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Determination of L- and D-amino acids in smokeless tobacco products and tobacco

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Abstract

Quantities of free L- and D-amino acids were determined by GC-SIM-MS in 25 European snuff tobaccos (from Germany, England and Sweden) and eight chewing tobaccos (from the Philippines, Africa and Denmark) and compared to those of cigar, cigarillo, and freshly harvested tobacco leaves of cultivars of *Nicotiana tabacum* L. Amino acids were isolated from tobacco samples by treatment with 70% aqueous methanol and purified by a cation exchanger. Next they were converted into their *N*(*O*)-pentafluoropropionylamino acid-(2)-propyl esters, and enantiomers separated and quantified by GC-SIM-MS on a Chirasil®-L-Val capillary column. Among L-amino acids the most abundant were Pro, Asx and Glx in the low milligram range (about 2–6 mg/g) whereas the other L-amino acids were in the submilligram range. Though native tobacco leaves contained low amounts of few D-AAs (0.2–1.9%), all processed tobacco samples had D-amino acids in varying amounts and patterns.

The D-enantiomers of Ala, Asx and Glx were detected in all samples approaching 34.0% D-Ala, 13.8% D-Asx, and 16.1% D-Glx in different samples. Large quantities of other D-amino acids were also detected in tobaccos approaching 32.2% D-Leu, 25.8% D-Phe and 18.0% D-Thr in various samples. Lower amounts of the D-enantiomers of D-Val, D-Ile, and D-Pro could also be determined in certain tobaccos.

It is assumed that D-amino acids are generated from fructose-L-amino acids (Amadori rearrangement products) which are formed in the course of the Maillard reaction.

Keywords: Amino acid enantiomers; Gas chromatography mass spectrometry; Snuff and chewing tobacco; Amino acid racemization; *Nicotiana tabacum* L.; Maillard reaction; Amadori rearrangement products

1. Introduction

1.1. Definition and use of smokeless tobacco

Tobacco stands for any of various plants of the genus *Nicotiana* of the nightshade family (*Solanaceae*) as well as the leaves of several of these plants, which are processed for smoking, chewing or snuffing. Cigarettes represent small rolls of cut tobacco wrapped in paper for smoking and cigars consist of rolls of tobacco leaves for smoking. Smokeless tobaccos are pulverized or shredded tobacco chewed or placed between cheek and gum or inhaled in small pinches of about 0.1 g amounts through the nostrils. That means that smokeless tobacco does not have to be lit as opposed to other tobacco products.

In the UK and continental Europe including Germany the name snuff or nasal snuff exclusively stands for products applied to the nostrils. Confusingly, in the USA the names snuff or even nasal snuff refers to smokeless products, which are used moist or dry as chewing tobacco.

Abbreviations: GC-SIM-MS, gas chromatography selected ion monitoring mass spectrometry; PFP, pentafluoropropionyl; Prp, propyl; amino acids were abbreviated to common three-letter nomenclature; Nle, norleucine (internal standard); GABA, γ-aminobutyric acid; Asx = (Asp + Asn); Glx = (Glu + Gln).

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These are products in powder form, which are placed in the mouth over a long period of time. The American moist snuff represents granulated tobacco mixed with water and used in the mouth whereas portion packed snuff is packed in small, porous bags (sachets). In the US a dry snuff is placed between the lower lip and the gum, or a moist form is used which is placed between the cheek and the gum.

Twisted smokeless tobacco is made of whole tobacco leaves that are twisted into a strand and cut into small pieces and to be used in the mouth.

1.2. Tobacco curing, aging and fermentation

Notably, the raw material for the production of smokeless tobacco products is the tobacco that is also grown for the production of smoke tobaccos. Briefly, tobacco production includes harvesting of the leaves of the plant, which are subjected to drying processes named curing in order to produce leaves of suitable physical properties and chemical composition. Various regimes of ventilation, temperature, and humidity control are employed for different types of tobacco. Since freshly cured tobacco leaves are unfit for use, in the following they are subjected to aging and fermentation procedures.

Aging is a mild fermentation process generally applied to cigarette tobacco carried out in a hogshead with control of moisture and temperature for several years. Fermentation, or sweating, is a more severe process carried out for several months or a few years and characterized by high initial moisture content, by heat generation and loss of weight. It is believed that the aging of flue-cured tobacco is essentially a chemical process, the main reaction being that between reducing sugars and amino components, with the formation of melanoidins and carbon dioxide. This process is known as non-enzymatic browning or the Maillard reaction.

1.3. Production and types of snuff and chewing tobacco

To make snuff, light varieties of tobaccos are blended which have been cured and aged in the normal, albeit varying, ways. Following reconditioning the tobacco is (or is not) cut into strips and repacked to undergo a severe fermentation. The fermented tobacco is dried completely and ground to a fine powder that is sieved through a silk cloth.

For the nasal snuffs, tobaccos are blended and milled in a high speed mill to produce very fine powder. The powder is moistened and fermented and matured in a cool storage room for about three to four weeks. After final blending flavorings such as peppermint oil, eucalyptus oil, menthol, or fruit extracts are added and moistening agents such as glycerol or paraffin oil.

German 'Schmalzler' is a traditional nasal snuff that is made from mainly Brazilian tobaccos with addition of stalks resulting from cigar manufacturing. To this blend a special preparation of Brazilian tobacco is added, named Mangote or Fresko, which represents selected Brazilian tobacco that was sorted and fermented for a long period of time, spun into ropes and then packed into animal skins. The raw materials altogether are processed as a general rule into a dry granulate.

This granulate is subjected to an important process called sossing. Water and sugar syrup are added as well as fruits such as prunes. The exact nature of the soss varies and is well-kept secret of manufacturers. The sossed material is filled into boxes and stored in fermentation rooms at varying temperatures. This maturing and fermentation process can take up to six months. This process is crucial for the final taste and aroma of the product. The matured material is carefully grinded on grinding stools. The resulting tobacco powders are blended and oils and other additives like aromas are added. In former times butter dripping was added, which explains the name 'Schmalzler'.

