amino acid standard was >99.9%. As internal standard for GC analysis L-norleucine (L-Nle, Fluka) was used.

2.3. Beers and raw materials

Beers (*n*=42) in bottles or cans (cf. Table 1) were purchased from local retail outlets or were provided by breweries. Barley grains (*n*=4), barley malts and wheat malts (*n*=9), and hops (*n*=3) were kindly provided by Dr. Hasan Taschan, Giessen, Germany. They were not specified with regard to cultivars, brand names, or origin.

2.4. Treatment of samples

For GC analysis aliquots (20 ml) of the beer samples were degassed in an ultrasonic device, Model Sonorex Super RK 106 (Bandelin, Berlin, Germany). To aliquots (1–5 ml) the internal standard L-Nle (100 µl of a 10 m*M* solution in 0.01 *M* HCl) was added and the pH was adjusted to 2.5 by addition of suitable amounts of 1 *M* HCl. Samples were subjected to Dowex 50W-X8 cation exchanger packed in glass columns (bed volume: 4 cm×0.5 cm I.D.). After washing with bidistilled water (10 ml) the amino acids adsorbed were eluted with 4 *M* NH$_3$ (3 ml) and the eluate was evaporated to dryness under reduced pressure.

Grains, malts and hops were milled with a Mortar Grinder, Model RM 1000 (Retsch, Haan, Germany). Aliquots (10 g) were suspended in 0.1 *M* HCl (50 ml) and the internal standard L-Nle (200 µl of a 10 m*M* solution in 0.01 *M* HCl) was added. After stirring for 20 min on a magnetic stirrer, samples were centrifuged at 3500 *g* for 15 min in a Model Labofuge 400 centrifuge (Heraeus, Hanau, Germany). Residues were suspended twice in 0.1 *M* HCl (20 ml) and the procedure was repeated. Supernatants were collected, residues were discarded. Proteins were precipitated by addition of TFA (10 ml). After centrifugation the residues were suspended twice in TFA [3 ml of a 10% (v/v) aqueous solution] and centrifuged. Supernatants were combined and extracted with *n*-hexane (3×25 ml). The organic phases were discarded and the aqueous phases were evaporated to dryness under reduced pressure on a rotary evaporator. The remaining

Table 1

Absolute (mg/kg or mg/l) and relative (%) amounts of amino acids in raw materials and beers determined by GC[a]; n = number of samples investigated (data are average of duplicate analyses of each sample)

	Raw materials (mg/kg)			Top-fermented beers (mg/l)			Bottom-fermented beers (mg/l)		
	Hops (n=3)	Barley (n=4)	Malt (barley and wheat) (n=9)	Lambic beers[c] (n=6)	Altbier and ales[d] (n=11)	Wheat beers[e] (n=11)	Pilsener and lagers[f] (n=7)	Black beers[g] (n=3)	Strong beers[h] (n=4)
(D+L)-Amino acids	2800–4000	300–480	600–3800	500–1500	500–2040	560–1080	1280–2260	750–1440	1440–5000
D-Amino acids	23.0–48.4	1.9–6.6	0–47.9	6.4–41.6	6.5–41.6	11.3–96.3	8.3–29.8	8.9–24.9	13.2–50.8
% D-Amino acids[b]	1.1–1.2	0.4–1.8	0–1.6	1.0–5.3	0.9–2.7	1.3–11.3	0.7–1.3	1.2–1.8	0.9–1.2

[a] Arg, His, Trp and Cys not determinable.

[b] Calculation of relative amounts was carried out according to the equation % D-amino acids = 100·D/(D+L), shown here are the lowest and highest values taken from the average amounts of the single samples.

[c] Including two kriek (cherry)-lambics, one pecheresse (peach)-lambic, one framboise (raspberry)-lambic and two geuze (blended)-lambics, all products of Belgium.

[d] Comprising five samples of German ''Altbier'', three English ales and three Irish ales.

[e] Including three samples of ''Helles Weissbier'', one sample of ''Kristall-Weizen'', three samples of ''Dunkles Weissbier'', all products of Bavaria, Germany, and four samples of ''Berliner Weisse'', Berlin, Germany.

[f] Comprising three samples of German Pilsener, one Pilsener of the Czech Republic, one Pilsener of Poland, one German lager and one American lager.

[g] Black beers (''Schwarzbier'') of Germany.

