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Chapter 4.4

GAS CHROMATOGRAPHIC DETERMINATION OF D-AMINO ACIDS IN FOOD AND BEVERAGES

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1. INTRODUCTION

In the past formation and analysis of D-amino acids in proteins or foodstuffs treated with alkali or subjected to otherwise harsh conditions attracted priority attention [1-3]. In the last two decades it was fully realized, however, that bacterial fermented foods and beverages are major sources of D-amino acid uptake with the diet [4-10]. Free D-amino acids have also been detected as natural constituents of plants, algae, bivalves, crustaceae and wild or domesticated animals [11-16]. Consequently, food of plant or animal origin, are additional sources of dietary D-amino acid uptake. Furthermore, we have recently shown that D-amino acids are formed on heating saccharides with L-amino acids, a reaction referred to as the Maillard reaction. It was postulated that D-amino acids are formed on decay of relatively stable intermediates of this reaction named the Amadori compounds [17-19].

For the analysis of D-amino acids in foodstuffs preferably high performance liquid chromatographic (HPLC) and gas chromatographic (GC) methods are used. For HPLC analysis precolumn derivatization of amino acids with Marfey's reagent (2,4-dinitrofluorobenzene-L-alanine amide) [20] and UV detection of the diastereoisomers formed were used for the investigation of proline isomers in wine [21]. For analyzing of a broad

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range of foodstuffs (cheese, honey, yeast spread) and beverages (wine, beer, fruit juices) derivatization of amino acids with o-phthaldialdehyde together N-isobutyryl-L-cysteine and resolution of the resulting fluorescent diastereomeric isoindol derivatives on ODS-stationary phases were used [12,22,23]. Ligand exchange chromatography and use of L-phenylalaninamide together with copper (II) acetate as chiral additive were employed for the HPLC analysis of food D-amino acids [8]. Capillary electrophoresis was used for the determination of DL-Asp in white and red wine [24].

The most frequently used chiral selector for gas chromatographic chiral amino acid analysis is Chirasil®-L-Val representing dimethylpolysiloxane functionalized with L-valine tert. butylamide. This is attributed to the thermal stability and commercial availability of this stationary phase [25-27]. The resulting fused silica capillary column was and still is intensively used for the investigation of fermented and nonfermented foods as well as alcoholic and nonalcoholic fermented beverages such as beer, wine, vinegars, fruit juices and plant products [5-7,11,28]. For GC analysis conversion of non-volatile amino acids into volatile derivatives is required. Trifluoroacyl or pentafluoropropionyl amino acid alkyl esters are usually employed. Recently the separation of *N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl esters of food DL-amino acids on Chirasil®-L-Val was reported [29].

Other commercially available chiral columns for GC such as functionalized γ-cyclodextrin (Lipodex® E) have also been used for food analysis, albeit much less frequently [7,10]. Finally it should be mentioned that laboratory designed trioxaundecanoyl tetramide (Phe-3-O-TA) was intensively used for the analysis of DL-amino acids in foods and drinks [8,30,31].

In the following we provide protocols and demonstrate the versatility of Chirasil®-L-Val for the detection of D-amino acids in food and beverages. Furthermore, we propose a mechanism for the generation of D-amino acids in the course of the Maillard reaction which is of major importance in the food industry.

2. MATERIALS

2.1. Equipment

1. Shimadzu 14 A gas chromatograph with flame ionization detector (FID) or GC 17 A coupled to a Model QP5000 mass spectrometer (MS) (Shimadzu, Kyoto, Japan).
2. Fused silica capillary column Chirasil®-L-Val (*N*-propionyl-L-valine *tert.* butylamide polysiloxane) (25 m x 0.25 mm id; film thickness 0.12 µm) (Varian-Chrompack, Darmstadt, Germany)
3. Reactitherm Heating/Stirring Module with 'Reacti-Vap' Evaporator Model 18780 (Pierce, Rockford, IL, USA) or Heating Module (Model BT 2000, Kleinfeld Labortechnik, Gehrden, Germany) and nitrogen distributor device.
4. Ultra-Turrax® homogenizer (Model T25, IKA Labortechnik, Staufen, Germany).
5. Vials (1 or 5 mL) with teflon-lined screw caps (ReactiVials®, Wheaton, NJ, USA).
6. Rotatory evaporator with vacuum pump (Model Laborata 4000; Heidolph Instruments, Schwabach, Germany).

7. Quartz distill for generating doubly distilled water (Model Destamat Bi 18^{E}; Heraeus Instruments, Osterode, Germany).
8. Centrifuge (Model Labofuge 400, Heraeus Instruments).
9. Microsyringes (Mikroliter # 701; Hamilton, Reno, NV, USA).
10. Pasteur capillary pipettes (15 cm x 0.5 cm id), outlet closed with glass wool plug.
11. Nitrogen supply.

2.2. Chemicals and Reagents

1. Solvents: methanol (MeOH), 1-propanol (1-PrOH), 2-propanol (2-PrOH), dichloromethane (DCM), petroleum ether (b.p. 40–70°C).
2. Derivatizing reagents: trifluoroacetic acid anhydride (TFAA), pentafluoropropionic acid anhydride (PFPAA), acetyl chloride (AcCl), 10% aqueous trichloroacetic acid (TCA).
3. AcCl/1-PrOH: 1-PrOH (9 mL) and AcCl (1 mL) are mixed with stirring and chilling.
4. BHT (10%, *g/v*) in 1-PrOH: BHT (50 mg) dissolved in 1-PrOH (5 mL).
5. Internal standard 10 mM L-Nle: Nle (13.1 mg) dissolved in 0.1 M HCl (10 mL).
6. Amino acid standard solution (AA-S-18; from Sigma Chemicals, St. Louis, MO, USA) containing 2.5 M L-amino acids: Ala, Arg, Asp, Cys (1.25 M), Glu, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val and achiral Gly; GABA and L-Nle or special amino acids have to be added.
7. A qualitative standard composed of D- and L-amino acids in the ratio 1:2 is prepared by mixing appropriate quantities of enantiomers (or addition of L-amino acids to DL-amino acids).
8. Dowex 50W X8-400 cation exchanger (200 – 400 mesh from Supelco Park, Bellefonte, Pennsylvania, USA).
9. Doubly distilled water from a quartz distill was used exclusively.

