Amino Acids (2003) 24: 43–55
DOI 10.1007/s00726-002-0322-8

Amino
Acids
Printed in Austria

Chromatographic determination of L- and D-amino acids in plants

H. Brückner and **T. Westhauser**†

Interdisciplinary Research Center, Institute of Nutritional Sciences, Department of Food Sciences, University of Giessen, Germany

Received January 1, 2002
Accepted May 30, 2002
Published online September 10, 2002;

Summary. Quantities of free L- and D-amino acids (L- and D-AAs) in plants (leaves of coniferous and decidious trees, fleshy fruits, leaf blades of fodder grasses, and seeds and seedlings of edible legumes) were determined. Amino acid (AA) enantiomers were converted into diastereomers using pre-column derivatization with *o*-phthaldialdehyde together with *N*-isobutyryl-L(or D)-cysteine followed by separation of the resulting fluorescent isoindol derivatives on an octadecylsilyl stationary phase using high-performance liquid chromatography. Relative amounts of D-AAs were also determined by enantioselective gas chromatography-mass spectrometry on Chirasil-L-Val®. Free D-AAs acids in the range of about 0.2% up to 8% relative to the corresponding L-AAs acids were found in plants. D-Asp, D-Asn, D-Glu, D-Gln, D-Ser and D-Ala could be detected in most of the plants, and D-Pro, D-Val, D-Leu and D-Lys in certain plants. As D-AAs were detected in gymnosperms as well as mono- and dicotyledonous angiosperms of major plant families it is concluded that free D-AAs in the low percentage range are principle constituents of plants.

Keywords: L- and D-amino acids – Chirality – High performance liquid chromatography – Gas chromatography – Mass spectrometry – Plant amino acids – Plant living fossils

Abbreviations: Amino acids are abbreviated according to the three-letter-nomenclature; L-*homo*-Arg, L-*homo*-arginine; GABA, γ-aminobutyric acid

Introduction

The biosynthesis of the proteinogenic L-amino acids (L-AAs) of higher plants proceeds via well established biochemical patheways resulting in AAs belonging to the so-called glutamate-, aspartate, pyruvate-, serin-, and shikimate biogenetic family (Singh, 1999). In the final steps of the biosynthesis usually an amino group is transferred by aminotransferases (transaminases) onto intermediately formed 2-oxocarboxylic acid. As these pathways are stereospecific this explains the presence and abundance of free L-AAs in plants. It was realized, however, that certain mirror images (enantiomers, or epimers if several chiral centers are concerned) of the protein L-amino acids, named D-AAs, do also occur in plants. Notably, it was recognized as early as 1960 that *N*-acylation of D-AAs, in particular *N*-malonylation of D-Trp, is common in mono- and dicotyledonous plants (Zenk and Scherf, 1963; 1964). Further, D-Ala, D-Asp, and D-Glu were detected in the free and conjugated form in pea seedlings (*Pisum sativum*) (Ogawa et al., 1977), barley grains (*Hordeum vulgare* L.) and hops blossoms (*Humulus lupulus* L.) (Erbe and Brückner, 2000), and D-Ala as well as D-Ala-D-Ala were found in pasture grass (*Phalaris tuberosa* L.) (Frahn and Illman, 1975) and wild rice (*Oryza australiensis* Domin) (Manabe, 1985). Conjugated D-α-amino-*n*-butyric acid was reported to occur in nine genera of legumes (Ogawa et al., 1976). Various free D-AAs were also detected in cured tobacco leaves (Kullman et al., 1999). However, most of the research on D-AAs focused on the metabolisation and conjungation of selected D-enantiomers administered to plants under experimental conditions.

The knowledge on the occurence of D-AAs in higher plants was compiled in few reviewes (Robinson, 1976; Gamburg and Rekoslavskaya, 1992). General considerations on AAs resulting from their chirality ("handedness"), including biosynthesis, chemistry, microbiology, and nutritional aspects, were treated e.g. by Davies (1977) and Friedman (1999).