[h] Including two French, one Belgian and one German beer.

residues were dissolved in bidistilled water (5 ml), pH 2.5 was adjusted by addition of 1 M NH₃ and samples were subjected to cation-exchange treatment as described above.

For HPLC analysis aliquots (5 ml) of the beers were degassed in an ultrasonic device and adjusted to pH 8–10 by addition of 1 M NaOH. Then MeOH–0.13 M sodium borate buffer (80:20, v/v) was added to give a final volume of 25 ml. Aliquots (1.5 ml) were centrifuged at 1500 g for 20 min in a Micro Centrifuge (Roth) and the supernatants were transferred to the autosampler vials.

2.5. Derivatization, analysis and quantification of amino acids

For GC analysis amino acid containing residues resulting from cation-exchange treatment (see above) were dissolved in 0.1 M HCl (500 µl) and transferred into 1 ml ''Reacti-Vials'' (Wheaton, Millville, NJ, USA). Samples were evaporated to dryness in a stream of N₂ at 70–80°C using a heating module (''Thermoblock'', Gebr. Liebisch, Bielefeld, Germany) combined with an evaporating unit (Model 18780, ''Reacti-Vap'', Pierce).

For analysis by GC–FID or GC–MS, amino acids of purified samples were converted into their corresponding $N(O)$-trifluoroacetyl amino acid 1-propyl esters or $N(O)$-pentafluoropropionyl amino acid 2-propyl esters with addition of antioxidant BHT as described [17]. BHT elutes after L-Nle (cf. Figs. 1 and 2). Note that Arg, His and cysteine (Cys) as well as tryptophan (if present in samples) could not be determined by GC as a result of the methodology and derivatization chemistry used, and Asn and Gln are hydrolyzed to Asp and Glu, respectively.

Relative amounts of D-amino acids were calculated according to Eq. (1):

$$\% \, D = 100 \cdot A_D / (A_D + A_L) \tag{1}$$

where % D is the relative amount of the D-enantiomer, and A_D and A_L are the peak areas of the D- or L-enantiomer, respectively.

For quantification response factors of amino acids were determined in relation to L-Nle. Equimolar amounts of amino acids, including the I.S., were injected into the GC–FID or GC–MS system. Response factors were calculated according to Eq. (2):
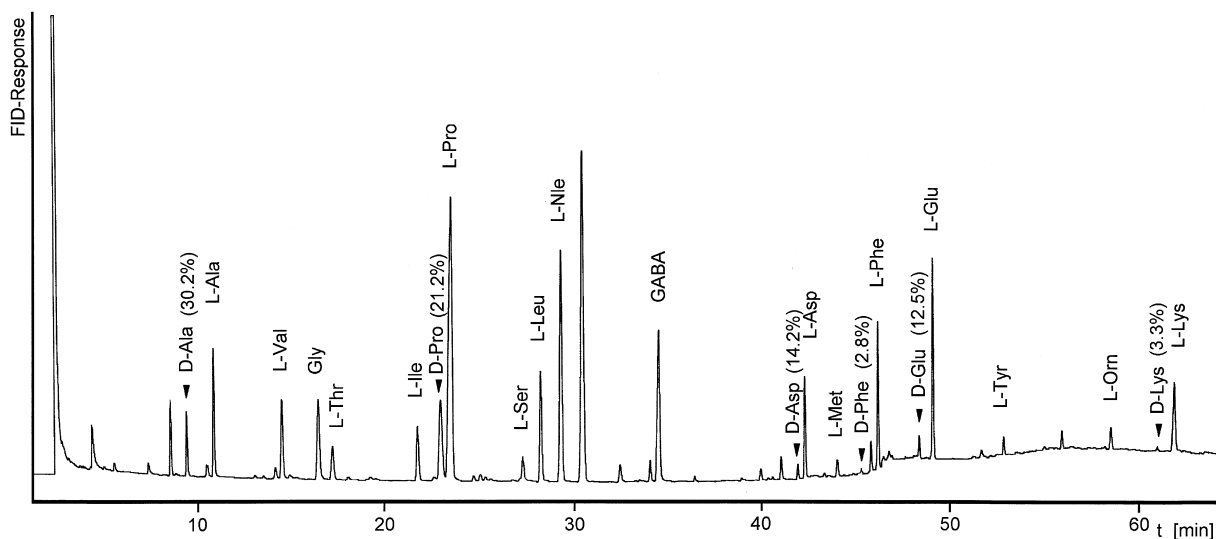
$$f_R = A_{AA} / A_{I.S.} \tag{2}$$