2.3. Sources of Foodstuffs

Cocoa beans: 'Forastero', Ivory Coast, roasted at 150°C for 120 min, provided by a major chocolate producer, Munich, Germany.
Forest honey: 'Waldhonig Auslese', commercial product, Germany.
Maple syrup: 'Spring Tree', Grad A, from Quebec, Canada.
Grape juice concentrate: Turkish 'Pekmez', imported product.
Raisin: 'Sweet Valley' product of USA.
Sherry: Jerez Fino 'Tio Pepe', from Jerez, Spain.
Cheddar: 'Farmhouse' cheddar, matured for 12 months, Somerset, UK.

3. METHODS

Treatment and analyses are described exemplarily in the following. Procedures can be applied to solid, syrupy and liquid foodstuffs.

3.1. Cacao

Cacao beans (10 g) were grinded in an electric coffee mill. To aliquots (0.5 g) petroleum ether, b.p. 50-70°C (3 x 4 mL) was added, the mixture was stirred for about 30 min and centrifuged (1200 *g*, 15 min). The procedure was repeated twice. The organic solvents were discarded and the remaining residues extracted with 0.1 M HCl (3 x 3 mL). The combined extracts were evaporated to dryness using a rotatory evaporator at 10 mbar and a bath temperature of 40°C. To the remaining residue 0.1 M HCl (2 mL) was added. To aliquots (100 µl) bidistilled water (2 mL) and the internal standard L-Nle (10 mM in 0.01 M HCl, 20 µl) was added and the pH was adjusted to 2.3 by addition of 0.5 M HCl. The solution was passed through a Pasteur pipette filled with activated Dowex 50W X8 cation exchanger (bed volume 0.5 cm x 5 cm length). The resin was washed with water (about 10 mL) until neutral and amino acids were eluted with 4 M aqueous ammonia (3 mL). The effluent was evaporated to dryness *in vacuo* in a conical flask (20 mL), then 0.1 M HCl (1 mL) was added and the solution transferred into a ReactiVial®. The solvent was removed in a stream of nitrogen. To the remaining residue 2.5 M HCl in 1-PrOH (0.5 mL) and BHT in 1-PrOH (20 µl) were added and the mixture heated in the tightly closed vial at 100 °C for 1 hour. Then reagents were removed at ambient in a stream of nitrogen, DCM (300 µl) and PFPAA (50 µl) were added and the mixture heated for 20 min at 100 °C and excess of reagents removed in a stream of nitrogen. Then DCM (100 µl) was added, solvent removed in a stream of nitrogen, the procedure repeated once and finally the remaining residue was dissolved in DCM (200 µl) and 0.5 to 1.0-µl aliquots were injected onto the GC column.

3.2. Honey, Maple Syrup and Grape Juice Concentrate

To aliquots (1 g) of foodstuffs water (5 mL) was added and resulting solutions were adjusted to pH 2.3 by addition of 0.01 M HCl. Analytes were passed through cation exchanger columns (bed volume 0.5 x 3.5 cm) and amino acids eluted, derivatized and analyzed as described above. After washing with water (10 mL) the effluent was evaporated to dryness, derivatized as described above and analyzed by GC-SIM-MS.

3.3. Raisins

Amounts (1 g) of raisins were briefly washed with ethanol in order to clean the surface and then minced with a lancet. To aliquots (1 g) 10% aqueous TCA (4 mL) was added and

the mixture was blended using of an Ultra-Turrax® homogenizer. Then the mixture was centrifuged, the precipitate was discarded and the supernatant transferred into a separatory funnel. The aqueous phase was extracted with diethyl ether (3 x 10 mL) in order to remove excess of TCA. Then the aqueous phase was adjusted to pH 2.3 by addition of 0.1 M HCl and passed through a Dowex 50W X8 cation exchanger. The effluent was evaporated to dryness and amino acids converted into derivatives and analyzed as described above for cacao.

3.4. Sherry Wine

An aliquots of the fortified wine (5 mL) was adjusted to pH 2.3 by addition of 0.01 M HCl. The resulting solution was passed without further treatment through the cation exchanger and amino acids were eluted, derivatized and analyzed as described above.

3.5. Cheese

The interior of Cheddar cheese (about 200 g) was finely grinded and to an aliquot (100 mg) water (5 mL) and 50 mM L-Nle in TFA (40 µl) were added with magnetic stirring. Additional TFA (1 mL) was added in order to precipitate proteins. The mixture was stirred for 15 min and then filtered through fluted paper. The filter was washed with water, the filtrate transferred to a separatory funnel and extracted with petroleum ether (2 x 20 mL). The organic phase containing lipids was discarded. Then the aqueous phase was evaporated to dryness, 5 mM HCl (2 mL) was added and the solution passed through the cation exchanger. The effluent was treated and analyzed as described above.

4. RESULTS

Chromatograms resulting from analyses of a standard and food samples are compiled in Figure 1. The GC-SIM-MS on Chirasil®-L-Val of derivatives of an amino acid standard composed of D- and L-amino acids (ratio about 1:2), including achiral Gly and GABA and the internal standard L-Nle, together with a peak resulting from the antioxidant BHT, is presented in Figure 1a. Under the conditions used D-Met and L-Asx (the latter actually representing L-Asp) are not resolved. Using SIM-MS this does not cause problems since co-eluting amino acid derivatives can be distinguished owing to their characteristic fragment ions.

Enantioselective GC-SIM-MS of roasted cacao beans is shown in Figure 1b. The high relative quantities of D-amino acids and their diversity are worth of noting and have been recognized previously [7,32]. On roasting of cocoa beans Amadori compounds (fructose amino acids) are formed and proceeding of the Maillard Reaction is indicated by colour change and formation of characteristic flavour compounds. Amadori compounds are precursors of D-amino acids (see Figure 2).