Chewing tobacco is made from fire cured (UK) or air-cured (US) tobaccos which are cut and granulated and sold as plug or loose leaf. Notably, the sossing procedure is also applied to the raw materials of chewing tobaccos. It is said that the soss is composed of extracts of orange, lemon, prunes, grape resins, figs, and may also contain sweeteners such as glucose, sucrose, corn syrup or molasses or honey and flavorings such as licorice or menthol. Further, spices, Jamaica rum and fortified wines such as Madeira or Samos may be added. Preparation of the soss at increased temperature and performing sossing at elevated temperature for a certain period of time is common. Sometimes tobacco extracts are added. Then the tobacco is spun and matured for 8–12 weeks. The final product, usually rich in sweeteners, is cut into suitable pieces for marketing.

For details of the biochemistry and technology of tobacco products we refer to the monographs of Tso (1972) and Wahlberg and Ringberger (1999).

1.4. Tobacco amino acid analysis

Free amino acids in various types of tobacco and oven-dried greenhouse tobacco were determined by Yang and Smetena (1993) using HPLC and fluorescence detection following derivatization with naphthalene-2,3-dicarboxaldehyde. For the quantification of amino acids released on total hydrolysis of brown pigments in tobacco a dedicated amino acid analyzer based on ion exchange chromatography (IEC) and the post-column ninhydrin reaction was used by Andersen, Vaughn, and Lowe (1970) for estimation of the concentration of proteins and amino acids in burley tobacco during air curing (Hamilton & Lowe, 1978). Amino acids in soluble tobacco pigment fractions were determined by GC after conversion into their *N*-trifluoroacetyl/*n*-butyl esters by Sheen and Burton (1978). Among the numerous papers on tobacco amino acids only few deal with their stereochemistry. The content of fructose amino acids on storage and ageing of tobacco was investigated by Noguchi, Satoh, Nishida, Andoh, and Tamaki

(1971). Conjugated 1-deoxy-1-L-prolino-D-fructose (fructose-L-Pro) from cured tobacco leaves was characterized by Tomita, Noguchi, and Tamaki (1965) and conjugated D-Ala-D-Ala was isolated from tobacco leaves by Noma, Noguchi, and Tamaki (1973). The enantiomeric composition of amino acids in processed American tobacco stan-

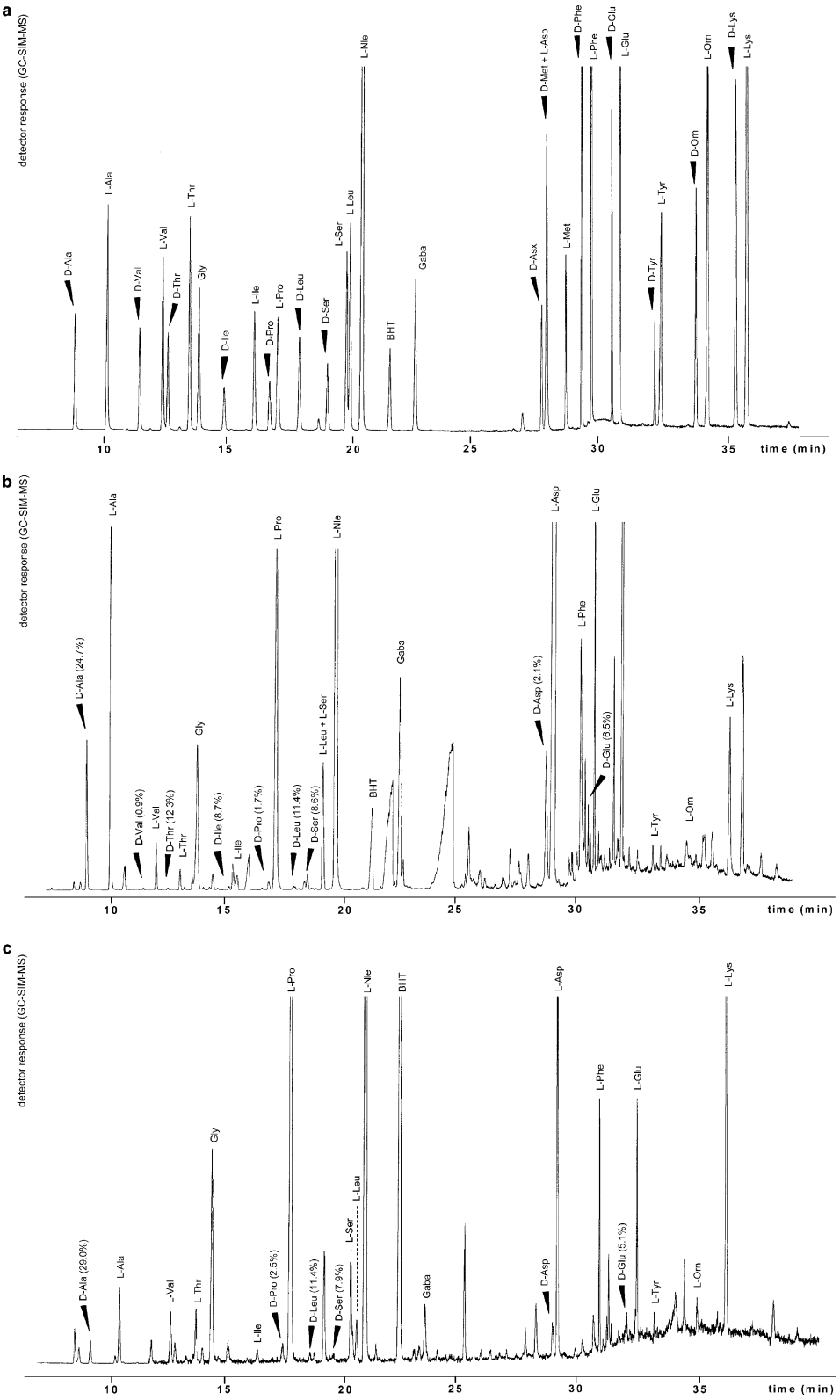

Fig. 1. GC-SIM-MS of PFP-amino acid-(2)-Prp esters resolved on Chirasil-L-Val of (a) standard of DL-amino acids (ratio D:L ca. 1:2), and amino acids extracted from (b) German snuff (no. 16), (c) English snuff (no. 22), (d) Philippine chewing tobacco (no. 25), (e) cigar (no. 33), (f) fresh tobacco leaf (no. 35).