Fig. 1. GC of a Berliner Weisse beer (No. 1), flavored with woodruff; amino acids were separated on Chirasil-L-Val as trifluoroacetyl amino acid 1-propyl esters; for chromatographic conditions see Experimental.

where f_R is the response factor of amino acid to be determined, A_{AA} the peak area of amino acid to be determined, and $A_{I.S.}$ the peak area of I.S. obtained from a standard amino acid mixture (standard H) to which L-Nle was added in equimolar concentration (2.5 mM).

Note that response factors depend on the instruments and detectors used for quantification of amino acids. Thus, they had to be determined separately for GC–FID and GC–MS.

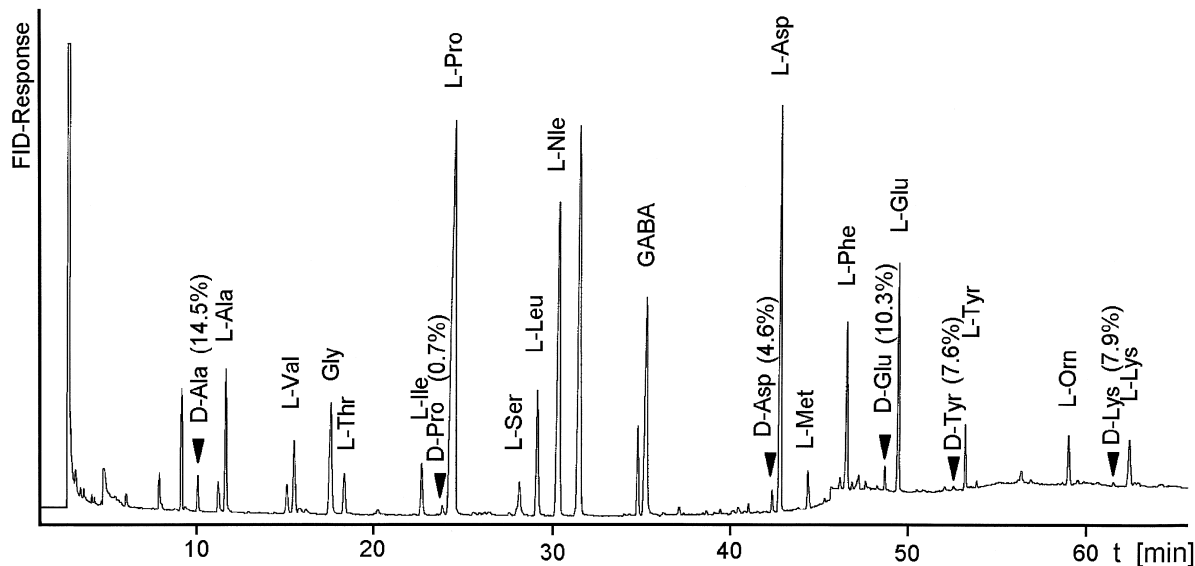Amounts of amino acid in beers were calculated according to Eq. (3):

Fig. 2. GC of a Kriek-lambic beer; amino acids were separated on Chirasil-L-Val as trifluoroacetyl amino acid 1-propyl esters; for chromatographic conditions see Experimental.

$$c_{AA} = \frac{A_{AA}c^*_{I.S.}f_{dil}M_{r,\,AA}}{A_{I.S.}f_R 1000} \tag{3}$$

where c_{AA} is the concentration of an amino acid in sample (mg/l), A_{AA} the peak area of amino acid, $A_{I.S.}$ the peak area of I.S., f_R the response factor of amino acid, $c^*_{I.S.}$ the concentration of I.S. (mol/l), f_{dil} the correction factor for dilution with HCl used for pH adjustment, and $M_{r,\,AA}$ the molecular mass of amino acid (g/mol).

The limit of quantitative determination of amino acids in beer and raw materials ranged from 0.57 mg/l (Thr) to 1.49 mg/l (Met) using FID and from 0.06 mg/l (Ala) to 0.39 mg/l (Met) using MS.