The chromatogram of a forest honey is shown in Figure 1c. Honey is produced from plant nectars by *Apis mellifera* L. Since honey is not subject of severe technological treatment the

presence of D-amino acids is somewhat unexpected. However, honey might be considered to represent a very concentrated solution of glucose and fructose in water which contains also free amino acids besides other minor constituents. Consequently, the Maillard reaction proceeds to a certain extent and presence of D-amino acids in honey is attributed to racemization of L-amino acids in the course of this reaction.

This result is supported by analysis of a maple syrup (Fig. 1d), representing a plant sap from the maple tree *Acer saccharum* L. which is commonly concentrated by boiling. Relative high amounts of D-amino acids can be seen in the chromatogram. Maple sap contains saccharose and amino acids. On heating and proton catalysis saccharose is converted partly into glucose and fructose. The former yields fructose amino acids (Amadori compounds), the latter glucose amino acids (Heyns compounds) on heating with amino acids.

The chromatogram of concentrated grape juice (Turkish 'Pekmez'), representing a dark brown syrup, indicating intensive Maillard reaction, is shown in Figure 1e. Large quantities of D-amino acids can be seen.

The chromatogram of amino acid enantiomers detected by GC-FID of raisins (dried grape fruits) is presented in Figure 1f. Here again large quantities of D-amino acids can be seen. Grapes are rich in glucose, fructose and amino acids. On drying concentrations increase and proceeding of the Maillard reaction is indicated by the typical brown colour of raisins.

The chromatogram of a Sherry wine, representing an alcoholic fermented beverage, is presented in Figure 1g. Sherry, like fortified wines in general, contains very high amounts of D-amino acids which approach or exceed 50% of the corresponding L-enantiomer in many cases [33].

Finally, the GC-FID of free amino acids isolated from an English cheddar cheese is presented in Figure 1h. Here, the large quantities of D-Pro are conspicuous. Cheese is a typical bacterial fermented food produced with the aid of lactobacilli and lactic acid streptococci. This explains the abundance of D-AAs in cheddar or fermented sour milk products in general [5].

These representative analyses demonstrate that D-amino acids are quite common in food and beverages and their presence, structural variety and formation requires explanations.

Formation of D-amino acids in foodstuffs that had been the subject of severe technological treatments (alkali, heat, pressure etc.) is well known for many years and has been reviewed in detail [3]. It has also been fully realized in recent years that all kinds of bacterial fermented food, i.e. food that attributes its appearance, texture, flavor and nutritional acceptance to the action of bacteria, contain D-amino acids. Bacteria have an abundance of racemases and contain D-amino acids in their cytoplasm and cell walls (peptidoglycan) [9].

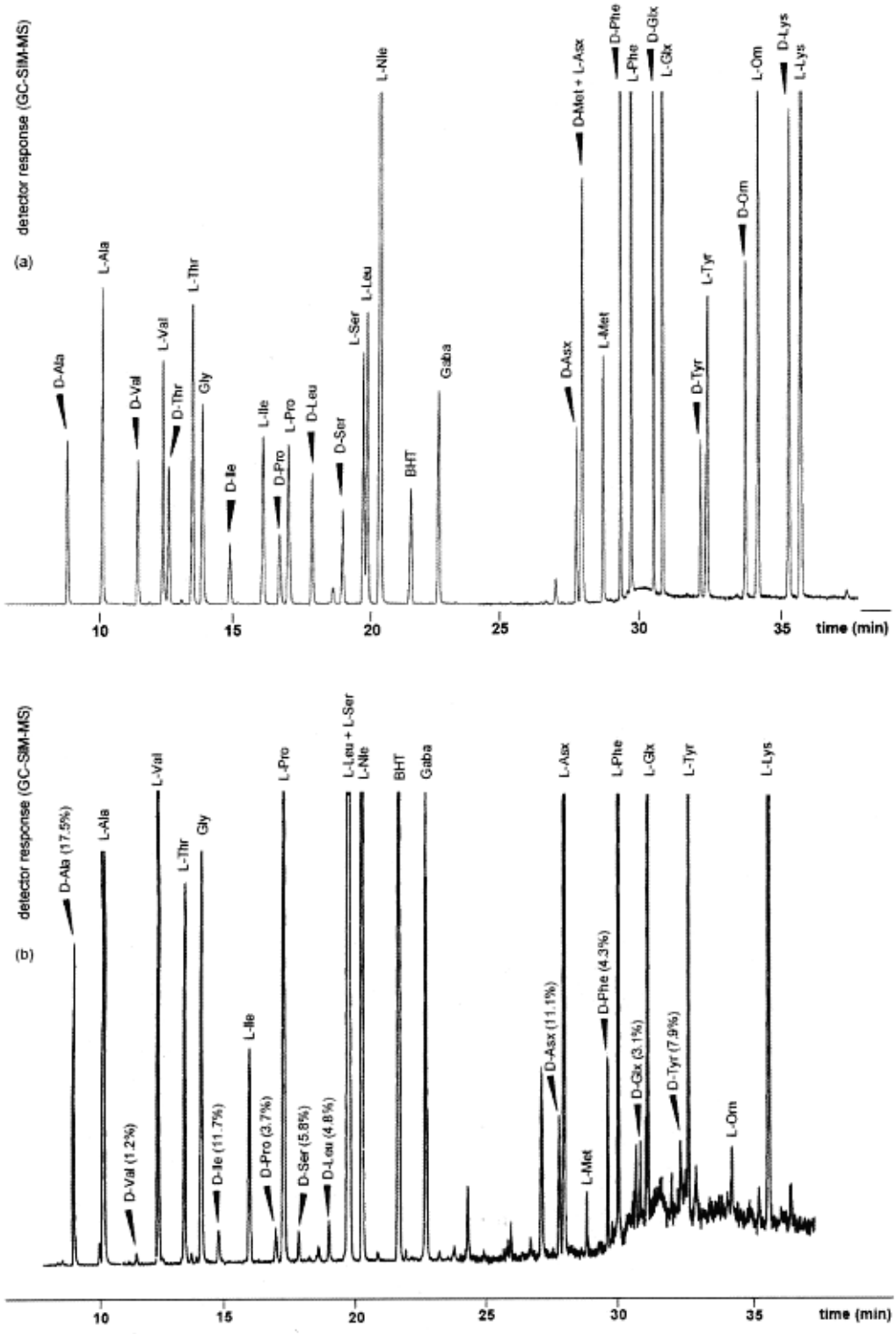
However, neither harsh technological treatment nor bacteria are satisfactory explanations for the occurrence of D-amino acids in honey and cocoa, or dried plant products such as raisins or fruit syrups.

