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Gas chromatographic determination and mechanism of formation of D-amino acids occurring in fermented and roasted cocoa beans, cocoa powder, chocolate and cocoa shell*

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Summary. Fermented cocoa beans of various countries of origin (Ivory Coast, Ghana, Sulawesi), cocoa beans roasted under defined conditions (100–150 °C; 30–120 min), low and high fat cocoa powder, various brands of chocolate, and cocoa shells were analyzed for their contents of free L- and D-amino acids.

Amino acids were isolated from defatted products using a cation exchanger and converted into volatile *N*(*O*)-pentafluoropropionyl amino acid 2-propyl esters which were analyzed by enantioselective gas chromatography mass spectrometry on a Chirasil®-L-Val capillary column. Besides common protein L-amino acids low amounts of D-amino acids were detected in fermented cocoa beans. Quantities of D-amino acids increased on heating. On roasting cocoa beans of the Forastero type from the Ivory Coast at 150 °C for 2 h, relative quantities of D-amino acids approached 17.0% D-Ala, 11.7% D-Ile, 11.1% D-Asx (Asp + Asn), 7.9% D-Tyr, 5.8% D-Ser, 4.8% D-Leu, 4.3% D-Phe, 37.0% D-Pro, and 1.2% D-Val. In cocoa powder and chocolate relative quantities amounted to 14.5% D-Ala, 10.6% D-Tyr, 9.8% D-Phe, 8.1% L-Asx, and 7.2% D-Ile. Lower quantities of other D-amino acids were also detected. In order to corroborate our hypothesis that D-amino acids are generated from Amadori compounds (fructose amino acids) formed in the course of the Maillard reaction, fructose-L-phenylalanine and fructose-D-phenylalanine were synthesized and heated at 200 °C for 5–60 min. Already after 5 min release of 11.7% D-Phe and 11.8% L-Phe in the free form could be analyzed. Based on the data a racemization mechanism is presented founded on the intermediate and reversible formation of an amino acid carbanion in the Amadori compounds.

Keywords: Amino acid enantiomers – Racemization – Gas chromatography mass-spectrometry – Chirasil®-Val – *Theobroma cacao* L. – Maillard reaction – Amadori rearrangement products

Introduction

Cocoa beans represent the seeds of the tropical cocoa tree, *Theobroma cacao* L., of the family *Sterculiaceae*. The names 'Criollo' and 'Forastero' refer to subspecies of *Theobroma*, the botanical name meaning 'Food for the Gods'. 'Criollo' trees provide highly aromatic cocoa beans but are much more sensitive and lower in yield in comparison to 'Forastero' trees. 'Trinitario' represents a cross breed among both. Names used together with cocoa products such as 'Arriba', 'Nouméa' or 'Tobago' originally refer to regions of origin and are associated with the quality of cocoa beans.

Since harvesting and processing of beans is crucial for understanding and discussing the structural changes of amino acids, essential procedures are described briefly in the following.

The about 20–50 almond-sized seeds of cocoa trees are embedded in a pod and are surrounded by a sweet mucilaginous pulp. The seeds are covered with a brittle seed coat (shell or hull) that comprises a germ with rootlets and two thick cotyledons, called nibs. At harvest the fully ripe pods are opened and the seeds with adhering pulp removed. The pulp is usually subjected to natural but controlled fermentation by wild yeasts such as *Kloeckera* and *Saccharomyces* spp., and species of bacteria of genera like *Lactobacillus*, *Pediococcus*, *Bacillus*, *Acetobacter* and *Gluconobacter* causing alcoholic and acetic acid/lactic acid fermentation. This procedure, together with an increase of the temperature, destroys the embryo and liquefies the pulp that drains away. In addition internal autolytic (enzymic) reactions proceed, resulting in breakdown of polysaccharides and proteins, flavor formation, and change of the color of seeds owing to enzymic browning reactions. Finally, the seeds are dried to a moisture content of about 6–8% and exported as cocoa beans which

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serve as raw materials for the production of cocoa products. Processing is commonly performed by consuming countries since advanced technologies are required.

Following purification, beans are roasted at 120–130 °C for 10–35 min. Roasted beans are transferred to winnowing machines in order to remove the germs, the germ rootlets and the hulls. The isolated and purified hulls contain the alkaloids caffeine and theobromine and are used for preparing a stimulating beverage (cocoa shell tea). After further purification the nibs are crushed and milled. At the end the resultant product is a homogeneous paste named cocoa mass or cocoa liquor that contains about 55% fat and is named cocoa butter.

To convert cocoa mass into cocoa powder it is subjected to an expeller press at a pressure of 400–500 bar and a temperature of 90–100 °C. Liquid cocoa butter is separated and the residual cocoa press cake is milled providing cocoa powder. Depending on the residual cocoa fat it is divided in high-fat cocoa powder (20–22% fat) as used in the household and low-fat cocoa powder (10–20% fat) which is used preferably in the industry.

Notably, before pressing the cocoa nibs or the cocoa mass might be subjected to an alkalization process ('dutching') comprising treatment with an dilute alkali solution at 75–100 °C, followed by neutralization and drying at a temperature above 100 °C.

Chocolate is nowadays made from non-alkalized cocoa mass by addition of sweeteners (sucrose, lactose, and glucose syrup), cocoa butter, milk or milk powder, and flavor components such as coffee paste.

The mixture is refined in roller machines and subjected to a ripening process at 45–50 °C for 24 h followed by a process named 'conching'. The latter process is performed nowadays in rotatory conches at 65–75 °C for totally about 2 days. The conching process removes unpleasant volatiles together with an excess of humidity and coats the cocoa particles uniformly with cocoa butter. At the very end the molten chocolate mass is subjected to a tempering process in order to initiate proper crystallization and then molded into the final product.