For the determination of the repeatability of the GC methods work-up and analysis of one sample was repeated three times. Using FID for quantification the relative standard deviations (RSDs) ranged from 1.1% to 4.8% for Asp, Met, Pro, Phe, Glu, Lys and Tyr. The other amino acids showed RSDs of 6.5–9.2%. In the MS mode the RSD values ranged from 1.1% (Asp) to 7.2% (Gly). Recoveries of the amino acids were determined by addition of standard H (250 μl) to a beer sample prior to the work-up procedure. Recoveries ranged from 76% (Met) to 110% (Phe).

Automated derivatization chemistry of amino acids using OPA–IBL(D)C, and analysis of derivatives by HPLC were performed as described previously [14]. Quantification was carried out via external standard calibrations. The limit of quantitative determination of amino acids using HPLC ranged from 0.04 mg/l (D-Thr) to 0.53 mg/l (L-Orn) for IBLC derivatives and from 0.08 mg/l (L-Thr) to 0.68 mg/l (L-Lys) for IBDC derivatives.

3. Results and discussion

A summary of quantities of the amino acids determined in beers and raw materials is given in Table 1. As can be seen D-amino acids were detected in all beers and in most of the raw materials. A remarkable exception was a black malt where no D-amino acids were found and amounts of free L-amino acids were low (598 mg/kg) in comparison to pale malts (2500–3842 mg/kg). This is attributed to the reaction of free L- and D-amino acids with reducing sugars. This leads to intense generation of Maillard products during the roasting process resulting in loss of amino acids and formation of black malt. In light-colored malts, however, D-amino acids were detected. No significant differences in the amino acid pattern and the enantiomeric distribution of amino acids were found among barley malts and wheat malts.

In comparison to barley and wheat malts, barley grains contained much lower amounts of free L- and D-amino acids (cf. Table 1). The major free amino acid in barley was L-Glu (60–85 mg/kg), followed by L-Phe (40–72 mg/kg) and L-Asp (28–62 mg/kg). In malt samples, however, L-Pro was the most abundant free amino acid (400–700 mg/kg), followed by L-Phe (350–630 mg/kg) and L-Glu (220–320 mg/kg). This increase is a result of the action of exo- and endopeptidases on grain proteins in the malting process.

Low but significant amounts of D-amino acids have been recognized to occur naturally in plants [6]. Therefore, the D-amino acids found in barley grains might be attributed to endogenous formation, or to uptake of D-amino acid from soils. It is also known that the grains used for malt production are always contaminated with microorganisms [8] which are potential sources of D-amino acids [10]. During steeping and germination, which is not carried out aseptically, not only grain-specific exo- and endopeptidases but also microbes and their racemases [9] are activated. The L-amino acids released from the grain proteins could be partly transformed into D-amino acids by microbial racemases. This could explain the higher relative and absolute amounts of D-amino acids determined in malts in comparison to grains. Further, free and conjugated D-amino acids have been detected in germinating seeds [20,21].

In the dried hop blossoms only low relative amounts of D-Asp and D-Glu, besides the common L-amino acids, were detected (cf. Table 1). Again, endogenous formation, uptake from soil and microbial contamination, alone or together, might be the explanations for the presence of D-amino acids. Since relatively low amounts of hops are added to the wort (0.1–0.5 kg per 100 l) the contribution to the D-amino acid content of beer is negligible.

In all beers the D-enantiomers of Ala, Asp and Glu

Table 2

Quantitative data of chiral amino acid analysis of selected beers obtained by GC analysis