dards was analyzed by Kullman, Chen, and Armstrong (1999). The authors established a sophisticated HPLC column switching system composed of non-chiral C-18 and modified chiral β-cyclodextrin phases followed by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.

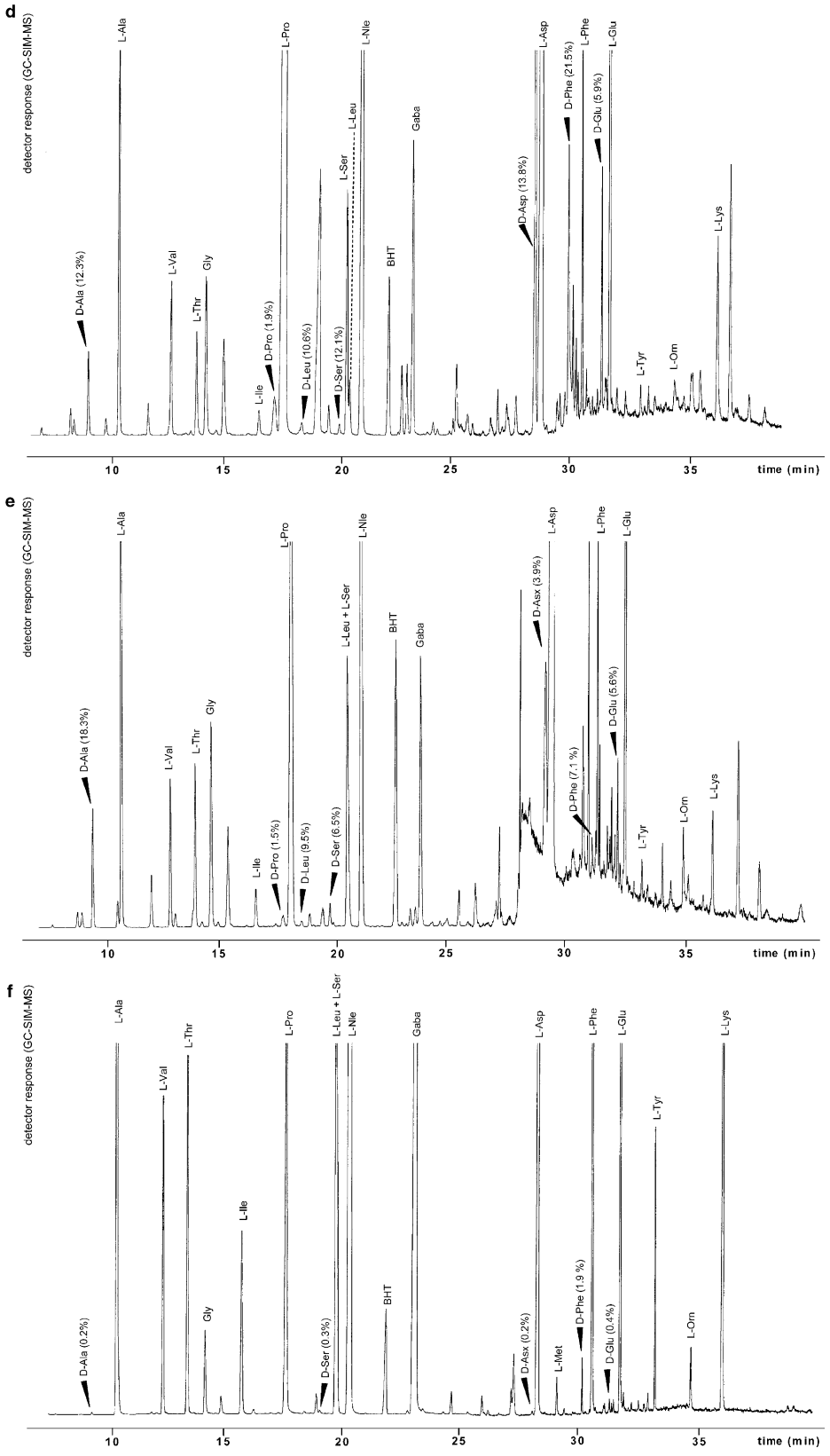

Fig. 1 (*continued*)

Table 1
Quantities of L-AA (mg/g tobacco) and relative amounts of D-AA (%*D*) in German snuffs (nos. 1-16)[a]