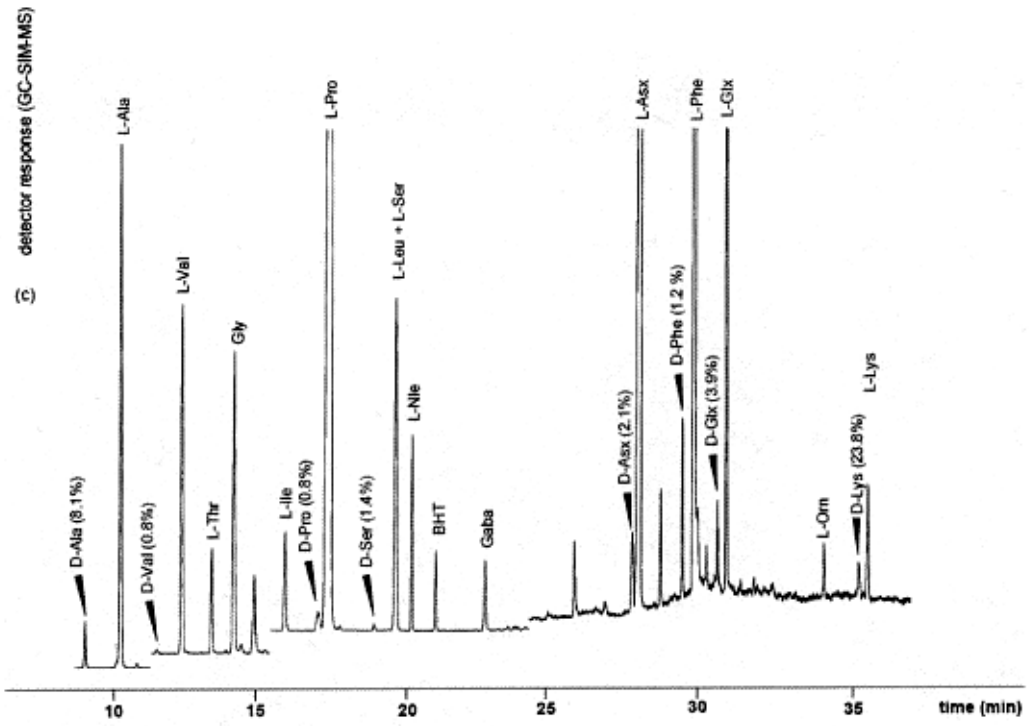
We had recognized in previous studies that heating of saccharides together with L-amino acids leads to the formation of D-amino acids [17]. This interaction is well known as non-enzymic browning or the Maillard reaction. Consequently, it is reasonable to assume that D-amino acids are generated in the course of the Maillard reaction. All conditions favoring the Maillard reaction will lead to the formation of D-amino acids. Kinetics of formation are dependent on concentrations and structures of reactants, temperature, pH, water activity, and

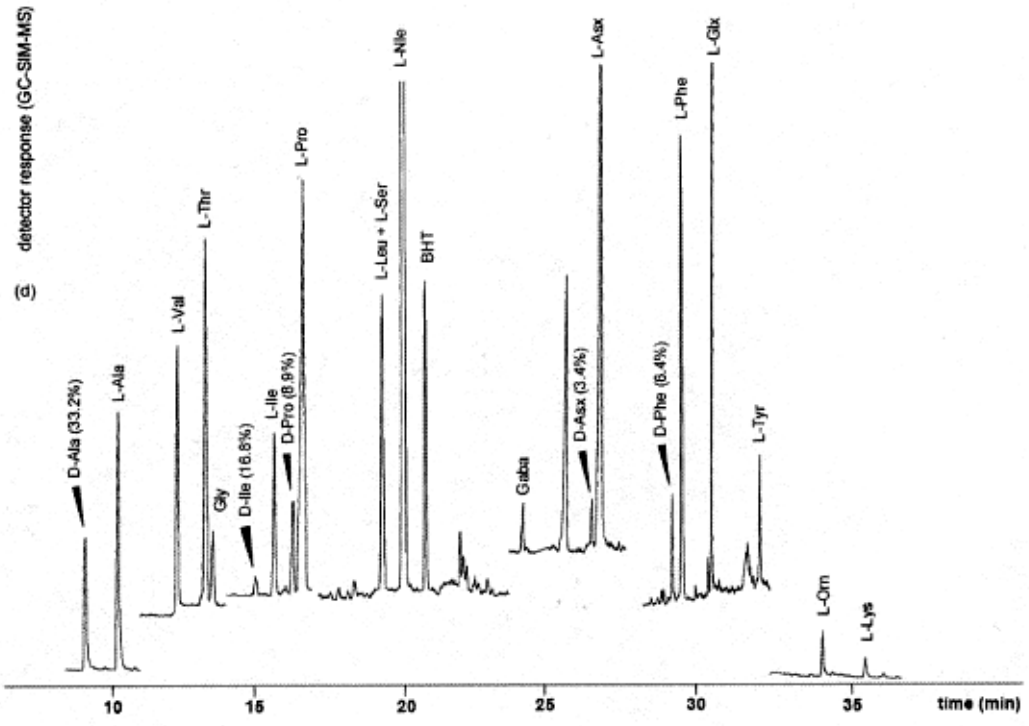
presence of catalysts. Notably, the Maillard reaction proceeds also at ambient temperature or in physiological fluids [34].