Amino acid	Berliner Weisse (No. 2)			Framboise lambic			Pilsener			Pale ale			Bavarian wheat beer			Strong beer		
	L (mg/l)	D (mg/l)	D (%)	L (mg/l)	D (mg/l)	D (%)	L (mg/l)	D (mg/l)	D (%)	L (mg/l)	D (mg/l)	D (%)	L (mg/l)	D (mg/l)	D (%)	L (mg/l)	D (mg/l)	D (%)
Ala	52.2	22.5	30.1	61.2	10.9	17.3	273.5	4.2	1.5	72.1	2.4	3.2	83.5	2.6	3.0	250.0	6.7	2.6
Val	30.9	n.d.	–	44.5	n.d.	–	128.3	n.d.	–	22.6	n.d.	–	73.2	n.d.	–	188.0	n.d.	–
Gly[a]	47.8	–	–	29.6	–	–	99.6	–	–	58.2	–	–	51.3	–	–	145.5	–	–
Thr	13.3	n.d.	–	20.2	n.d.	–	9.3	n.d.	–	9.2	n.d.	–	7.9	n.d.	–	15.5	n.d.	–
Ile	22.9	n.d.	–	24.7	n.d.	–	35.6	n.d.	–	9.0	n.d.	–	26.3	n.d.	–	44.5	n.d.	–
Pro	194.3	51.9	21.1	91.7	0.6	0.7	623.7	1.7	0.3	381.7	1.5	0.4	393.8	0.8	0.2	662.5	2.0	0.3
Ser	12.0	n.d.	–	22.1	n.d.	–	30.2	n.d.	–	41.1	n.d.	–	15.5	n.d.	–	38.9	n.d.	–
Leu	45.2	n.d.	–	41.6	n.d.	–	60.7	n.d.	–	19.9	n.d.	–	68.4	n.d.	–	84.8	n.d.	–
GABA[a]	77.0	–	–	28.1	–	–	123.5	–	–	30.6	–	–	83.8	–	–	193.6	–	–
Asp[b]	30.1	5.2	14.7	622.2	10.3	1.6	43.3	3.9	8.3	27.6	4.3	13.5	33.2	3.1	8.5	68.3	7.3	9.7
Met	9.7	n.d.	–	13.6	n.d.	–	14.4	n.d.	–	5.4	n.d.	–	11.5	n.d.	–	21.1	n.d.	–
Phe	39.3	1.3	3.2	27.2	n.d.	–	75.7	n.d.	–	18.5	1.5	7.5	84.7	1.2	1.4	101.9	3.4	3.2
Glu[c]	53.9	6.6	10.9	36.4	2.2	5.7	96.1	3.1	3.1	41.0	2.5	5.7	70.0	4.6	6.2	116.5	5.9	4.8
Tyr	5.7	n.d.	–	14.6	1.0	6.4	70.0	n.d.	–	20.9	n.d.	–	55.4	1.7	3.0	71.3	n.d.	–
Orn	6.5	n.d.	–	3.1	n.d.	–	6.1	n.d.	–	7.1	n.d.	–	5.8	n.d.	–	10.2	n.d.	–
Lys	31.6	1.3	4.0	8.6	n.d.	–	85.4	n.d.	–	19.9	n.d.	–	15.5	n.d.	–	52.4	n.d.	–
Σ	672.4	88.8	11.7	1089.4	25.0	2.3	1775.4	12.9	0.7	784.8	12.2	1.5	1079.8	14.0	1.3	2065.0	25.3	1.2

[a] Non-chiral amino acid; n.d.=not detected; Arg, His, Trp and Cys not determinable.

[b] Sum of (Asp+Asn) (see Experimental).

[c] Sum of (Glu+Gln) (see Experimental).

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were detected. These D-amino acids are considered as chemical markers for microbial activity [14,17,18]. Small amounts of these D-amino acids might originate from the grains and/or malts being fermented (cf. Table 1). However, since the raw materials are used in amounts of about 12% (w/w) for the production of common beers comprising ca. 5% (v/v) alcohol [1,2] their contribution to the D-amino acid content of beer is probably low.

Individual groups of beer showed very deviating and characteristic amino acid pattern including ratios of enantiomers. Highest amounts of D-amino acids were determined in a special German beer called Berliner Weisse. This is a top fermented wheat beer, low in alcohol (2.5–3.7%, v/v), which is subjected to a secondary lactic fermentation in order to make it tart and spritzy [2]. The chromatogram of Berliner Weisse beer No. 1, with artificial woodruff flavor added, is presented in Fig. 1. Very high amounts of D-Pro were detected, as well as high relative amounts of D-Ala, D-Asp and D-Glu. Quantitative data of Berliner Weisse No. 2, from the same manufacturer, with artificial raspberry flavor added, are shown in Table 2.

Interestingly, in another Berliner Weisse (No. 3) only traces of D-Pro were detected, but high relative amounts of other D-amino acids. The latter special beer was produced by a different manufacturer using differing fermentation conditions. The manufacturer of Berliner Weisse Nos. 1 and 2 uses mixed strains of top fermenting yeast together with mixed strains of *Lactobacillus delbrueckii*. These mixed cultures are added to equal parts of barley malt and wheat malt, together with water and a proportion of wort matured for 3–6 months. Then, fermentation is conducted for 3–4 days at 20–25°C. After maturation for 3–6 months at 13–25°C the brew is centrifuged. Then an inoculum named *Kräusen* is added, which represents a portion of partly fermented wort, together with *Lactobacillus delbrueckii*. Finally, the beer is bottled and warm conditioning at ≥20°C for 3–4 weeks follows before release [1].