From a chemical point of view the fat-free dry cocoa powder is composed mainly of starch and other polysaccharides, mono and oligosaccharides, proteins, amino acids, polyphenols and their oxidation products, and some carboxylic acids. On roasting intensive interaction of amino acids and reducing sugars with formation of flavor compounds and colour, named the Maillard reaction, occur. Furthermore, thermal degradation of proteins with formation of diketopiperazines, and Strecker degradation of amino acids with formation of aroma intensive Strecker aldehydes proceed. For full details on the technology of cocoa and chocolate production, and for chemical changes occurring during fermentation and processing we refer to the literature (Hurst and Martin, 1980; Martin, 1987; Biehl et al., 1993; Kleinert, 1997; Schieberle, 2000).

Much of the analytical work on cocoa deals with the determination of amino acids and saccharides (Kirchhoff et al., 1989; Offem, 1990; Puziah et al., 1998; de Brito et al., 2000) since these compounds and their interactions are of great importance for the quality of cocoa end products in terms of flavor, color and sensorial acceptance by consumers.

Few works, however, are concerned with the chirality of amino acids in native and processed cocoa beans and cocoa products. i.e. the occurrence of the mirror images of the common L-amino acids, named D-amino acids or D-enantiomers. This process, changing the stereochemistry of amino acids, is commonly named isomerization or racemization.

Some time ago we had recognized that cocoa powder, in accordance with roasted coffee, contains relatively large amounts of D-amino acids (Brückner and Hausch, 1990). In continuation of this work we recently analyzed processed cocoa products for the presence of D-amino acids (Pätzold and Brückner, 2005a; Pätzold et al., 2005). We now show that D-amino acids are common in cocoa products and present data on their generation from Amadori rearrangement products which represent stable intermediates formed in the course of the Maillard reaction. Based on these data we present a plausible mechanism for amino acid racemization in the course of the Maillard reaction.

Materials and methods

Instrumental

Enantioselective separations of derivatized cocoa amino acids were performed on a chiral Chirasil®-L-Val fused silica capillary column (*N*-propionyl-L-valine *tert*. butyl amide polysiloxane), 25 m length × 0.25 mm I.D., film thickness 0.12 µm (from Varian Inc., Darmstadt, Germany) (Frank et al., 1978; Pätzold and Brückner, 2005b). The column was installed in a Model A17 gas chromatograph coupled to a Model QP5000 mass spectrometer. The carrier gas was helium at an inlet pressure of 5 kPa, purge flow was 3 ml min^{-1} and flow rate was 0.5 ml min^{-1}. Injector and interface temperatures were 250 °C and split ratio 1:30. The temperature program was 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.

The pressure of carrier gas was 5.0 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 selected ion monitoring appropriate ion sets were selected and characteristic mass fragments of the PFP/2-Prp esters of the amino acids were used (m/z): Ala (190, 191, 235), Val (203, 218, 219), Thr (202, 203), Gly (176, 177), Pro (216), Leu (190, 232, 233), Ser (188, 189), Asp (189,

234, 235, 262), Met (203, 221, 263), Phe (91, 148, 190, 266), Glu (202, 230, 248, 276), Tyr (253, 266), Lys (176, 230), GABA (176, 204, 232, 249).

Chemicals

Methanol (MeOH), dichloromethane (DCM), 2-propanol (2-PrOH), petroleum ether (b.p. 50–70 °C), acetyl chloride (AcCl), aqueous ammonia (25%), and aqueous HCl (36%), were obtained from Merck, Darmstadt, Germany. Cation exchanger Dowex 50W X8, practical grade, 200–400 mesh (0.037–0.075 mm particle size) was from Sigma, Deisenhofen, Germany. Pentafluoropropionic acid anhydride (PFPAA) and amino acids standards were from Pierce, Rockford, IL, USA, and antioxidant 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) was from Fluka, Buchs, CH. For esterification of amino acids a mixture of AcCl in 2-PrOH (1:9 v/v; mixed with chilling) was used. For quantitation and determination of response factors of amino acids a protein hydrolysate standard containing 2.5 µmol/L-amino acid per ml 0.1 M HCl from Sigma was used (product no. AA-S-18) and appropriate amounts of achiral γ-aminobutyric acid (GABA) and internal standard L-norleucine (Nle) were added. For testing the column and optimization of chiral resolution a mixture of D- and L-amino acids (ratio D:L∼1:2) was prepared and analyzed (Pätzold and Brückner, 2005b).