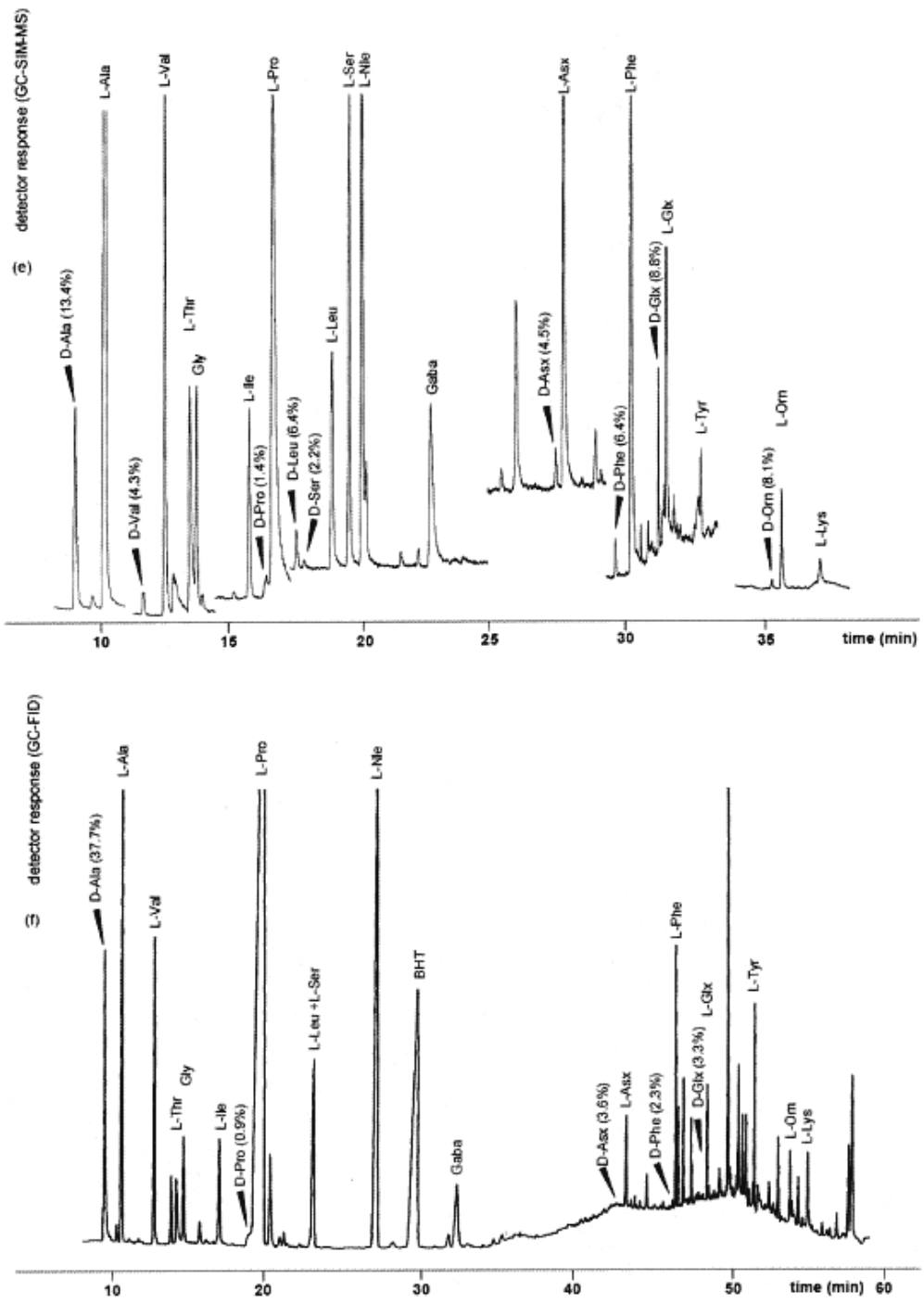
Relative stable intermediates of the Maillard reaction are the Amadori and Heyns compounds (fructose amino acids and glucose amino acids). Indeed, Amadori compounds (fructose amino acids) are formed and have been detected in many foodstuffs such as milk powder, roasted cacao beans, fruit and vegetable powders, dried apricots and raisins, fruit juice concentrates and tobacco [32,35-37]. Heating of synthetic Amadori compounds containing L-amino acids leads to the release of D-amino acids and *vice versa*. A tentative racemization mechanism via intermediate formation of a planar carbanion has been suggested [18,19] and is presented in Figure 2. Proceeding of the Maillard reaction in foodstuffs will be accompanied inevitably with the formation of D-amino acids [38,39].

Relevance of D-amino in foodstuffs and uptake therefrom is still a matter of debate and also discussed in detail in this volume by M. Friedman. Peptide bonded D-amino acids without doubt prevent or hamper digestibility of food proteins and thus reduce nutritional value. With regard to free D-amino acids corresponding to the protein L-amino acids and under conditions of realistic food intake we do not expect adverse health effects on normal human beings. This view is corroborated by data on clinical administration of large quantities of racemic amino acid mixtures to infants. Owing to the expenses of pure amino acid enantiomers such clinical administration was common at the beginning of parenteral nutrition [9,37,40]. Reports on nephrotoxic effects of D-Ser administered in high doses to rat should be discussed in the light of the natural occurrence of D-Ser in man and animal brain [16,41,42] and pharmaceutical use of DL-Ser as chelating agent for iron or use of the magnesium salt of DL-Asp as cardioprotective drug [43]. Further, D-amino acids are converted by mammalian D-amino acid oxidases to α-keto acids which are further metabolized. Notably, an abundance of D-amino acids are excreted with the urine. Consequently, research on physiological D-amino acids has changed to their endogenous biosynthesis and the specific roles which D-amino acids may play in living organisms [41,44,45].