AA[b]	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16	
	L	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*
Ala	0.56	14.6	0.13	13.3	0.11	32.2	0.35	17.1	0.20	20.0	0.15	20.0	0.08	16.9	0.30	13.8	0.16	13.0	0.22	13.0	0.11	11.7	0.14	14.2	0.06	19.3	0.05	11.7	0.02	14.3	0.25	24.7
Val	0.04	0.0	0.01	0.0	0.01	0.0	0.02	0.0	0.02	0.0	0.11	0.0	0.01	0.0	0.02	0.0	0.01	0.0	0.01	0.0	0.01	0.0	0.01	3.9	0.01	0.0	0.00	0.0	0.00	0.0	0.04	0.9
Thr	0.05	0.0	0.01	0.0	0.01	18.0	0.05	0.0	0.03	0.0	0.04	0.0	0.01	0.0	0.02	10.6	0.01	0.0	0.02	0.0	0.01	0.0	0.01	0.0	0.01	0.0	0.00	0.0	0.00	0.0	0.02	12.3
Gly[c]	0.31	–	0.06	–	0.04	–	0.12	–	0.14	–	0.08	–	0.02	–	0.07	–	0.02	–	0.04	–	0.04	–	0.04	–	0.04	–	0.04	–	0.06	–	0.08	–
Ile	0.01	0.0	0.00	0.0	0.02	0.0	0.00	0.0	0.01	0.0	0.09	0.0	0.01	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.01	11.5	0.01	0.0	0.00	–	0.00	0.0	0.00	0.0	0.03	8.7
Pro	6.31	0.1	4.40	0.6	2.60	0.1	5.78	0.1	3.50	0.7	3.30	2.6	1.14	1.1	4.00	0.2	2.34	0.7	2.88	0.3	2.05	1.0	1.05	0.8	0.93	2.2	0.63	0.9	0.79	0.8	0.66	1.7
Leu	0.03	11.7	0.00	0.0	0.00	0.0	0.20	4.2	0.18	8.4	0.00	0.0	0.01	14.4	0.02	2.2	0.01	32.2	0.02	19.4	0.01	11.3	0.00	0.0	0.01	0.0	0.01	0.0	0.01	0.0	0.23	0.0
Ser	0.02	0.0	0.04	0.0	0.05	2.5	0.23	0.0	0.20	3.4	0.24	13.2	0.07	6.4	0.03	5.4	0.03	0.0	0.02	0.0	0.00	0.0	0.07	0.0	0.03	11.1	0.01	0.0	0.00	0.0	0.20	8.6
GABA[c]	0.18	–	0.10	–	0.06	–	0.13	–	0.13	–	0.02	–	0.02	–	0.10	–	0.04	–	0.03	–	0.05	–	0.03	–	0.02	–	0.02	–	0.02	0.0	0.07	–
Asx	3.12	2.8	1.97	3.1	2.15	1.7	7.95	1.0	3.05	3.0	2.48	6.0	1.02	3.2	2.00	2.3	1.30	2.8	1.48	2.1	1.18	2.2	2.08	5.9	0.64	4.2	1.23	3.0	1.57	4.0	1.41	2.1
Met	0.00	0.0	0.00	0.0	0.01	0.0	0.02	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.02	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.01	0.0
Phe	0.20	12.1	0.22	17.9	0.12	8.3	0.47	7.0	0.17	22.7	0.10	7.6	0.06	20.5	0.21	11.8	0.08	10.0	0.16	5.4	0.07	25.1	0.19	11.8	0.05	25.8	0.07	19.4	0.10	20.0	0.05	0.0
Glx	2.26	2.0	1.75	2.6	0.78	1.6	1.93	1.1	1.36	3.0	1.20	8.0	0.39	2.4	1.00	1.9	0.56	2.2	0.76	2.2	0.46	2.0	1.11	4.5	0.52	2.8	0.54	3.1	0.73	3.0	0.75	6.5
Tyr	0.19	0.0	0.00	0.0	0.04	0.0	0.13	0.0	0.08	0.0	0.04	0.0	0.01	0.0	0.06	0.0	0.00	0.0	0.02	0.0	0.00	0.0	0.02	0.0	0.00	0.0	0.03	0.0	0.00	0.0	0.12	0.0
Orn	0.06	0.0	0.03	0.0	0.01	0.0	0.06	0.0	0.00	0.0	0.00	0.0	0.03	0.0	0.07	0.0	0.04	0.0	0.01	0.0	0.05	0.0	0.03	0.0	0.03	0.0	0.01	0.0	0.00	0.0	0.00	0.0
Lys	0.00	0.0	0.00	0.0	0.06	0.0	0.19	0.0	0.00	0.0	0.00	0.0	0.04	0.0	0.05	0.0	0.05	0.0	0.04	0.0	0.06	0.0	0.00	0.0	0.03	0.0	0.19	0.0	0.07	0.0	0.02	0.0

[a] Data in Tables 1–4 are average of each two amino acid (AA) analyses of two independent analyte preparations; SDS 5–10%.
[b] AAs in tables are listed according to their order of elution from GC-column.
[c] Non-chiral AA.

Here, we present data on quantities of D- and L-amino acids in European snuffs and international chewing tobaccos and compare results to cigar, cigarillo and amino acids occurring in native tobacco leaves. Further, we furnish a plausible hypothesis on the genesis of D-amino acids in processed tobaccos.

2. Materials and methods

2.1. Instrumental

Enantioselective separation of derivatized tobacco amino acids was performed on a chiral fused silica capillary column Chirasil®-L-Val (*N*-propionyl-L-valine *tert*-butyl amide polysiloxane) (Pätzold & Brückner, 2005), 25 m length × 0.25 mm ID, film thickness 0.12 μm (from Varian Inc., Darmstadt, Germany). The column was installed in a Model A17 gas chromatograph coupled to a Model QP5000 mass spectrometer (Shimadzu, Kyoto, Japan). 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 2.5 °C/min to 150 °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, 5 min isobaric.

For selected ion monitoring appropriate ion sets were selected and characteristic mass fragments (*m*/*z*) of the PFP/2-Prp esters of the amino acids were used: Ala (190, 191), Val (218, 203), Thr (203, 202), Gly (176, 177), Pro (216), Leu (190, 232), Ser (188, 189), Asp (234, 216), Met (263, 203), Phe (91, 148), Glu (202, 203), Tyr (253, 266), Orn (216), Lys (230), GABA (232, 176), Nle (182, 126).