Table 1. Characterization of cocoa products analyzed

No.	Cocoa products
1	Cocoa beans, fermented, 'Forastero', Ivory Coast, Africa
2	Cocoa nibs, 'Forastero', Ivory Coast, Africa
3	Cocoa beans, roasted at 100 °C for 100 min, 'Forastero', Ivory Coast, Africa
4	Cocoa beans, roasted at 120 °C for 70 min, 'Forastero', Ivory Coast, Africa
5	Cocoa beans, roasted at 150 °C for 120 min, 'Forastero', Ivory Coast, Africa
6	Cocoa beans, fermented, 'Forastero', Ghana, Africa
7	Cocoa nibs, 'Forastero', Ghana, Africa
8	Cocoa beans, roasted at 120 °C for 70 min, 'Forastero', Ghana, Africa
9	Cocoa beans, fermented, 'Forastero', Nigeria, Africa
10	Cocoa nibs, 'Forastero', Nigeria, Africa
11	Cocoa beans, roasted at 120 °C for 70 min, 'Forastero', Nigeria, Africa
12	Cocoa beans, fermented, 'Trinitario', Sulawesi, Indonesia
13	Cocoa nibs, fermented, 'Trinitario', Sulawesi, Indonesia
14	Cocoa beans, roasted at 140 °C for 30 min, 'Trinitario', Sulawesi, Indonesia
15	Cocoa beans, roasted at 130 °C for 60 min, 'Trinitario', Sulawesi, Indonesia
16	Cocoa powder, 'Bensdorp®', lightly defatted, Germany
17	Cocoa powder, 'Poeder®', lightly defatted, Germany
18	Cocoa powder, 'Sarotti®', extensively defatted, Germany
19	Cocoa powder, 'Trumpf®', lightly defatted, Germany
20	Chocolate powder, 'Sarotti®', minimum 32% cocoa, Germany
21	Chocolate powder, 'Poulain®', minimum 32% cocoa, France
22	Chocolate powder, 'Poulain®', minimum 60% cocoa, France
23	Chocolate, 35% cocoa, Rausch 'Nouméa Edelvollmilch Schokolade', Germany
24	Chocolate, 39% cocoa, Rausch 'Madagaskar Edel-Vollmilch Schokolade', Germany
25	Chocolate, Rausch 37% cocoa, 'Java Edel-Vollmilch Schokolade', Germany
26	Chocolate, 60% cocoa, Rausch 'Amazonas Edel-Bitter Schokolade', Germany
27	Chocolate, 70% cocoa, Rausch 'Arriba Extra-Bitter Schokolade', Germany
28	Chocolate, 75% cocoa, Rausch 'Tabago Extra-Bitter Schokolade', Germany
29	Chocolate, 99% cocoa, Lindt 'Exellence Noirissime', Germany
30	Chocolate, 70% cocoa, Lindt 'Exellence Edel-Bitter', Germany
31	Cocoa shells, S. Frey, Bietigheim-Bissingen, Germany

Sources of cocoa products

Fermented and roasted cocoa beans and nibs, were obtained from a major international chocolate manufacturer. Cocoa powders, chocolate powders, chocolate and cocoa shells were commercial products purchased from retail outlets. Details are compiled in Table 1.

Treatment and analysis of cocoa products

Cocoa beans, nibs and chocolate were frozen and minced with a lancet. To 0.5–1 gram amounts of analytes petrol ether (10 ml) was added and the mixture treated in an ultrasound bath for 10 min. Then the mixture was centrifuged at $3500 \times g$ for 15 min and the supernatant was collected. The remaining residue was treated twice as described and the supernatants were combined and evaporated to dryness on a rotary evaporator at 30–40 mbar at a bath temperature of 40 °C. The remaining residue was dissolved in 0.01 M HCl (3 ml), and adjusted to pH 2.3 if required by addition of 0.01 M HCl. Then L-Nle (10 µL; 10 mmol) was added as internal standard and samples were put on columns filled with Dowex 50W X8 cation exchanger; washed until neutral, and amino components eluted with 4 M aqueous ammonia. After evaporation to dryness amino acids were converted into 2-propyl esters by adding AcCl in 2-PrOH (200–400 µl) and 1% BHT in DCM (10 µl) followed by heating at 100 °C for 1 h. After removal of reagents in a stream of nitrogen acylation was performed by adding DCM (200 µl) and PFPAA (50 µl) and heating at 100 °C for 20 min followed by removal of reagents in a stream of nitrogen. Depending on amino acid concentrations amounts of 50–500 µl DCM were added and aliquots of 0.5–1 µl were analyzed by GC-MS.

Gas chromatographic mass spectrometric quantification of amino acid enantiomers

Relative amounts of D-amino acids were calculated according to Eq. (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 of the D- or L-enantiomer, respectively, determined by GC-MS. For quantification response factors of amino acids 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 Eq. (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 L-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 Eq. (3)

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

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 concentration of the IS added to the sample.

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 quantity of a D-amino acid c_{DAA}, if required, can be calculated according to Eq. (4).

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

Synthesis of the fructosyl-amino acids (Amadori compounds)

N-(1-Deoxy-D-fructosyl)-L-phenylalanine (fructose-L-phenylalanine, Fru-L-Phe) and *N*-(1-deoxy-D-fructosyl)-D-phenylalanine (fructose-D-phenylalanine, Fru-D-Phe) were synthesized according to the literature (Yaylayan and Huyghues-Despointes, 1994) by refluxing D-glucose (30 mmol) and L-Phe or D-Phe (30 mmol) in MeOH (250 ml) for 8 h. Crude compounds containing unreacted amino acids were purified by preparative HPLC similar to a protocol reported (Moll and Gross, 1981). Fractions containing the pure fructose-amino acids were combined, organic solvents removed in vacuo and the remaining aqueous phases were freeze-dried. The compounds were characterized by electrospray ionization mass spectrometry (ESI-MS) in the negative and positive ion mode using a LCQ instrument (Thermo-Finnigan, San José, CA, USA). Amadori compounds Fru-L-Phe and Fru-D-Phe (molecular weights 327.0 g/mol) yielded identical mass spectra and diagnostic ions (m/z): 325.8 $(M–H)^-$, most intensive; 308.1 $(M–H_2O–H)^-$, 350.6 $(M + Na)^+$, most intensive; 328.4 $(M + H)^+$. The negative ion MS of Fru-L-Phe is shown in Fig. 1. Hydrolysis (6 M HCl, 100 °C, 18 h) of synthetic fructose-amino acids and enantioselective analyses showed that 99.2% L-Phe and 0.8% D-Phe were released from Fru-