(a)
detector response (GC-SIM-MS)
D-Ala
L-Ala
D-Val
L-Val
D-Thr
L-Thr
Gly
D-Ile
L-Ile
D-Pro
L-Pro
D-Leu
D-Ser
L-Ser
L-Leu
L-Nle
BHT
Gaba
D-Asx
D-Met + L-Asx
L-Met
D-Phe
L-Phe
D-Glx
L-Glx
D-Tyr
L-Tyr
D-Orn
L-Orn
D-Lys
L-Lys
10
15
20
25
30
35
time (min)
(b)
detector response (GC-SIM-MS)
D-Ala (17.5%)
L-Ala
D-Val (1.2%)
L-Val
D-Thr
L-Thr
Gly
D-Ile (11.7%)
L-Ile
D-Pro (3.7%)
L-Pro
D-Ser (5.8%)
D-Leu (4.8%)
L-Leu + L-Ser
L-Nle
BHT
Gaba
D-Asx (11.1%)
L-Asx
L-Met
D-Phe (4.3%)
L-Phe
D-Glx (3.1%)
L-Glx
D-Tyr (7.9%)
L-Tyr
L-Orn
L-Lys
10
15
20
25
30
35
time (min)


(c)
detector response (GC-SIM-MS)
D-Ala (8.1%)
L-Ala
D-Val (0.8%)
L-Val
L-Thr
Gly
L-Ile
D-Pro (0.8%)
L-Pro
D-Ser (1.4%)
L-Leu + L-Ser
L-Nle
BHT
Gaba
D-Asx (2.1%)
L-Asx
D-Phe (1.2 %)
L-Phe
D-Glx (3.9%)
L-Glx
L-Orn
D-Lys (23.8%)
L-Lys
10
15
20
25
30
35
time (min)


(d)
detector response (GC-SIM-MS)
D-Ala (33.2%)
L-Ala
L-Val
L-Thr
Gly
D-Ile (16.6%)
L-Ile
D-Pro (8.9%)
L-Pro
L-Leu + L-Ser
L-Nle
BHT
Gaba
D-Asx (3.4%)
L-Asx
D-Phe (6.4%)
L-Phe
L-Glx
L-Tyr
L-Orn
L-Lys
10
15
20
25
30
35
time (min)


(e)
detector response (GC-SIM-MS)
D-Ala (13.4%)
L-Ala
D-Val (4.3%)
L-Val
L-Thr
Gly
L-Ile
D-Pro (1.4%)
L-Pro
D-Leu (6.4%)
D-Ser (2.2%)
L-Leu
L-Ser
L-Nle
Gaba
D-Asx (4.5%)
L-Asx
D-Phe (6.4%)
L-Phe
D-Glx (8.8%)
L-Glx
L-Tyr
D-Orn (8.1%)
L-Orn
L-Lys
10
15
20
25
30
35
time (min)
(f)
detector response (GC-FID)
D-Ala (37.7%)
L-Ala
L-Val
L-Thr
Gly
L-Ile
D-Pro (0.9%)
L-Pro
L-Leu +L-Ser
L-Nle
BHT
Gaba
D-Asx (3.6%)
L-Asx
D-Phe (2.3%)
L-Phe
D-Glx (3.3%)
L-Glx
L-Tyr
L-Orn
L-Lys
10
20
30
40
50
time (min)
60