2.2. Chemicals

Aqueous ammonia (25%), aqueous HCl (36%), dichloromethane (DCM), 2-propanol (2-PrOH), methanol (MeOH), acetyl chloride (AcCl) 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 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) 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 (product no. AA-S-18) was used and appropriate amounts of L-Orn, GABA, and L-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 (cf. Fig. 1(a)).

2.3. Sources of tobaccos samples

German snuffs (nos. 1–16) were products of Pöschl Company, 84141 Geisenhausen, Germany. According to manufacturers declaration snuffs nos. 1–15 represented snuffs which were flavored with menthol, eucalyptus, columbia oil, peppermint or various fruit flavors; no. 5 was prepared from double fermented tobacco leaves, no. 6 was a combination of 'Schmalzler' and snuff, and no. 16 represented a typical Bavarian 'Schmalzler'. A Pöschl product designated as 'snuffy' was a white powder defined as tobaccoless product declared as being composed of glucose with menthol added (not included in Table 1).

English snuffs (nos. 17–23) ('Smith's Snuffs'; no manufacturer given) represented light to dark brown fine powders and were purchased from a tobacconist in London, UK.

Swedish snuff (no. 24) was a brown powder named 'Rumney's Export Snuff' and is distributed by Swedish Match, Germany.

Philippine chewing tobaccos (nos. 25–27) were leaves provided from manufacturers in the Philippines.

Sudan chewing tobacco sample (no. 28), labeled as toombak, was from Khartoum, Sudan and purchased in Cairo/Egypt.

Table 2
Quantities of L-AA (mg/g tobacco) and relative amounts of D-AA (%*D*) in English snuffs (nos. 17–23) and Swedish snuff (no. 24)

	17		18		19		20		21		22		23		24	
	L	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*
Ala	0.10	26.0	0.34	10.5	0.09	16.1	0.28	16.1	0.13	30.6	0.14	29.0	0.03	17.3	0.03	18.7
Val	0.09	0.0	0.13	0.0	0.11	0.0	0.03	0.0	0.08	0.0	0.12	0.0	0.02	0.0	0.00	0.0
Thr	0.07	0.0	0.30	0.0	0.10	0.0	0.02	0.0	0.09	0.0	0.12	0.0	0.02	0.0	0.05	0.0
Gly	0.14	–	0.19	–	0.19	–	0.05	–	0.03	–	0.32	–	0.05	–	0.01	–
Ile	0.03	0.0	0.04	0.0	0.05	0.0	0.02	0.0	0.04	0.0	0.05	0.0	0.02	0.0	0.00	0.0
Pro	0.73	2.4	2.71	0.9	1.31	3.8	0.30	2.6	3.33	3.3	2.62	2.5	0.53	2.0	1.03	0.8
Leu	0.40	0.0	0.00	0.0	0.11	5.2	0.03	0.0	0.12	9.4	0.05	11.4	0.06	1.5	0.00	0.0
Ser	0.19	13.6	0.49	3.8	0.30	4.3	0.08	5.3	0.27	0.0	0.21	7.9	0.16	0.0	0.03	9.6
GABA	0.02	–	0.10	–	0.05	–	0.01	–	0.05	–	0.01	–	0.01	–	0.01	–
Asx	1.34	3.3	6.78	2.2	1.12	9.4	0.58	7.9	1.70	13.2	5.07	0.0	0.70	7.1	3.22	5.7
Met	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.01	0.0
Phe	0.15	0.0	7.16	0.0	0.26	0.0	0.12	0.0	0.27	0.0	0.35	0.0	0.08	0.0	0.17	19.7
Glx	1.00	8.5	4.74	2.3	1.44	16.1	0.32	13.8	1.33	8.8	1.40	5.1	0.36	5.7	0.99	4.8
Tyr	0.19	0.0	0.44	0.0	0.54	0.0	0.45	0.0	0.04	0.0	0.08	0.0	0.03	0.0	0.04	0.0
Orn	0.21	0.0	0.14	0.0	0.56	0.0	0.00	0.0	0.16	0.0	0.14	0.0	0.09	0.0	0.03	0.0
Lys	0.10	0.0	0.33	0.0	0.22	0.0	0.03	0.0	0.04	0.0	0.00	0.0	0.03	0.0	0.01	0.0

Table 3
Quantities of L-AA (mg/g tobacco) and relative amounts of D-AA (%*D*) in chewing tobaccos from the Philippines (nos. 25–27), Sudan (no. 28), Egypt (nos. 29 and 30), and Denmark (nos. 31 and 32)

	25		26		27		28		29		30		31		32	
	L	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*
Ala	0.18	12.3	0.26	14.9	0.06	18.5	0.31	34.0	0.16	4.0	1.69	1.8	0.83	8.7	0.86	10.2
Val	0.08	0.0	0.12	0.0	0.02	0.0	0.02	0.0	0.03	0.0	0.06	0.0	0.03	0.0	0.03	0.0
Thr	0.03	0.0	0.10	0.0	0.03	0.0	0.02	0.0	0.09	0.0	0.01	0.0	0.11	0.0	0.07	0.0
Gly	0.01	–	0.08	–	0.05	–	0.03	–	0.04	–	0.01	–	0.14	–	0.20	–
Ile	0.03	0.0	0.03	0.0	0.01	0.0	0.01	0.0	0.03	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Pro	1.72	1.9	2.68	2.2	0.73	2.2	0.51	5.0	1.41	1.2	4.12	0.4	2.42	1.3	6.21	1.7
Leu	0.07	10.6	0.02	11.4	0.00	10.4	0.09	11.0	0.15	0.0	0.01	0.0	0.00	0.0	0.00	0.0
Ser	0.09	12.1	0.20	10.7	0.05	6.0	0.04	5.6	0.00	0.0	0.00	0.0	0.27	5.1	0.32	3.5
GABA	0.02	–	0.01	–	0.02	–	0.00	0.0	0.28	–	0.00	–	0.17	–	0.00	–
Asx	4.50	13.8	3.65	13.7	0.95	9.9	1.57	10.7	0.31	10.5	0.29	3.5	3.81	4.0	6.69	3.7
Met	0.03	0.0	0.04	0.0	0.00	0.0	0.00	0.0	0.10	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Phe	0.23	21.5	0.14	19.4	0.13	18.6	0.58	0.2	0.32	12.3	0.21	3.4	0.21	11.9	0.41	12.6
Glx	1.49	5.9	2.07	5.5	1.02	4.3	1.35	6.2	0.51	8.0	0.32	2.1	0.44	3.7	1.34	3.4
Tyr	0.00	0.0	0.05	0.0	0.02	0.0	0.17	0.0	0.00	0.0	0.00	0.0	0.07	0.0	0.02	0.0
Orn	0.05	0.0	0.02	0.0	0.03	0.0	0.34	0.0	0.08	0.0	0.00	0.0	0.00	0.0	0.00	0.0
Lys	0.03	0.0	0.08	0.0	0.02	0.0	0.11	0.0	0.13	0.0	0.04	0.0	0.09	0.0	0.08	0.0