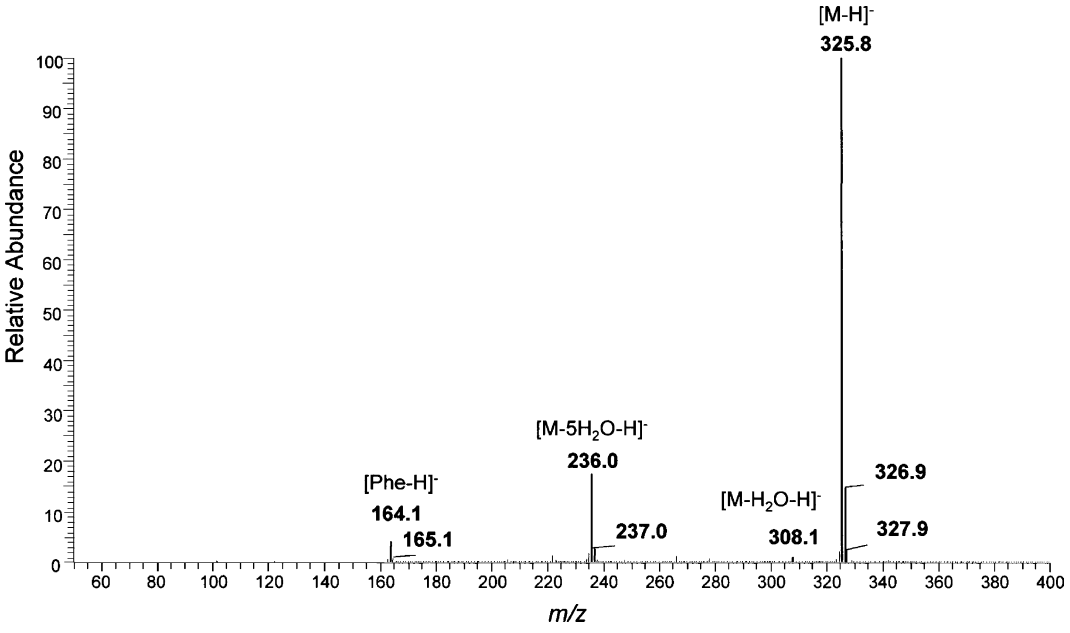

Fig. 1. ESI-MS (negative ion mode, direct injection) of synthetic fructosyl-L-phenylalanine used for heating experiments

Table 2. Quantities of free L-amino acids (mg/100 g product) in cocoa beans and nibs (for relative quantities see Table 3)

	Ivory Coast					Ghana			Nigeria			Sulawesi			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
L-Ala	27.5	25.8	31.3	22.3	16.6	31.5	41.8	16.0	27.1	34.3	34.9	35.8	21.2	19.6	18.5
L-Val	24.7	22.0	28.9	18.3	10.7	23.3	31.3	13.7	17.7	24.9	28.3	27.9	18.3	15.1	17.9
L-Thr	27.4	27.0	31.4	21.0	11.5	28.6	33.6	19.3	21.2	28.0	33.4	24.1	19.2	13.4	11.1
Gly	10.4	9.4	11.6	7.4	6.0	12.9	14.4	7.1	8.6	11.3	12.2	8.5	6.3	4.3	3.9
L-Pro	19.2	18.9	21.2	15.6	9.4	21.1	21.2	12.7	19.5	20.5	19.8	24.5	19.7	13.5	11.3
L-Ile	12.3	11.5	13.9	9.7	4.6	11.1	14.3	7.7	10.6	13.1	15.1	17.0	10.9	9.6	7.4
L-Leu	41.6	39.9	48.8	33.6	13.3	47.5	56.7	31.0	39.6	52.7	52.7	34.0	29.1	16.3	14.8
GABA	38.8	40.5	39.0	32.2	10.9	51.0	34.8	22.8	44.9	33.1	33.1	85.5	71.0	50.5	41.1
D-Asx	39.5	38.2	41.1	26.9	28.8	40.9	40.6	26.6	41.4	41.3	41.3	51.1	37.2	29.3	25.7
L-Met	6.0	4.4	6.7	4.2	1.4	10.1	6.3	2.9	5.0	7.9	7.9	6.8	4.9	1.9	1.2
L-Phe	44.2	38.0	42.8	35.2	11.9	53.0	52.2	31.7	39.9	20.1	20.1	47.1	30.5	13.1	11.8
L-Glx	43.5	38.5	36.3	29.4	12.9	46.1	36.8	19.0	38.5	49.1	49.1	60.5	40.7	24.6	16.6
L-Lys	24.9	22.9	24.6	20.3	8.6	28.7	25.6	12.8	18.9	20.9	20.9	16.1	17.0	8.2	6.8

Data are average of two measurements of two separate sample analyses, L-Ser and L-Tyr, could not be quantified and are omitted, Asx = sum of Asn and Asp, Glx of Gln and Glu, respectively

Table 3. Relative quantities of D-amino acids (%D) in cocoa beans and nibs (sample nos. 1–15, see Materials and methods)

	Ivory Coast					Ghana			Nigeria			Sulawesi			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D-Ala	1.5	3.4	3.7	3.0	17.5	1.4	1.3	3.3	1.4	1.4	3.6	1.1	2.0	3.7	4.5
D-Val	0.1	0.1	n.d.	n.d.	1.2	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	0.3	n.d.	n.d.
D-Pro	n.d.	n.d.	n.d.	n.d.	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
D-Ile	2.2	3.4	2.2	2.5	11.7	2.4	n.d.	2.7	n.d.	n.d.	2.4	5.2	4.8	6.9	7.2
D-Ser	n.d.	0.5	1.1	1.0	5.8	n.d.	n.d.	2.4	n.d.	n.d.	1.0	n.d.	0.5	1.8	1.7
D-Leu	0.2	0.3	0.5	0.6	4.8	n.d.	n.d.	2.2	n.d.	n.d.	0.8	n.d.	0.4	0.9	0.9
D-Asx	1.3	n.d.	n.d.	2.4	11.1	n.d.	n.d.	1.7	1.7	2.1	2.5	n.d.	n.d.	2.7	3.3
D-Phe	n.d.	n.d.	n.d.	n.d.	4.3	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	1.9
D-Glu	n.d.	n.d.	n.d.	2.2	3.1	n.d.	n.d.	n.d.	n.d.	n.d.	1.9	n.d.	n.d.	1.6	2.1
D-Tyr	n.d.	1.6	n.d.	n.d.	7.9	2.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.9	n.d.	n.d.

n.d. Not detected (<0.05%)

L-Phe, and 99.1% D-Phe and 0.9% L-Phe from Fru-D-Phe. The data prove that the configuration of Phe was retained in the synthetic fructose amino acids prior to heating.