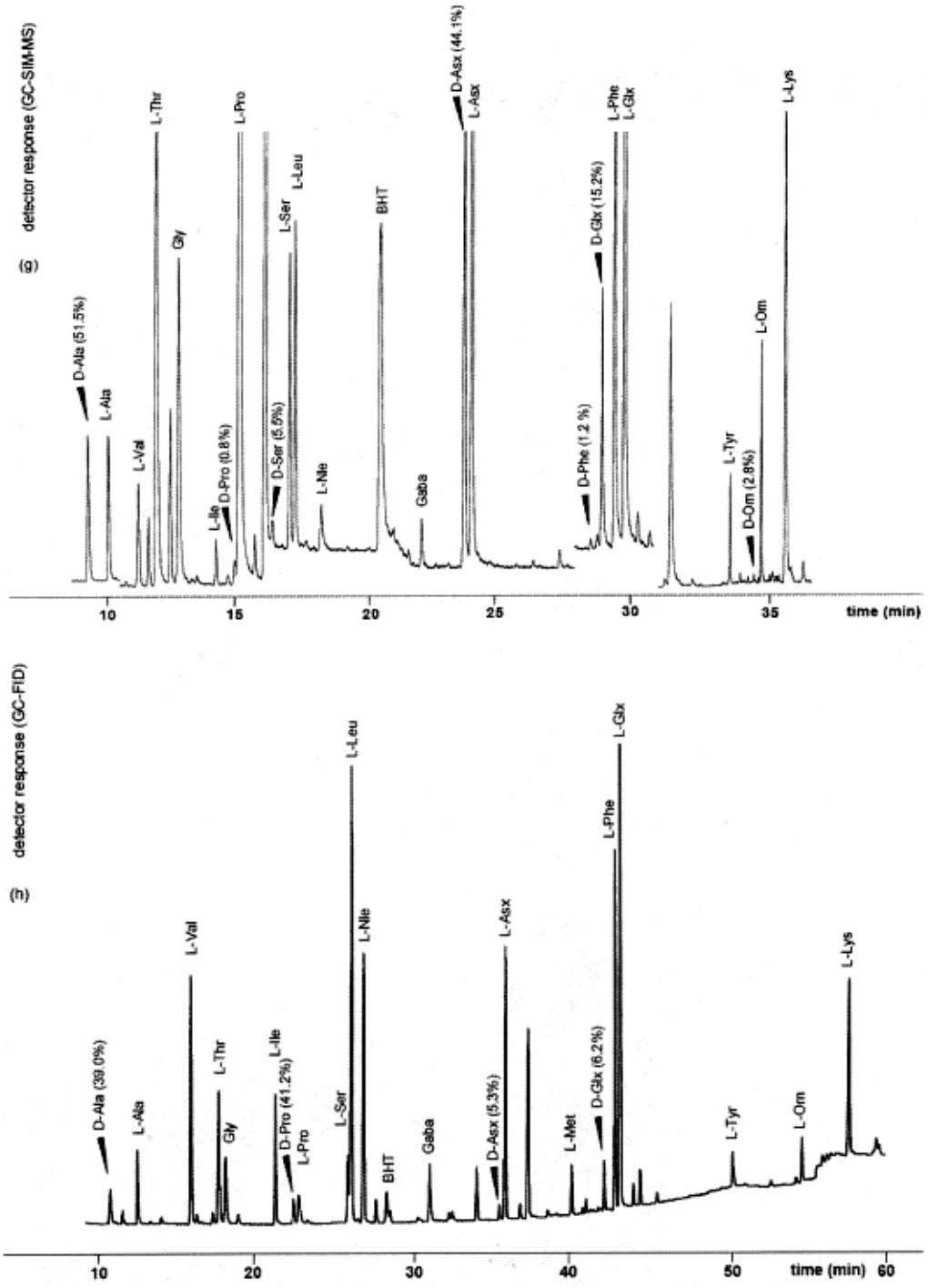


Figure 1. GC-SIM-MS of PFP-amino acid-(2)-Prp esters resolved on Chirasil®-L-Val of (a) standard of DL-amino acids (ratio D:L ca. 1:2), and amino acids extracted from (b) roasted cocoa from Ivory Coast, (c) forest honey, (d) maple syrup, (e) Pekmez, and GC-FID of PFP-amino acid-(2)-Prp esters from (f) raisin 'Sweet Valley', and GC-FID of PFP-amino acid-(1)-Prp esters from (g) Sherry, and (h) cheddar cheese. For chromatographic conditions, see note 7 and 8.

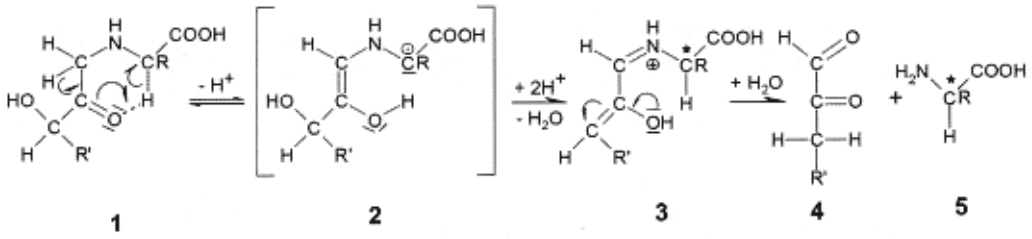


Figure 2. Proposed racemization mechanism of amino acids in Amadori compounds 1 formed in the course of the Maillard reaction. C^{α}–proton abstraction from bonded amino acids is favored by formation of an intramolecular hydrogen bridge as indicated in 1, followed by 1,2-enaminolisation and generation of an intermediate carbanion 2 that is capable of being racemized on reprotonation. Dehydratisation yields the imin cation 3 which decomposes on heating with formation of 3-deoxyosone 4 and release of (partially) racemized amino acid 5. R = amino acid side chain; R' = sugar residue; asterisk refers to racemized amino acid.

5. Notes

Based on many years of experience concerning gas and liquid chromatographic determination of D-amino acids in biosamples we provide some notes related to analytical methodology.

1. Sample Treatment

- Samples should be adjusted to pH 2.3 before subjected to ion exchange.
- Loss of aspartic acid might occur at pH<2 or on loading large analytical volumes
- Triglycerides and lipids from analytes should be removed by extraction with diethyl ether *n*-hexane or ethyl acetate.
- In some cases it is of advantage to extract amino acids with 70% ethanol or methanol. In these solvents proteins and polysaccharides are not or sparingly soluble.
- Proteins can be precipitated and removed by centrifugation using trichloroacetic acid, picric acid or sulfosalicylic acid.

2. Ion Exchange

- Batches of the cation exchanger should be carefully washed successively with doubly distilled water, 2 M sodium hydroxide, water, 2 M HCl, 5 M aqueous ammonia and finally with water until neutral. The ion exchanger should be stored in 0.1 M HCl and washed with water until neutral prior to use. Ion exchangers should be stored protected from light. Used ion exchanger should be discarded and not regenerated.

-Water should be of highest purity in order to avoid contamination with amino acids. We prefer doubly distilled water from a quartz distill as we have observed that traces of D-amino acids may occur even in commercially available water 'for chromatography' produced by reversed osmosis. D-amino acids may result from bacterial biofilms.

3. Glasware and Derivatization Procedure

- Precaution: reagents and solvents should be checked regularly for contamination with D-amino acids in a blank run.

- All kinds of bacterial contamination (including dust adjacent to glassware) will lead to the presence D-amino acids.

- If trace amounts of amino acids have to be determined glassware should be wrapped in aluminum folia and heated in a muffle furnace at 500 °C.

- A small crystal of BHT (or 25 µl of a 0.5% solution in the alcohol used for esterification) should be added to the analyte prior to derivatization.

- It is recommended to prepare a qualitative standard for calibration by mixing appropriate amounts of solid D- and L-amino acids (or addition of L-amino acids to the corresponding DL-amino acid) and fine grinding of the resulting standard mixture in a mortar. Thus gram amounts of a solid standard of almost infinite lifetime can be produced and aliquots handed over to coworkers and students in order to avoid contamination. A skilled analyst will use about 2 mg aliquots for derivatization and calibration.

- To aliquots of this standard special amino acids can be added, if required (e.g. hydroxyproline stereoisomers, Orn etc.).

- For quantitative purposes commercially available standard mixtures of L-amino acids can be used and L-Nle added since GC response factors for derivatives of L- and D-enantiomers are equal (see Experimental).