The development of sensitive and effective gas- and liquid chromatographic methods for the direct and

† Deceased

indirect separation of L- and D-AAs outdate more cumbersome methods for the determination of plant D-AAs such as combined enzymatic chromatographic procedures (Aldag et al., 1971). Thus GC and HPLC made possible the detection of D-AAs permanently present in unprocessed vegetables and fruits in the range of about 0.5–3% relative to their L-enantiomers (Brückner and Westhauser, 1994). Microbial contamination, or controlled microbial fermentation of edible plants or plant juices, has been demonstrated to increase amounts and kinds of D-AAs (Brückner et al., 1995). Consequently, it has been suggested that quantities of certain D-AAs in processed fruit juices exceeding significantly the natural level might serve as molecular markers for bacterial contamination (Brückner and Lüpke, 1991; Gandolfi et al., 1992; Brückner and Westhauser, 1994).

In continuation of our work on the natural occurrence of free D-AAs in vegetables and fruits we now present data on their presence in plants in general and discuss mechanisms on their formation.

Materials and methods

Instruments

Since the HPLC instrument and the fully automated derivatization and separation procedures of amino acid derivatives have been described already in detail (Brückner et al., 1991, 1992, 1995; Brückner and Westhauser, 1994) only a brief description of the analytical methodology is given in the following.

For HPLC a HP 1090 Series L instrument (Hewlett-Packard, Waldbronn, Germany) was used comprising a binary solvent-delivery system, autoderivatizer and programmable fluorescence detector set at 230 nm excitation wavelength and 445 emission wavelength. For the separation of amino acid derivatives (see below) a 250 mm × 4 mm ID column and 20 mm × 4 mm ID guard column were used filled with Hypersil ODS 5 μm stationary phase (Shandon, Astmoor, Runcorn, Cheshire, U.K.). Columns filled with this stationary phase are available from Agilent. For elution of derivatives a linear gradient generated from (A) 23 mM sodium acetate (pH 5.95) and (B) a mixture of CH_3OH and acetonitrile was applied. For the derivatization of amino acids mixtures of *o*-phthaldialdehyde (OPA) together with either *N*-isobutyryl-L-cysteine (IBLC) or *N*-isobutyryl-D-cysteine (IBDC) in potassium borate buffer (pH 10.4) were used. The reagents IBLC and IBDC (as well as OPA) are available e.g. from subsidiaries of Fluka/Sigma-Aldrich Company. The material used in this work was from Novabiochem (Läufelfingen, Switzerland) and had an enantiomeric purity of >99.96% (IBLC) and >99.89% (IBDC).

For calibration and quantification of amino acid enantiomers an external standard mixure containing 100 pmol L-amino acids (including achiral Gly and GABA) and 5 pmol D-amino acids was prepared in our laboratory. For quantitative considerations using this approach see Brückner et al. (1992; 1995).

For comparison and completion of data, relative amounts of D-AAs in selected samples were determined by gas chromatography-mass spectrometry (GC-MS) using an instrument comprising a GC-17 A gas chromatograph equipped with a 25 m × 0.25 mm I. D. Chirasil-L-Val® fused silica capillary column coupled to a QP 5000 mass spectrometer (Shimadzu, Kyoto, Japan). Derivatization procedures and *N*(*O*)-pentafluoropropionyl amino acid 2-propyl esters as described previously were used (Brückner and Schieber, 2000). This method made also possible the determination of D- and L-Pro in analytes.

Sources of plants

Fruits and cactus. Perfect fruits were selected and purchased in retail outlets with the exception of apples. Cultivars of apple (*Malus sylvestris* var. *domestica* (Borckh. Mansf.), pineapple (*Ananas comosus* (L.) Merr.), water melon (*Citrullus lanatus* (Thunb.) Matsum. et Nakai), papaya (*Carica papaya* L.), mango (*Mangifera indica* L.), and yellow passion fruit (*Passiflora edulis* var. *flavicarpa* Sims) were investigated. The prickly pear cactus (*Opuntia ficus-indica* (L.) Mill.) was from Lanzarote (Canary Islands), and coconut milk was drawn from freshly imported coconut (*Cocos nucifera* L.).