Egyptian chewing tobacco samples (nos. 29 and 30) were purchased in Cairo, Egypt. Sample no. 25 represented a wet product owing to treatment with cherry-flavored syrup.

Denmark chewing tobaccos (nos. 31 and 32) represented twisted tobaccos and were products of Oliver Twist Comp., Odense, Denmark.

Cigar no. 33 ('Black Gold') was a product of Havatampa Inc., FL, USA and *cigarillo* no. 34 ('Cohiba') was from the Republic of Cuba.

Tobacco plants (*Nicotiana tabacum* L.) var. Bel. W3 (no. 35) and var. Bel. B3 (no. 36) were grown in the greenhouse of the Institute of Plant Ecology, Giessen University. Analyzed were two freshly harvested leaves.

2.4. Isolation of amino acids from native and processed tobaccos

The surface of freshly harvested tobacco leaves was cleaned several times with 70% aqueous ethanol, stalkers were removed, and leaves were minced with a lancet. The other tobaccos were analyzed as obtained. To 0.5–1 g amounts of tobacco (or 2 g of leaves) 70% MeOH (2.5 ml) were added and the mixture was ultrasonicated for 25 min at room temperature. Then the mixture was centrifuged at 3500*g* for 20 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 (1 ml), and 250 µl-aliquots were adjusted to pH 2.3 by addition of 0.01 M HCl. Then samples were put on Pasteur pipettes filled with Dowex 50 WX 8 cation exchanger (bed volume 5×0.5 cm ID); washed until neutral, and amino components eluted with 4 M aqueous ammonia. After evaporation to dryness a solution of 5% antioxidant BHT in 2-PrOH (50 µl) was added and amino acids were converted into *N*(*O*)-pentafluoropropionylamino acid-(2)-propyl esters by treatment with AcCl in 2-PrOH for 1 h at 100 °C. Reagents were removed in a steam of nitrogen, followed by addition of PFPAA (50 µl) in DCM (300 µl) and heating at 100 °C for 20 min.

Note that under the routine conditions employed for derivatization and analyses of tobacco amino acids, His and Arg are not eluted from the GC column and Asn and Gln are converted into Asp and Glu, respectively.

2.5. Gas chromatographic mass spectrometric quantification of amino acid enantiomers

Relative amounts of D-amino acids were calculated in the following equation:

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

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 in the following equation:

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

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 in the following equation:

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

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 sam-

Table 4
Quantities of L-AA (mg/g tobacco) and relative amounts of D-AA (%*D*) in cigar (no. 33), cigarillo (no. 34) and freshly harvested tobacco leaves (nos. 35 and 36)

	33		34		35		36	
	L	%*D*	*L*	%*D*	*L*	%*D*	*L*	%*D*
Ala	0.35	18.3	0.11	29.1	1.88	0.2	0.51	0.4
Val	0.14	0.0	0.03	0.0	0.66	0.0	0.27	0.0
Thr	0.15	0.0	0.05	0.0	0.11	0.0	0.10	0.0
Gly	0.12	–	0.10	–	0.00	–	0.00	–
Ile	0.05	0.0	0.01	0.0	0.57	0.0	0.18	0.0
Pro	1.09	1.5	1.06	3.7	0.58	0.0	1.26	0.0
Leu	0.03	9.4	0.20	0.0	0.72	0.0	0.42	0.0
Ser	0.24	6.5	0.08	7.1	0.89	0.3	0.89	1.3
GABA	0.09	–	0.04	–	1.35	–	1.98	–
Asx	1.81	3.9	1.26	4.7	0.17	0.2	5.39	0.8
Met	0.00	0.0	0.00	0.0	0.18	0.0	0.25	0.0
Phe	1.03	7.1	0.10	6.5	0.95	1.9	1.33	1.6
Glx	1.33	5.6	0.80	6.5	4.39	0.4	4.11	1.8
Tyr	0.10	0.0	0.04	0.0	0.40	0.0	0.51	0.0
Orn	0.02	0.0	0.01	0.0	0.15	0.0	0.29	0.0
Lys	0.19	0.0	0.09	0.0	0.78	0.0	0.99	0.0

ple, A_{IS} the peak area of the IS added to the sample and c_{IS} the amount 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 1–4, the absolute quantities of D-amino acid c_{DAA} can be calculated, if required in the following equation:

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

3. Results

3.1. General

The absolute and relative quantities of free L-and D-amino acids of 24 snuff tobaccos and 8 chewing tobaccos were determined and compared to a cigar and cigarillo and those extracted from freshly harvested tobacco leaves. The data resulting from quantitative enantioselective GC-SIM-MS are compiled in Tables 1–4 and chromatograms (GC-SIM-MS) of a standard and of selected tobacco samples are presented in Fig. 1. Note that L-Leu and L-Ser are resolved on a new capillary column but coelute on aged columns. GC-SIM-MS, however, enables their differentiation owing to specific fragment ions.