Heating of the Amadori compounds and cocoa powder

Experiment 1

Aliquots of 1.5 mg Fru-L-Phe or Fru-D-Phe were heated in the dry state in open vials at 200 °C for various periods of time (5–60 min). Samples were hydrolyzed (6 M HCl/18 h/110 °C), evaporated to dryness and subjected to ion-exchange and derivatization as described above for cocoa products.

Experiment 2

Aliquots of 1.5 mg Fru-L-Phe or Fru-D-Phe were heated in the dry state in open vials for 5 min. Then 0.01 M HCl (1 ml) was added and the solution subjected directly to the ion-exchange and derivatization procedure. The configuration of the free Phe released was determined by GC-SIM-MS on Chirasil-L-Val as described above for cocoa products.

Experiment 3

Aliquots of 5 g cocoa powder (no. 18) were heated in the dry state in open bakers for 3 and 18 hours at temperatures of 100 and 180 °C, respectively. Aliquots of 0.5 g were treated and analyzed as described above for cocoa products.

Results

Quantities of L-amino acids determined by GC-SIM-MS in fermented and roasted cocoa beans and nibs from Africa and Indonesia are presented in Table 2 and the relative quantities of D-amino acids in these products are shown in Table 3. If required, absolute amounts of D-amino acids can be calculated from the data according to Eq. (4) in Materials and methods.

Quantities and kinds of L-amino acids and change on heating are in agreement with those reported in the literature (Hurst and Martin, 1980; Kirchhoff et al., 1989; Offem, 1990; de Brito et al., 2000; Puziah et al., 1998). Restrictions are that Arg and His could not be determined by the method used and Asn and Gln are hydrolyzed to Asp and Glu, respectively. With regard to the aim of the work in the following we focus on D-amino acids.

As can be seen (Tables 3 and 4) relative quantities of D-amino acids increase on roasting whereas quantities L-amino acids decrease on roasting.

Whereas fermented cocoa beans and nibs and beans roasted at relatively low temperatures contained D-amino

Table 4. Ranges of L-amino acids (mg per 100 g) and of relative amounts of D-amino acids (%D) in cocoa and cocoa products[a]

AA	Cocoa and chocolate powder products ($n = 7$; nos. 16–22)		Chocolate ($n = 8$; nos. 23–30)		Cocoa shell (no. 31)	
	mg/100 g	%D	mg/100 g	%D	mg/100 g	%D
L-Ala	1.5–18.9	5.5–14.3	1.0–21.0	6.0–14.5	2.2	12.4
L-Val	3.0–22.4	n.d.–0.6	1.9–24.2	n.d.–0.6	2.0	n.d.
L-Thr	0.9–19.1	n.d.	1.9–14.6	n.d.	4.0	n.d.
Gly	1.1–6.3	–	2.6–9.1	–	12.2	–
L-Pro	5.0–15.8	n.d.–0.9	7.2–24.1	n.d.	6.9	n.d.
L-Ile	1.2–6.3	n.d.–7.2	1.9–8.8	n.d.–6.7	31.3	15.9
L-Leu	3.5–20.3	n.d.–0.9	2.3–23.0	n.d.–1.3	1.8	8.2
L-Ser[b]	–	n.d.–3.3	–	n.d.–4.2	–	2.3
Gaba	2.8–27.5	–	11.1–32.5	–	2.1	–
L-Asx	6.5–30.5	3.1–8.1	11.9–23.4	3.5–4.3	2.3	6.1
L-Met	n.d.–4.2	n.d.	2.6–8.3	n.d.	0.1	n.d.
L-Phe	5.7–25.9	1.6–6.3	5.1–36.4	n.d.–9.8	1.7	3.0
L-Glx	7.4–26.6	1.9–4.9	4.8–32.2	n.d.–3.9	2.4	5.9
L-Tyr	–	n.d.–0.9	–	n.d.–10.6	–	6.2
L-Lys	2.1–16.6	n.d	4.7–54.7	n.d.–1.8	1.0	n.d.

[a] Data are average of two measurements of two separate sample analyses
[b] Absolute quantities of L-Ser and L-Tyr not sufficiently precise determinable
n.d. Not detected (<0.05%)

acids in the low percentage range, heating at 150 °C for 120 min lead to a drastic increase of relative quantities of D-amino acids. This is most obvious for sample no. 5 in which highest relative quantities of 17.5% D-Ala, 11.7% D-Ile, and 11.1% D-Asx, among other D-amino acids, could be determined.

The ranges of L-amino acids and the relative quantities of D-amino acids determined in cocoa powders ($n = 4$) together with chocolate powders ($n = 3$), chocolate of varying contents of cocoa ($n = 8$), and a sample of cocoa shells are presented in Table 4.

As can be seen highest relative amounts of D-amino acids of these products do not differ very much and altogether range from 5.5 to 14.5% D-Ala, 6.7–7.2% D-Ile, 10.6–12.3% D-Tyr, and 6.3–9.8% D-Phe besides lower quantities of other D-amino acids. Notably, high relative amounts of D-amino acids, approaching 15.9% D-Ile and 12.4% D-Ala, could be detected in an aqueous infusion of cocoa shells.

In Fig. 2a–d representative chromatograms on Chirasil-L-Val are shown demonstrating presence of D-amino acids in (a) fermented, low roasted cocoa beans (no. 3), (b) fermented, high roasted cocoa beans (no. 5), (c) low fat cocoa powder (no. 17), and (d) chocolate containing 70% cocoa (no. 30).