Trees and soil. Trees were adult plants from the arboretum of Hohenheim University, Germany. Investigated were ginkgo (maidenhair tree) (*Ginkgo biloba* L.), metasequoia (*Metasequoia glyptostroboides* Hu et Cheng), spruce tree (*Picea abies* (L.) Karst.), and maple tree (*Acer platonoides* L.). The apple cultivar "Golden Delicious" (together with leaves and soil samples) were from an orchard of Stuttgart area (Germany). Surface soil samples were taken close to the tree, mixed, and aliquots analyzed at the Landesanstalt für Landwirtschaftliche Chemie, Hohenheim University. According to particle-size analysis the loamy soil of pH 7.0 was composed of medium sand (9.5%), medium silt (46.6%), and clay (43.9%). Carbon was 2.56% and nitrogen analysis provided 119 mg N/kg fresh soil. For AA analysis of this soil see below.

Grasses. The fodder grasses Meadow Foxtail (*Alopecurus pratensis* L.), Black Bent Grass (*Agrostis gigantea* Roth), and leaves of maize (*Zea mays* L.) were obtained from the Institute of Plant Physiology, Hohenheim University.

Seeds. Plant seeds were obtained from the Institute of Plant Breeding, Hohenheim University. Investigated were runner beans cv. "Rapid" (*Phaseolus vulgaris* L. var *vulgaris*), soy bean cv. "Lunja" (*Glycine max* (L.) Merr.), lucerne (alfalfa) (*Medicago sativa* (L.), garden cress *Lepidum sativum* L.), and water cress (*Nasturtium officinale* R. Br.).

Treatment of samples for analysis

General procedures

Analytical procedures and chemicals, including cation exchanger, were tested regularly in order to reveal possible contaminations with D-AAs. Glassware was heated at 500°C prior use and bidistilled water was distilled again in a quartz distill. Juicer, lancets etc. were washed with distilled water and 70% EtOH prior to use and plants were peeled or surfaces washed with distilled water and 70% EtOH. Fruit juices (2 μl aliquots) were analyzed directly by HPLC after centrifugation and filtration using disposable 0.2 μm membrane filters. For analysis of samples by GC-MS cation exchanger treatment procedures already described were used (see e.g. Brückner et al., 1995). Samples were adjusted to pH 2 and passed through glass columns filled with Dowex 50W X8 cation exchanger (bed volume 1 × 5 cm). The AAs adsorbed were eluted with 4 M aqueous ammonia, eluates were dried *in vacuo*, and AAs derivatized for GC-MS analysis.

Fleshy fruits, cactus and coconut. Fruits were washed and brushed with water and the surface was cleaned with 70% aqueous ethanol.

Then fruits were peeled, sliced and subjected to an automatic juicer based on the centrifugal principle. Cores and seeds of fruits had been removed if appropriate. The juices obtained were filtered using fluted filter-papers, the filtrates were centrifuged at 1.650 × *g*, and supernatants were filtered using disposible membrane filters of 0.45 μm and 0.2 μm pore size. The joints of the cactus *Opuntia* (botanically a stem succulent) were treated analogously. For coconut milk the endocarp of the nut (botanically a stony fruit) was punched, the juice drawn, an aliquot centrifuged at 1.650 × *g*, the supernatant filtered using membrane filters, and aliquots (2 μl) were analysed by HPLC.