In contrast, the manufacturer producing Berliner Weisse No. 3 uses pure cultures of both yeast and *Lactobacillus delbrueckii* for fermentation. Prior to bottling only *Kräusen* but no lactic cultures are added followed by maturation of the brew for at least 3 months [1].

Thus, it is most likely that the strains of *Lactobacillus delbrueckii* added for secondary fermentation (bottle conditioning) are responsible for the racemization of L-Pro in the Berliner Weisse No. 1. Indeed, there is evidence that certain strains of *Lactobacillus* have a Pro-racemase, for high amounts of Pro have been detected in fermented dairy products [14,22].

In lambic beers the typical markers D-Ala, D-Asp, D-Glu and D-Pro were detected, as well as D-Tyr, D-Arg, D-Ser and/or D-Lys. High amounts were observed in particular in so-called fruit lambics (cf. Figs. 2 and 3 and Table 2). Due to the addition of fresh fruits or fruit juices, the amino acid pattern of lambics is distinct from that of common beers. In beers exclusively made from barley and/or wheat or their respective malts, L-Pro is the major amino acid. In contrast, L-Asp or L-Asn are the most abundant L-amino acids in fruit beers manufactured with addition of cherries (kriek-lambic, Fig. 2), peaches (pecheresse lambic, cf. Fig. 3), or raspberries (frambiose lambic, Table 2).

GC analysis of amino acids cannot discriminate between Asp and Asn originally present in samples (as well as between Glu and Gln) due to the hydrolysis of the amides under the acidic derivatization conditions used (see Experimental). Therefore, amino acid analysis of fruit lambics via HPLC after derivatization with OPA–IBLC or OPA–IBDC [14] was carried out. It revealed that L-Asn was the major amino acid (356–912 mg/l), at least in these beers (cf. Fig. 3).

In comparison to special beers, the common lagers, ales and Pilsener beers contain lower absolute and relative amounts of D-amino acids (cf. Table 2). Therefore highly sensitive and specific GC–selected ion monitoring (SIM) MS was used for quantification.

For GC–FID, TFA derivatives were analyzed in order to circumvent problems arising from the presence of impurities (e.g., PFPA contains frequently ca. 1% TFAA). This problem does not occur in the SIM-MS detection because of preselection of characteristic fragments for the PFP derivatives only.

The typical chromatogram of a German lager beer is shown in Fig. 4. The total amino acid content of German lagers and Pilseners ranged from 1275 to 2260 mg/l, whereas British ales had considerably
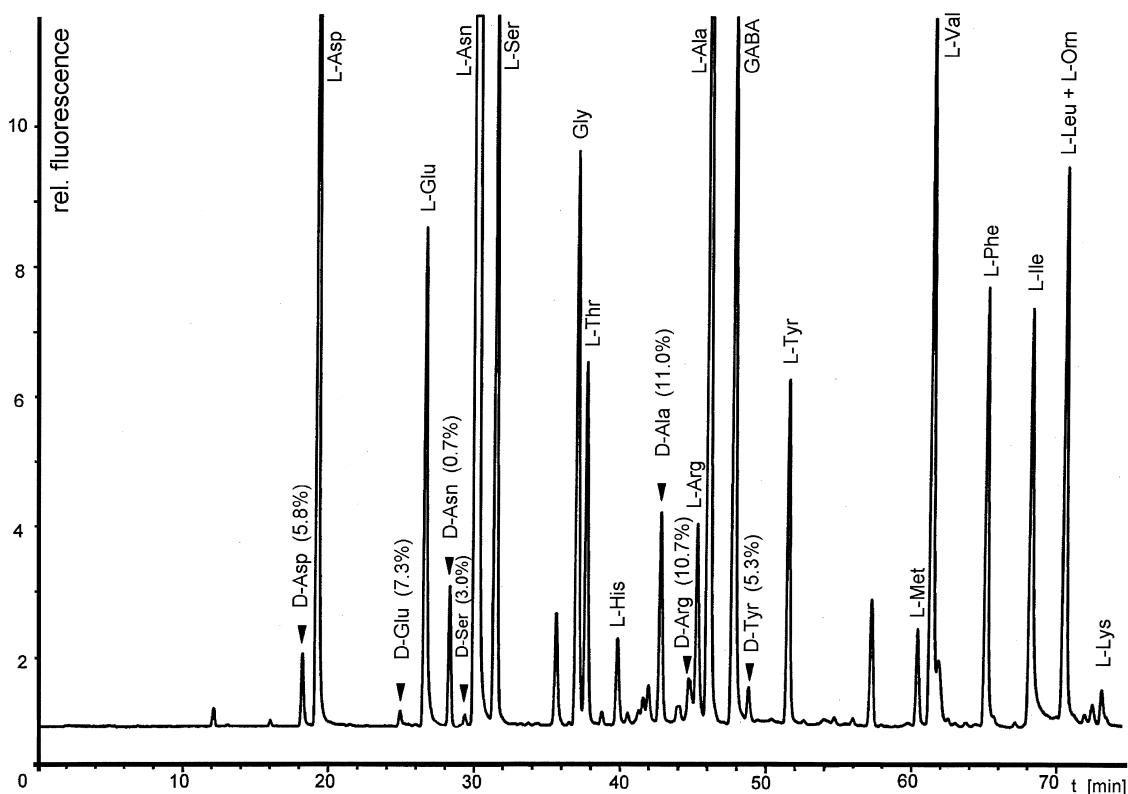
Fig. 3. HPLC of a Pecheresse-lambic beer; amino acids were separated on ODS2 Hypersil as OPA–IBLC derivatives; for chromatographic conditions see Experimental.