Data on heating of synthetic Amadori compounds at 200 °C (Experiment 1) and quantities of enantiomers of Phe in hydrolysates are compiled in Table 5. As can be seen enantiomers of the opposite configuration are released within a few minutes.

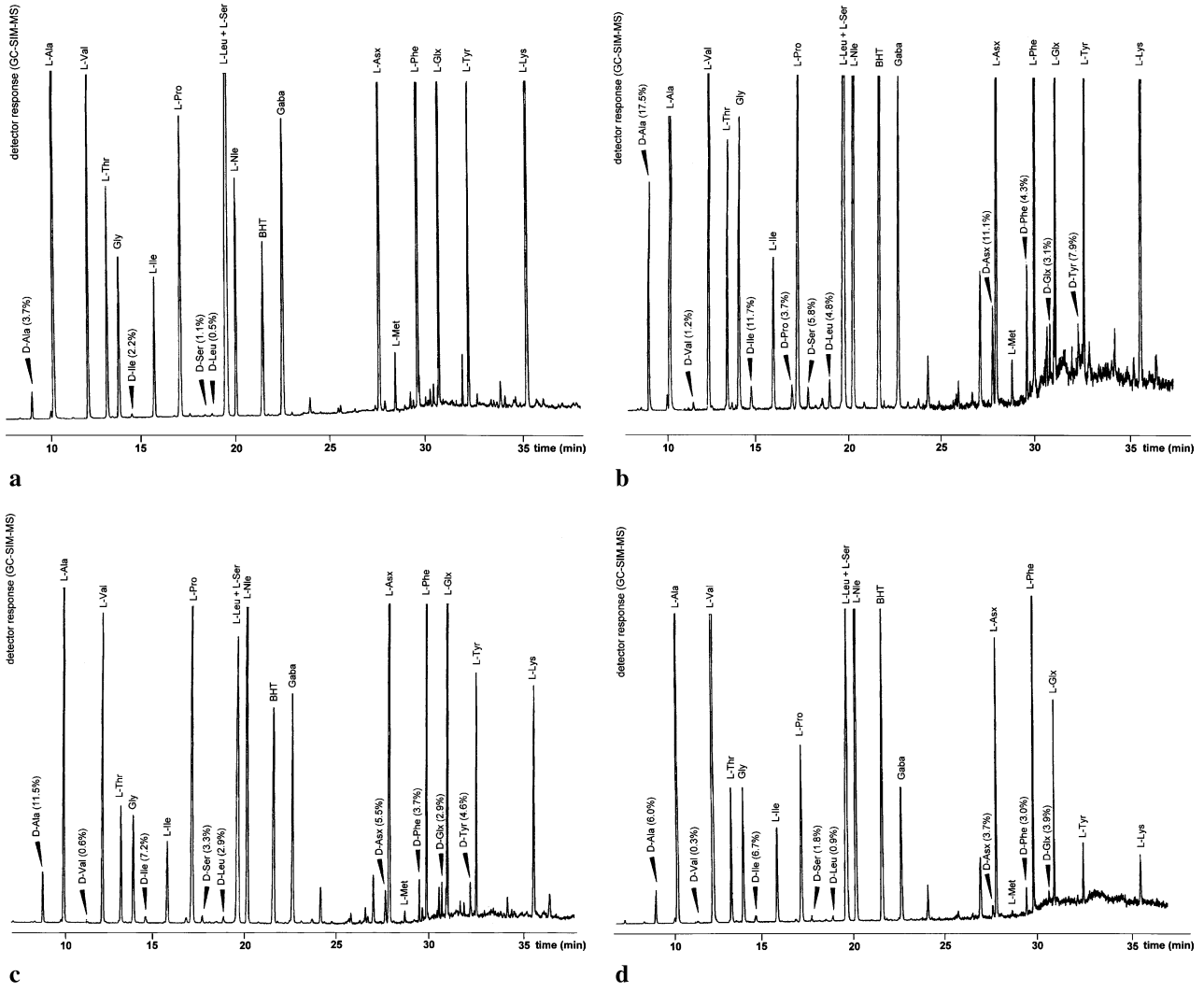

Fig. 2. GC-SIM-MS of derivatives (PFP/2-propyl esters) of amino acid enantiomers resolved on Chirasil-L-Val; **a** fermented cocoa beans roasted at 100 °C for 100 min (no. 3), **b** fermented cocoa beans roasted at 150 °C for 120 min (no. 5), **c** lightly defatted cocoa powder (no. 17), **d** chocolate with 70% cocoa (no. 30). For chromatographic conditions see Materials and methods

Table 5. Relative quantities (%) of Phe determined after heating (200 °C; 5–60 min) and total hydrolysis of (a) fructose-L-Phe and (b) fructose-D-Phe (Experiment 1)

Min	(a) %D	(b) %L
0	0.9	0.8
5	17.5	17.7
10	18.2	22.7
20	23.5	30.6
30	23.9	26.8
40	23.8	27.7
50	30.0	30.7
60	24.3	27.5

Data are average of each two independent heating experiments and two injections of analytes; t = 0 min refers to blank, i.e. relative quantities of Phe enantiomers released on hydrolysis of unheated fructose-L-(or D)-phenylalanine

Chromatograms (GC-SIM-MS) resulting from heating of synthetic Amadori compounds (Fru-L-Phe and Fru-D-Phe) and detection of free Phe enantiomers are presented in Fig. 3 (Experiment 2). Already after heating for 5 min at 200 °C quantities of 10.7% D-Phe were released from Fru-L-Phe and 10.8% L-Phe from Fru-D-Phe. After 40 min no more free L- nor D-Phe could be detected. This indicates that amino acids are destroyed at advanced stages of the Maillard reaction.