Tree leaves and blades of grasses. The surface of leaves was cleaned several times with 70% aqueous ethanol, stalkers were removed, and leaves were minced with a sterile lancet. To aliquots (2 g) 15 ml of 70% EtOH were added and the mixture was mashed with the aid of a homogenizer. The mixture was sonicated for 15 min. at 70°C, centrifuged at 1,650 × g and the supernatant collected. The remaining residue was treated twice as described, the combined supernatants were adjusted to pH 2 with addition of 0.1 M HCl and filtered. The filtrate was extracted in a separatory funnel with a mixture of light petroleum/diethyl ether (1 : 1, v/v) (3 × 20 ml), then the aqueous phase was evaporated to dryness. To the residue 0.01 M HCl (2 ml) was added, and aliquots (100 μl) were filtered and analyzed (2 μl) by HPLC. The remaining solution was subjected to cation exchange treatment and AAs eluted were analyzed by GC-MS (Brückner and Schieber, 2000).

Seeds and seedlings. The surface of seeds (2.0 g) was rinsed with 70% aqueous ethanol and conditioned with 70% ethanol for 15 h at ambient temperature. Seeds were chopped with a lancet and homogenized. The homogenate was treated as described above for plant leaves. Seeds were rinsed with 70% aqueous ethanol and sterilized for 20 min. in 1% sodium hypochlorite under a laminar flow. The hypochlorite solution was discarded, seeds were rinsed several times with sterile 0.825% sodium chloride. Then seeds were allowed to germinate for five days in the laminar flow in the dark on filter papers soaked with sterile sodium chloride. Germinated seeds (2 g) were minced with a lancet, 70% ethanol (15 ml) was added and the mixture was homogenized and treated as described for leaves and analysed by HPLC and GC-MS.

Soil sample. To the wet soil (25 g) amounts of 70% ethanol (50 ml) were added, the mixture was sonicated for 30 min. and centrifuged at 1.650 × *g* for 20 min. The supernatant was removed, to the remaining residue 70% ethanol (50 ml) was added and the suspension sonicated and centrifuged as described before. These procedures were repeated twice and supernatants were combined. Then 0.1 M HCl (50 ml) was added, the mixture was sonicated and centrifuged as above. The supernatant was evaporated to drynes, 0.01 M HCl (4 ml) was added and the solution was subjected to cation exchanger treatment and analysis by HPLC and GC-MS.

Results

Quantities of enantiomers of free L- and D-AAs (μmol per liter juice or per kg fresh plant materials) and relative amounts of D-AAs (% D) calculated from peak areas of diastereomers of plant's AAs investigated by HPLC are presented in Tables 1–4. Relative amounts of D-amino acids in plants determined by GC-MS are presented in Table 5. A typical chromatogram demonstrating the performance of the liquid chromatographic method used for the indirect separation of AA enantiomers via formation of fluorescent diastereomers by derivatization with OPA/IBLC is shown in Fig. 1. The secondary AA Pro can not be analysed using this approach.

Chromatograms of AAs of the apple "Golden Delicious" and demonstration of the reversal of the elution order using OPA/IBDC in place of OPA/IBLC are shown in Fig. 2. Note that the fluorescence of diastereomers resulting from derivatization with these reagents differ for certain amino acids up to 10%.

Using derivatization with OPA/IBLC and OPA/IBDC in the juice of apples of the cultivar "Golden Delicious" relative amounts of 0.4% D-Asp, 7% D-Asn, 0.5% D-Glu, 1.7% D-Ser, and 2.7% D-Ala were detected (Fig. 2). In leaves of this tree 7.9% D-Asp, 3.1% D-Glu, 11.7% D-Ser, and 1.5% D-Ala were determined, and in orchard soil collected close to this apple tree 8.5% (8.2%) D-Asp, 7.1% (4.3%) D-Glu, 8.3% (2.3%) D-Ser, and 15.5% (16.7%) D-Ala were determined by HPLC (Fig. 3) and GC-MS (data in parentheses). Chromatograms resulting from HPLC analysis of leaves of ginkgo tree and metasequoia tree are presented in Fig. 4. From the data of the Tables 1–4 it is evident that amounts of free L-amino acids vary considerably among plants and represent just their physiological state when samples were taken. Notably, free D-Asp, D-Glu and D-Ala could be determined in all plants, D-Asn, D-Gln, D-Ser were found in many plants, and D-Lys in few plants, but D-Val and D-Leu only in coconut milk.