- We have realized that change of the splitting ratio of the GC injector influences response factors Therefore, concentration or dilution of samples by otherwise unchanged analytical conditions are recommended.

4. Chirasil®-L-Val and Chirasil®-D-Val

- Manufactures columns are tested with TFA/OMe esters of few DL-amino acids using hydrogen as carrier gas and isothermal temperature. We use PFP/2-Prp esters in most cases owing to the best overall separation in particular with regard to DL-Pro and enantiomers of DL-Glu and DL-Phe.

- Various companies offer Chirasil®-L-Val columns but only Macherey-Nagel the Chirasil®-D-Val having D-Val as chiral selector in place of L-Val. This reverses the elution order of enantiomers [11,10,27]. If using an FID detector use of both columns makes results very reliable. If SIM is used, this is not necessary. Furthermore, overlapping peaks can be distinguished according to specific fragment ions.

- The critical amino acid that is worst separated on Chirasil®-L-Val is DL-Pro. We have observed that this might lead to problems using the Permabond® column (i.e. crosslinked) Chirasil®-L-Val column from Machery-Nagel. Notably, DL-Pro is best resolved on γ-cyclodextrins such as Lipodex® E [7,10].

- Stereoisomers of special collagen amino acids such as hydroxyproline or hydroxylysine, bacterial 2,4-diaminopimelic acid or lanthionine and enantiomers of dipeptides are also resolvable on Chirasil®-Val [46-48].

- It should be noted that many data published by the inventors of the Chirasil®-L-Val and Chirasil®-D-Val columns have been obtained from laboratory made and coated glass (not quartz) columns using hydrogen as carrier gas [25]. These columns were also fine-tuned and selected with regard to best enantioresolution. Commercially available columns show somewhat different elution behavior of enantiomers.

5. Problematic Amino Acids

- Problematic amino acids are Arg (not derivatized), His (imidazol group requires special derivatization using e.g. ethyloxycarbonylchloride); Asn, Gln (amides are hydrolyzed to acids), Ser/Thr (low stability of esters), Cys_2 (reduction to cystein required); Ile, Thr (*allo*–forms may lead to problems owing to assignment of stereoisomers); Trp (partly destroyed).

6. Relative and Absolute Quantification of Amino Acid Enantiomers

- Relative amounts of D-amino acids were calculated according to equation (1)

$$\%D = 100\, A_D / (A_D + A_L) \quad (1)$$

where %D is the relative amount of the D-amino acid to be determined, and A_D and A_L are the peak areas (ion abundances) of the D- or L-enantiomer, respectively, determined by GC-SIM-MS. For quantification response factors of amino acids in a standard mixture (for composition see above) were determined in relation to the internal standard (IS) L-Nle. Equimolar amounts of amino acids of the standard mixture, including L-Nle, were injected into the GC-MS system.

- Response factors were calculated according to equation (2)

$$f_R = A_{LAA}/A_{IS} \quad (2)$$

where f_R is the response factor of the amino acid to be determined, A_{LAA} the peak area of amino acid to be determined, and A_{IS} the peak area of the IS obtained from the standard amino acid mixture. Amino acids in samples were quantified according to equation (3)

$$c_{LAA} = (1/f_R \, x \, A_{LAA})/(A_{IS} \, x \, c_{IS}) \quad (3)$$

where c_{LAA} is the amount of L-amino acid, f_R the response factor, A_{LAA} the peak area of the L-amino acid in the sample, A_{IS} the peak area of the IS added to the sample and c_{IS} the amount of the IS added to the sample. If required, from the relative quantities of D-amino acids *(%D)* and the quantities of L-amino acids c_{LAA} presented in Tables 2-4, the absolute quantities of D-amino acid c_{DAA} can be calculated according to equation (4).

$$c_{DAA} = c_{LAA}\, (\%D) / (100 - \%D) \quad (4)$$

7. Conditions for GC-SIM-MS

- The conditions described here for GC-MS and GC-FID have been used for foodstuffs shown in Figure 1.

- Gas chromatographic conditions are dependent on the instruments and chiral columns used, together with the carrier gas and amino acid derivatives employed.

-Carrier gas helium at an inlet pressure of 5 kPa, purge flow 3 mL /min and flow rate 0.5 mL /min. Injector and interface temperatures 250°C and split ratio 1:30. The temperature program 70 °C for 1 min, then 2.5 °C/min to 100 °C, 2 min isothermal, then 3.5°C /min to 135°C, then 5 °C/min to 150 °C, then 20°C /min to 190°C, and 8 min isothermal.

- Pressure of carrier gas 5 kPa for 1 min, then 0.2 kPa/min to 7.0 kPa, 2 min isobaric; then 0.3 kPa/min to 10.8 kPa, then 1.4 kPa/min to 13.0 kPa, then 2.4 kPa/min to 15.0 kPa, 8 min isobaric.

- For SIM appropriate ion sets of the PFP/2-Prp esters of the amino acids were selected and characteristic mass fragments (*m/z*) were used: Ala (190, 191, 235), Val (203, 218, 219), Thr (202, 203), Gly (176, 177), Pro (216), Leu (190, 232, 233), Ser (188, 189), Asx (189, 234, 235, 262), Met (203, 221, 263), Phe (91, 148, 190, 266), Glx (202, 230, 248, 276), Tyr (253, 266), Orn (216), Lys (176, 230), GABA, (176, 204, 232, 249).

8. Conditions for GC-FID

- Carrier gas helium at an inlet pressure of 46.8 kPa, constant flow 17.9 mL /min and split flow 15.0 mL /min; injector and detector temperatures 250 °C and split ratio 1:30. Hydrogen flow adjusted to 30.0 mL /min and synthetic air flow to 300.0 mL /min.

- Temperature program 2.5 °C/min from 85 °C to 100 °C, 2 min isothermal, then 0.6 °C/min to 115 °C, then 9.0 °C/min to 130 °C, then 6.0°C/min to 180°C, then 5.0°C/min to 190°C and 12.0 min isothermal.

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