Quantities of L- and D-amino acids in cocoa powder no. 18 and change of amounts on heating at 200 °C (Experiment 3) are presented in Table 6. As can be seen in dependence of temperature and heating time absolute quantities decrease and relative amounts of D-amino acids increase.

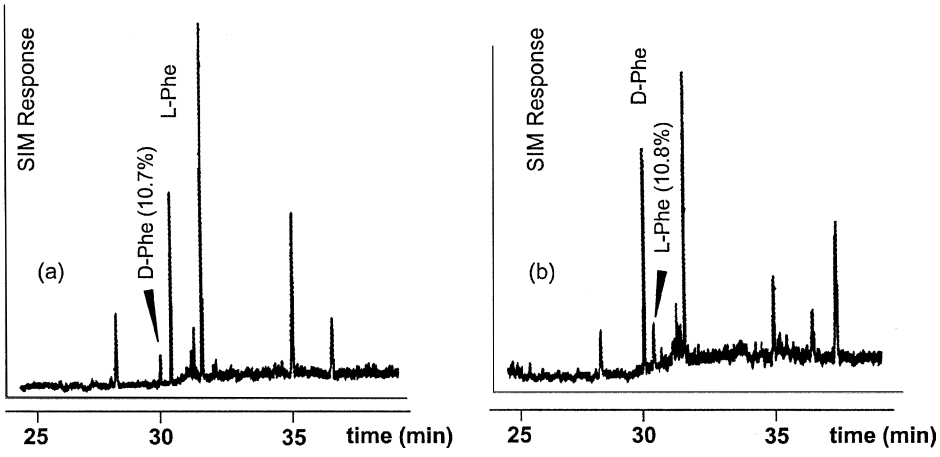

Fig. 3. GC-SIM-MS of amino acid enantiomers released on heating of **a** fructose-L-phenylalanine, **b** fructose-D-phenylalanine (Experiment 2)

Table 6. D-amino acids released on heating cocoa powder no. 18 (Experiment 3)

AA	Blank		3 h/100 °C		18 h/100 °C		3 h/180 °C		18 h/180 °C	
	mg/100 g	%D	mg/100 g	%D	mg/100 g	%D	mg/100 g	%D	mg/100 g	%D
L-Ala	18.9	9.2	18.3	10.2	18.8	11.3	1.3	10.4	1.1	17.3
L-Val	19.9	0.3	16.8	0.4	16.4	0.4	–	n.d.	–	0.3
L-Thr	19.1	n.d.	19.5	n.d.	16.3	n.d.	0.9	n.d.	0.3	n.d.
Gly	6.3	–	7.3	–	7.9	–	2.0	–	1.6	–
L-Pro	11.4	n.d.	17.9	n.d.	17.1	n.d.	0.4	n.d.	0.6	n.d.
L-Ile	9.2	6.3	8.2	6.3	7.3	8.3	0.1	n.d.	0.8	n.d.
L-Ser	–	1.4	–	2.0	–	2.3	–	3.4	–	n.d.
L-Leu	27.4	1.5	24.9	2.1	22.5	2.3	1.0	n.d.	0.7	10.2
Gaba	27.5	–	27.7	–	20.9	–	1.6	–	2.2	–
L-Asx	30.5	3.1	28.1	4.2	24.2	4.4	1.9	9.8	1.2	17.4
L-Met	4.2	n.d.	6.2	n.d.	2.7	n.d.	n.d.	n.d.	n.d.	n.d.
L-Phe	25.9	2.3	23.8	3.1	19.9	2.5	0.6	18.2	0.5	29.3
L-Glx	18.2	2.8	16.5	3.4	15.8	3.4	3.2	12.2	2.0	23.1
L-Tyr	–	6.3	–	2.7	–	n.d.	–	n.d.	–	n.d.
L-Lys	16.6	n.d.	14.3	n.d.	11.5	n.d.	0.8	n.d.	0.6	n.d.

Blanks refer to cocoa powder not heated; *n.d.* not detected; – not determinable

Discussion

From the quantitative data it is obvious that free L-amino acids are abundant in roasted cocoa and cocoa products. Only low amounts of D-amino acids, typically for plants (Brückner and Westhauser, 2003), can be detected in fermented cocoa beans. This indicates that microbial fermentation of the pulp does not or to a very low extend contribute to the formation of D-amino acids detected in fermented cocoa beans although bacteria are important sources of D-amino acids in fermented foodstuffs (Brückner et al., 1993). This also indicates that bacteria do not penetrate the cocoa beans.

The alkalization ('dutching') process is also a potential source of D-amino acids for cocoa powders since peptide bound and free amino acids easily racemize under alkaline conditions (Friedman and Masters, 1982). If applied, alkalization might contribute to the formation of D-amino acids in cocoa powders. This process, however, is not always used and 'dutched' cocoa powder is rarely used for chocolate manufacturing.

However, the data resulting from heating cocoa beans and model compounds prove that quantities of D-amino acids increase very much on roasting and that this process is a major source of D-amino acids.

Since roasted cocoa beans are used for the production of cocoa powder and cocoa products like chocolate, relatively high amounts of D-amino acids are also detectable in these products (cf. Table 4).

This is confirmed by heating cocoa powder at various periods of time and investigation of absolute and relative quantities of amino acids. Relative quantities of D-amino acids increase whereas absolute quantities of both L and D-amino acids decrease very much. This indicates irreversible destruction known from advanced stages of the Maillard reaction (cf. Table 6).

A definitive proof for the generation of free amino acids of the opposite configuration is provided by heating synthetic Amadori compounds (fructose amino acids) followed by chiral amino acid analysis. From Fru-L-Phe free D-Phe, and from Fru-D-Phe free L-Phe are released (cf. Table 6 and Fig. 3).