No racemization was observed under conditions of derivatization for GC of the standard of L-amino acids used for the determination of response factors.

Non-chiral Gly and GABA, the latter a characteristic neuroactive amino acid, were also detected in all tobacco samples. In processed tobaccos quantities ranged for Gly from 0.01 to 0.32 mg/g and for GABA from 0.00 to 0.28 mg/g. In two fresh tobacco leaves quantities of GABA of 1.35 and 1.98 mg/g, respectively, were detected, but no Gly (see Table 4).

3.2. German snuffs

In the German snuffs (nos. 1–16) L-Pro ranged from 0.63 to 6.31 mg/g, L-Asx from 0.64 to 7.95 mg/g, and L-Glx from 0.39 to 2.26 mg/g. Quantities of the other L-amino acids in all snuffs were much lower (see Table 1). Varying patterns of D-amino acids could be detected in all snuff tobaccos.

D-Pro, ranging from relative 0.1% to 2.6% was detected in all samples, as well as D-Asx (1.0–6.2%) and Glx (1.1–8.0%). However, the most abundant relative quantities were measured for D-Ala (11.7–32.2), D-Val (0.0–3.9%) and D-Ile (8.7–11.5%) were detected in two snuffs and D-Thr (10.6–18.0%) in three snuffs. Whereas quantities of D-Ser ranged from 0.0% to 13.2% no D-Tyr, D-Orn, or D-Lys could be detected in snuffs. For a GC-MS of German snuff no. 16, see Fig. 1(b).

One sample among the German snuffs was declared as 'tobaccoless' snuff and thus actually represents a snuffy powder. Indeed, no or just background levels of few amino acids could be detected in this sample. Therefore, data are not included in Table 1.

3.3. British snuffs and Swedish snuff

Quantities of amino acids in seven British snuffs (nos. 17–23) and a Swedish snuff (no. 24) are presented in Table 2. Amounts of L-Pro ranged from 0.30 to 3.33 mg/g, L-Asx from 0.58 to 6.78 mg/g and Glx from 0.32 to 4.74 mg/g. In the snuffs from Great Britain and Sweden D-Ala was most abundant, ranging from 10.5% to 30.6%. D-Leu ranged from 0.0% to 11.4% and D-Phe (19.7%) was detected only in the Swedish and German but not in the British snuffs.

The other D-amino acids detected in these snuffs were D-Pro (0.8–3.8%), D-Ser (0.0–13.6%), D-Asx (0.0–13.2%), and D-Glx (2.3–16.1%). No D-enantiomers could be detected of D-Val, D-Thr, D-Ile, D-Met, D-Tyr, D-Orn and D-Lys. For a representative GC-MS of an English snuff (no. 22), see Fig. 1(c).

3.4. Chewing tobaccos

Quantities of L-amino acids and relative quantities of the corresponding D-amino acids determined in chewing tobaccos are presented in Table 3. Among L-amino acids, L-Pro (0.51–6.21 mg/g), L-Asx (0.29–6.69 mg/g) and L-Glx (0.32–2.07 mg/g) were the most abundant. Among D-amino acids, highest relative amounts, detectable in all samples, were determined for D-Ala (1.8–34.0%), D-Phe (0.2–21.5%), D-Asx (3.5–13.8%), and D-Glx (2.1–8.0%). Further, D-Pro (0.4–5.0%), D-Leu (0.0–11.4%) and D-Ser (0.0–12.1%) could be detected but no D-enantiomers of the other amino acids. For a typical GC-MS of a Philippine chewing tobacco (no. 25), see Fig. 1(d).

3.5. Cigar, cigarillo and native tobacco leaves

A cigar and cigarillo were analyzed for comparison with smokeless tobaccos and two native tobacco leaves from a greenhouse. Data are presented in Table 4. L-Asx, L-Glx and L-Pro ranging from 0.17 to 5.39 mg/g were the most abundant among L-amino acids. Highest amounts among D-amino acids were determined for D-Ala in cigar and cigarillo, amounting to 18.3% and 29.1%, respectively. A variety of other D-amino acids (D-Pro, D-Leu, D-Ser, D-Asx, D-Phe, and D-Glx) could also be detected ranging from relative 0.2% to 9.4%.

In the tobacco leaves no or low amounts of D-amino could be detected (see Table 4). The abundance of D-amino acids in a cigar (no. 33) in comparison to native tobacco leaf (no. 35) is illustrated with the GC-MS data presented in Figs. 1(e) and (f), respectively.

4. Discussion

From the data, it is evident that varying amounts and kinds of L- and D-amino acids together with achiral Gly and GABA could be detected in processed, i.e. cured, fermented and, in part, sossed tobacco samples. In freshly harvested tobacco leaves L-amino acids were detected in

the low milligram range and certain D-amino acids in the low percentage range. This is in agreement with plant D-amino acids in general (Brückner & Westhauser, 2003).

Absolute quantities of L-amino acids and Gly and GABA in tobaccos were in the low milligram range for the most abundant amino acids (L-Pro, L-Asx and L-Glx) or sub milligram range for the other proteinogenic amino acids. Release of free L-amino acids from proteins and decrease during ageing has been explained by enzymic and chemical processes, which have been intensively investigated (Hamilton & Lowe, 1978).

Our quantitative data on free amino acids in processed and native tobaccos are in good agreement with those reported in the literature (Yang & Smetena, 1993).

As outlined above the stereochemistry of amino acids in tobaccos attracted little attention partly as result of need for equipment suitable for chiral amino acid analysis. From the data presented in tables and figures, however, it can be seen that D-amino acids, in part in high relative amounts, are detectable in tobacco products.