lower amounts of 553–785 mg/l. In wheat beers moderate amounts of amino acids ranging from 560 to 1080 mg/l were detected (cf. Tables 1 and 2).

Absolute amounts of D-amino acids only exceeded 30 mg/l in Berliner Weisse beers, certain lambic beers and strong beers. In the latter beers, however, the relative amounts of D-amino acids were as low as in common lager beers. This indicates that the higher absolute amounts of D-amino acids of strong beers are correlated with the higher original gravities of these beers (cf. Table 2).

In a previous study the amino acid content of wort and beer was investigated by HPLC [23]. Although the method applied did not provide enantiomeric separation of amino acids, and Pro could not be determined by the methodology used, the results are in good agreement with our quantitative data.

In another investigation the enantiomers of Pro, Leu and Phe were determined quantitatively in beers

[16]. Limitations on chiral resolution of three hydrophobic amino acids are attributed to the two-dimensional HPLC method used. Total amounts of these amino acids, in particular those of Pro, reported in Ref. [16] were much lower in comparison to the data presented in this work and those reported in the literature [23–25].

In a pale ale 2 mg/l total Pro, 1 mg/l total Phe, and 0.4 mg/l total Leu were determined [16]. In the same brand of beer (cf. Table 2) quantities of Leu, Phe and Pro, determined by GC, were 20-fold and 150-fold, respectively, higher than those reported for pale ale [16].