Relative quantities of D-enantiomers (% D) in certain plants (cf. Table 5) were also determined by GC/MS and agree excellent or satisfactorily with the HPLC approach with the advantage that enantiomers of Pro could be determined. Enantiomers of D-Pro, D-Val and D-Lys could be detected in most germinated seeds by GC-MS (Table 5).

Discussion

The data demonstrate that D-enantiomers of certain AAs occur in the free state in all plants. Relative amounts were in the low percentage range. Taking the complex matrix into account the liquid chromatographic approach applied is reliable in particular when data are confirmed by reversal of the elution order of diastereoisomers by derivatization using the OPA/IBLC and the OPA/IBDC reagent (cf. Fig. 2). This elegant approach has also been extended on the deter-

Table 1. Quantities of amino acid (AA) enantiomers in fleshy fruits and cocoa nut

	Apple 'Golden Delicious'[a)]			Pineapple			Water melon			Papaya			Mango			Yellow passion fruit			Coconut milk		
AA	L-AA μmol/l	D-AA μmol/l	% D	L-AA μmol/l	D-AA μmol/l	% D	L-AA μmol/l	D-AA μmol/l	% D	L-AA μmol/l	D-AA μmol/l	% D	L-AA μmol/l	D-AA μmol/l	% D	L-AA μmol/l	D-AA μmol/l	% D	L-AA μmol/l	D-AA μmol/l	% D
Asp	797	3.2	0.4	382	3.6	0.9	1,611	8.8	0.5	217	3.1	1.4	263	3.9	1.5	3,065	16.2	0.5	657	7.5	1.1
Glu	677	3.4	0.5	723	3.0	0.4	215	–	–	n.d.	n.d.	n.d.	1,169	4.9	0.4	3,558	17.1	0.5	1,769	8.6	0.5
Asn	2,071	14.6	0.7	3,109	24.7	0.8	731	–	–	5,034	33.9	0.7	93	–	–	195	–	–	314	15.5	4.7
Ser	197	3.4	1.7	795	–	–	916	–	–	375	3.1	0.8	266	–	–	2,962	17.1	0.6	1,357	12.2	0.9
Gln	33	–	–	929	–	–	4,045	8.5	0.2	456	1.7	0.4	2,439	7.3	0.3	4,336	–	–	265	–	–
Thr	64	–	–	145	–	–	472	–	–	292	–	–	49	–	–	297	–	–	554	–	–
Gly	18	–	–	177	–	–	280	–	–	1,528	–	–	12	–	–	1,317	–	–	689	–	–
His	8	–	–	143	–	–	–	–	–	70	–	–	72	–	–	480	–	−1.2	131	–	–
Ala	105	2.9	2.7	1,247	–	–	667	4.0	0.6	402	3.6	0.9	1,920	8.4	0.4	975	12.0	0.8	5,582	43.7	0.8
Arg	12	–	–	98	–	–	3,122	12.5	0.4	584	5.5	0.9	2,100	6.8	0.3	752	5.9	–	483	–	–
Tyr	4	–	–	155	–	–	410	–	–	63	–	–	29	–	–	142	–	–	102	–	–
Val	24	–	–	202	2.3	1.1	520	–	–	103	–	–	127	–	–	456	–	–	414	2.6	0.6
Met	3	–	–	72	–	–	187	–	–	34	–	–	12	–	–	113	–	–	102	–	–
Trp	2	–	–	22	–	–	174	–	–	26	–	–	11	–	–	92	–	–	35	–	–
Phe	17	–	–	122	–	–	375	–	–	126	–	–	38	–	–	344	–	–	104	–	–
Ile	13	–	–	93	–	–	434	–	–	76	–	–	47	–	–	346	–	–	245	–	–
Leu	9	–	–	100	2.5	2.4	313	–	–	125	–	–	79	–	–	186	–	–	293	1.4	0.5
Lys	15	–	–	96	–	–	407	–	–	104	–	–	138	–	–	356	–	–	403	–	–