The data presented are in principal agreement and extend those reported for cigarettes, a loose-leaf chewing tobacco, and each a dry and moist American snuff, all products serving as standards for American tobacco products (Kullman et al., 1999). Owing to the GC-SIM-MS methodology used, additional D-amino acids such as D-Thr, D-Ser and D-Glx could be determined. We could not detect D-Met in smokeless tobaccos. Met, however, is a very minor amino acid in tobaccos.

The range of relative quantities is compared in Table 5. As can be seen the data agree favourably.

Since native tobacco leaves contain very low amounts of D-amino acids (see Table 4 and Fig. 1(f)) the specific procedures applied to tobacco leaves must be responsible for the generation of D-enantiomers and their abundance in tobacco products. As outlined above the manufacturing process includes harvesting of various cultivars of tobacco, curing, fermentation, blending, sossing, and manufacturing of the final product.

Plant or bacterial amino acid transaminases or racemases, owing to their stereoselectivity, are not likely candidates as their presence does not explain the variety of D-amino acids formed and since heat curing reduces microorganisms and enzyme activity. Established physico-chemical or age dependent racemization of free tobacco L-amino acid is also not likely to occur as conditions are not severe enough.

We have recently shown, however, that racemization of amino acids occurs in the course of the Maillard reaction (Brückner, Justus, & Kirschbaum, 2001). This reaction can be defined as reaction of reducing sugars with amino acids leading finally to the formation of a multitude of flavor compounds and brown, polymeric products, named melanoidins (Ames, 1998). Relative stable intermediates of this very complex reaction are the fructose-amino acids resulting from the Amadori rearrangement. Indeed, fructose-amino acids have been detected in and isolated from foods such as freeze-dried apricots and peaches, milk powder, and dried vegetables (Anet & Reynolds, 1956; Cremer & Eichner, 2000; Sanz, del Castillo, Corzo, & Olano, 2001; van Boekel, 1998).

Notably, fructose-amino acids (Fru-amino acids) were detected in flue-cured and stored tobacco leaves (Noguchi et al., 1971) the most abundant of which also represent the most abundant D-amino acids in tobaccos, i.e. Ala, Asx and Glx, together with Ser and Phe. These are also the amino acids which racemize fast under experimental conditions of the Maillard reaction (Brückner et al., 2001). For comparison data of fructose-amino acids have been included in Table 5.

Quantities and types of D-amino acids released from fructose-amino acids are expected to depend on the amino acid residue therein and parameters such as their concentration, and temperature and storage time, pH, presence of other components such as humificants governing the water activity, flavor agents or catalysts. Release of amino acids from Amadori compounds is reversible until the amino acids are finally transferred at advanced stages of the Maillard reaction irreversibly into heterocyclic or polymeric compounds.

Consequently, generation of D-amino acid from Amadori compounds (or, analogously, Heyns compounds resulting from fructose and amino acids) is postulated to be a major route for their formation in tobaccos. A tentative mechanism via formation of a carbanion in the Amadori compound has been presented (Pätzold & Brückner, 2004; Pätzold, Kirschbaum, & Brückner, 2002). This general route for the generation of D-amino acids has been extended to other foods rich in reducing sugars and amino acids. Examples are fortified wines (Pätzold, Nieto-Rodriguez, & Brückner, 2003), balsamic vinegars (Erbe & Brückner, 2000), and fruit juice and dietary plant sap

Table 5
Range of relative quantities of D-amino acids (%*D*) determined in (a) 27 European snuffs (present study) in comparison to (b) three American smokeless tobacco standards (Kullman et al., 1999) and (c) fructose amino acids identified in cured tobacco leaves (Noguchi et al., 1971)

D-amino acid	%*D*		(c)
	(a)	(b)	
Ala	1.8–34.0	9.25–50.00	Fru-Ala (+)
Val	0.0–3.9	1.18–8.89	Fru-Val
Thr	0.0–18.0	n.d.	Fru-Thr
Ile	0.0–15.5	n.d.	n.d.
Pro	0.1–5.5	0.50–0.91	Fru-Pro (+)
Leu	0.0–32.2	10.78–23.14	n.d.
Ser	0.0–13.6	n.d.	n.d.
Asx (Asp + Asn)	0.0–13.8		
Asp	n.d.	3.60–4.42	
Asn	n.d.	2.26–5.14	Fru-Asn (+)
Met	n.d.	1.97–12.08	n.d.
Phe	0.0–25.8	8.08–12.77	Fru-Phe
Glx (Glu + Gln)	1.1–16.1	n.d.	Fru-Gln (+)
Tyr	n.d.	n.d.	Fru-Tyr
Orn	n.d.	n.d.	n.d.
Lys	n.d.	n.d.	n.d.

n.d., not detected or not determined/determinable; (+) indicates most abundant fructose-amino acid.

concentrates (Pätzold & Brückner, 2002, 2004). Consequently, fruit syrups and concentrates used for sossing tobacco products are additional sources of D-amino acids. The Maillard reaction also explains the occurrence of D-amino acids in roasted coffee and cacao (Casal, Mendes, Oliveira, & Ferreira, 2005; Kutz, Pätzold, & Brückner, 2004).

Finally, from a physiological point of view it is to be anticipated that both L- and D-amino acids in snuffs and chewing tobaccos are adsorbed by the mucosa of the nose and mouth, respectively and will appear in the blood of consumers. Although certain physiological effects of free D-amino acids have been compiled and data discussed in the literature (Friedman, 1999) it is not assumed that they have adverse effects on human beings (Schieber et al., 1997). Concentrations of D-amino acids are low, effective D-amino acid oxidases in kidney and tissues will oxidize D-amino acids rapidly and the remaining ones will be rapidly excreted with the urine (Pätzold, Schieber, & Brückner, 2005).

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