Amounts of total Pro, Leu and Phe reported in the literature are as follows: barley malts 720–786 mg Pro/kg, 134–180 mg Leu/kg and 102–126 mg Phe/kg [25]. Analyses of wort provided values of 122.5 mg Leu/l and 92.1 mg Phe/l (Pro not determinable as a result of the method used) [23]. Common beers
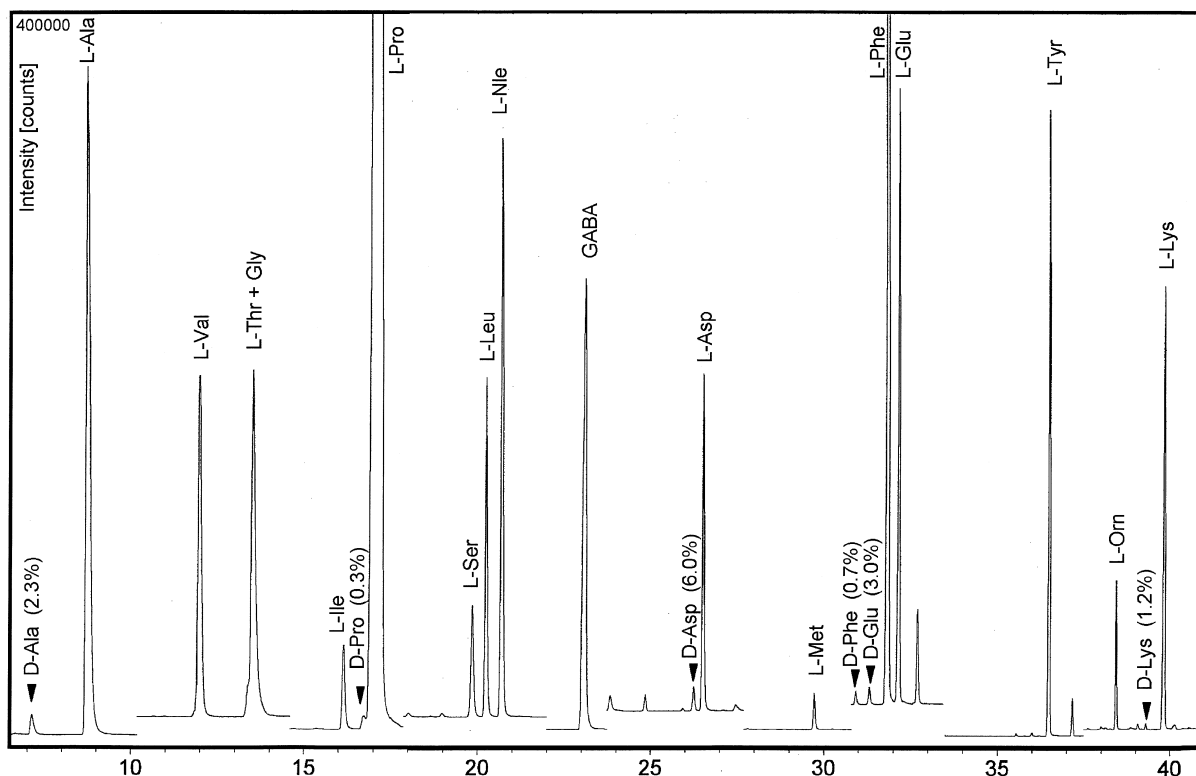
Fig. 4. GC–SIM-MS of a German lager beer; amino acids were separated on Chirasil-L-Val as pentafluoropropionyl amino acid 2-propyl esters; for chromatographic conditions see Experimental; varying baseline levels are the consequence of programmed ionsets resulting from differing numbers of selected characteristic mass fragments.

contain ca. 400 mg Pro/l, 3–60 mg Leu/l and 5–99 mg Phe/l [24]. Further, in our investigations we could not detect D-Leu in any beer sample ($n=42$) by sensitive and specific GC methods.

4. Conclusions

GC on chiral stationary phases is highly suitable for the quantitative determination of amino acid enantiomers in beers and raw materials used for manufacturing. Although L-amino acids were most abundant, certain D-amino acids were detected in all beers.

Quantities and pattern of amino acid enantiomers are strongly dependent on raw materials used and, in particular, on brewing processes employed and microorganisms involved. Raw materials contribute to a minor (grains, malt) or negligible (hops) extent

to the D-amino acid content of beer. Possibly, D-amino acids are also formed in the course of the non-enzymatic browning or Maillard reaction on heating malt, mash and wort [26].

Highest amounts of D-amino acids were determined in special beers (Berliner Weisse) subjected to long maturation and bottle conditioning with lactic cultures. Exceptionally high amounts of D-Pro in these beers are attributed to the action of a bacterial racemase [9]. Belgian fruit beers fermented with wild yeasts and a complex bacterial flora also comprise high amounts of D-amino acids. Further, addition of fresh cherries, peaches or raspberries, or their respective juices, alters the amino acid composition of these beers significantly. Thus, quantities, relative amounts and pattern of amino acid enantiomers can serve as indicatives for authenticity and quality of beers.

Finally, it should be emphasized in this context

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that negative health claims possibly drawn from the occurrence of D-amino acids in beers, or foods in general, taking quantities and realistic food intake into account, lacks experimental evidence [27,28]. D-Amino acids are common in the diet and occur, in part in high amounts, in particular in fermented foods. D-Amino acids ingested with the diet are effectively oxidized by D-amino acid oxidase localized in particular in the kidneys, as well as other organs and tissues of the body, providing 2-oxo acids which are further metabolized. Varying amounts of D-amino acids are also permanently excreted with the urine [28–30].

Acknowledgements

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