Amadori compounds of many common amino acids have been detected in unroasted and roasted cocoa beans and an increase and change of quantities in dependence of roasting time and temperature was observed (Heinzler and Eichner, 1991a, b; Oberparleiter and Ziegleder, 1997).

Consequently the question arises on the mechanism of the generation of D-amino acids in cocoa and cocoa products and their increase on heating.

From a chemical point of view cocoa and products are excellent candidates for the Maillard reaction. The raw materials are rich in reducing sugars or precursors of reducing such as sucrose and polysaccharides. Further, free amino acids together with proteins are abundant in cocoa. Free amino acids are released from proteins by proteases during fermentation. Cocoa and cocoa products are subjects of thermal treatment at varying temperatures and periods of time resulting in changing water activities and pH values at certain stages of processing (see Introduction).

These conditions favor the reversible formation of Amadori compounds but are not severe enough to destroy amino acids completely. We had previously shown that heating of L-amino acids together with reducing sugars leads to the formation of large amounts of D-amino acids (Brückner et al., 2001). This reaction is known as the Maillard reaction (Ledl and Schleicher, 1990) or non-enzymic browning reaction (Friedman, 1996). We had postulated that D-amino acids are generated from relatively stable intermediates of this reaction, named the Amadori and Heyns compounds (Brückner et al., 2002; Pätzold and Brückner, 2004a, b). Proceeding of the Maillard reaction with formation of Amadori products (fructose amino acids) in cocoa is well documented in the literature (Schnee and Eichner, 1987; Heinzler and Eichner, 1991a, b). Since heating of synthetic Amadori compounds such as fructose L-phenylalanine or fructose-D-phenylalanine lead to the formation of the opposite amino acid enantiomers and release in the free form (i.e. D-Phe from Fru-L-Phe and L-Phe from Fru-D-Phe) there is little doubt that the Amadori compounds (or structurally related Heyns compounds) are major precursors of D-amino acids in cocoa. These considerations can be extended on foodstuffs rich on saccharides and amino acids and of low water activity (Eichner and Karel, 1972; Eichner and Ciner-Doruk, 1979; Erbe and Brückner, 2000; Pätzold et al., 2003; Ali et al., 2006).

With regard to a racemization mechanism it is assumed that an intermediate carbanion in the reversibly formed Amadori compound and which can be protonated from opposite sides leads to the formation of (partially) racemized amino acids. At advanced stages of the Maillard reaction amino acids are irreversibly converted into heterocyclic compounds (Ledl and Schleicher, 1990). These considerations can be extended on foodstuffs such as honey and plant juice concentrates (Pätzold and Brückner, 2005d; 2006). A tentative racemization mechanism is presented in Fig. 4.

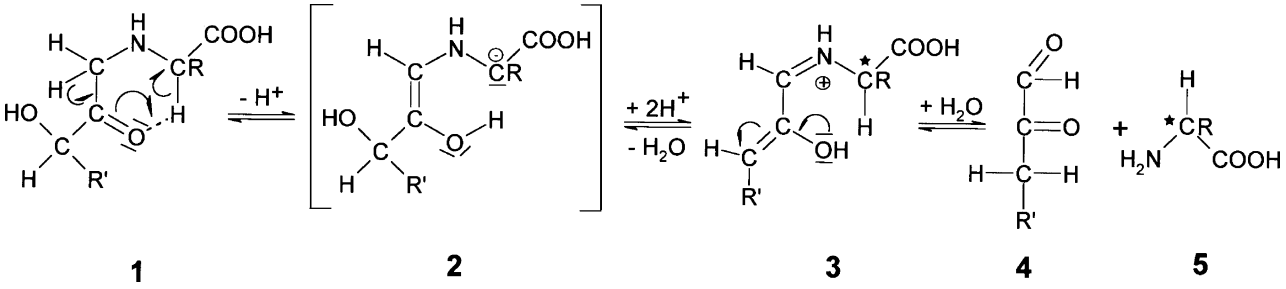

Fig. 4. Proposed mechanism for the reversible release of D-amino acids (or, vice versa, L-amino acids) from Amadori compound (fructosyl amino acids) (*1*) via intermediate formation of carbanion (*2*) and imin cation (*3*), followed by dehydratisation providing 3-deoxyosone (*4*) and (partially racemized) amino acid (*5*); R′ refers to sugar residue. The reaction is reversible until amino acids are destroyed at advanced stages of the Maillard reaction with formation of e.g. Strecker aldehydes, heterocyclic flavor compounds, and melanoidins

Conclusions

In cocoa beans low relative quantities of D-amino acids can be detected as in plants in general. Quantities in beans and cocoa products increase on roasting or heating. Consequently, quantities of D-amino acids might serve as indicators for the intensities or technology of roasting procedures. However, since additives rich on saccharides (invert sugar, glucose syrup, lactose) and amino acids (protein hydrolyzates) are legally used at certain stages of cocoa bean processing a definite evaluation of the relevance of D-amino acids in end products of commerce is difficult.

Proceeding of the Maillard reaction and formation and stability of Amadori compounds is dependent on the chemical nature and concentration of reducing sugars and amino acids, temperature, time, pH, water activity of the system, and presence of catalysts or inhibitors. Formation of D-amino acids in cocoa products is promoted by high concentrations of free amino acids and reducing sugars, low water activity and roasting at moderate temperature of about 130 °C. In addition varying heat treatments at various stages of cocoa product processing favor the reversible formation of Amadori compounds and, consequently, D-amino acids.

Notably, the Maillard reaction does also proceed to a certain extend at ambient temperature and under physiological condition (Ledl and Schleicher, 1990) and thus might contribute under favorable conditions to the pool of amino acids detectable in physiological samples (Pätzold and Brückner, 2005c). From the data it is concluded that D-amino acids will be formed inevitably in food, beverages or biosystems under conditions favoring the Maillard reaction.

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