[a)] For data (% D) of leaves of 'Golden Delicious' and soil of orchard see Fig. 3
n.d. not determinable

Table 2. Quantities of amino acid (AA) enantiomers in leaves[a)] of trees and cactus

AA	Ginkgo			Metasequoia			Spruce tree			Maple tree			Cactus		
	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D
Asp	957	8.9	0.9	175	2.2	1.2	895	1.9	0.2	212	13.7	6.1	159	1.0	0.6
Glu	425	10.6	2.4	937	9.0	1.0	1,243	4.5	0.4	302	4.8	1.6	180	1.4	0.8
Asn	1,253	23.5	1.8	200	2.4	1.2	49	–	–	249	–	–	118	0.8	0.7
Ser	1,478	10.8	0.7	315	5.1	1.6	203	1.8	0.9	3,568	16.6	0.5	306	1.6	0.5
Gln	3,220	51.8	1.6	214	–	–	434	3.3	0.8	431	–	–	1,186	7.0	0.6
Thr	339	–	–	62	–	–	100	–	–	416	–	–	171	–	–
Gly	400	–	–	139	–	–	55	–	–	366	–	–	71	–	–
His	108	–	–	193	–	–	31	–	–	779	–	–	127	–	–
Ala	1,570	14.9	0.9	304	8.3	2.7	275	8.5	3.0	403	7.7	1.9	69	1.6	2.3
Arg	73	–	–	1,447	–	–	273	–	–	356	–	–	140	–	–
Tyr	37	–	–	31	–	–	26	–	–	113	–	–	41	–	–
Val	130	–	–	46	–	–	63	–	–	412	–	–	313	–	–
Met	26	–	–	14	–	–	7	–	–	81	–	–	91	–	–
Trp	33	–	–	14	–	–	14	–	–	140	–	–	27	–	–
Phe	49	–	–	50	–	–	31	–	–	153	–	–	104	–	–
Ile	36	–	–	16	–	–	41	–	–	125	–	–	145	–	–
Leu	60	–	–	32	–	–	28	–	–	290	–	–	102	–	–
Lys	51	–	–	40	–	–	67	–	–	225	–	–	173	–	–

[a)] For data of leaves of ‘Golden Delicious’ see Fig. 3

Table 3. Quantities of amino acid (AA) enantiomers in grasses

AA	Black Bent grass			Meadow Foxtail			Maize		
	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D
Asp	700	2.8	0.4	1,195	9.2	0.8	405	4.7	1.1
Glu	620	4.1	0.7	605	10.3	1.7	1,179	8.3	0.7
Asn	152	9.4	5.8	887	41.4	4.5	267	15.1	5.3
Ser	654	6.4	1.0	1,545	9.3	0.6	1,043	7.4	0.7
Gln	709	10.0	1.4	2,821	33.3	1.2	997	19.1	1.9
Thr	327	–	–	812	–	–	312	–	–
Gly	158	–	–	712	–	–	1,903	–	–
His	32	–	–	49	–	–	77	–	–
Ala	1,476	28.4	1.9	3,463	54.4	1.5	2,139	30.1	1.4
Arg	83	–	–	105	–	–	68	–	–
Tyr	70	–	–	80	–	–	161	–	–
Val	208	–	–	201	–	–	311	–	–
Met	30	–	–	43	–	–	46	–	–
Trp	17	–	–	16	–	–	4	–	–
Phe	111	–	–	110	–	–	93	–	–
Ile	72	–	–	79	–	–	92	–	–
Leu	142	–	–	132	–	–	123	–	–
Lys	119	1.0	0.8	127	1.9	1.5	81	2.3	2.8

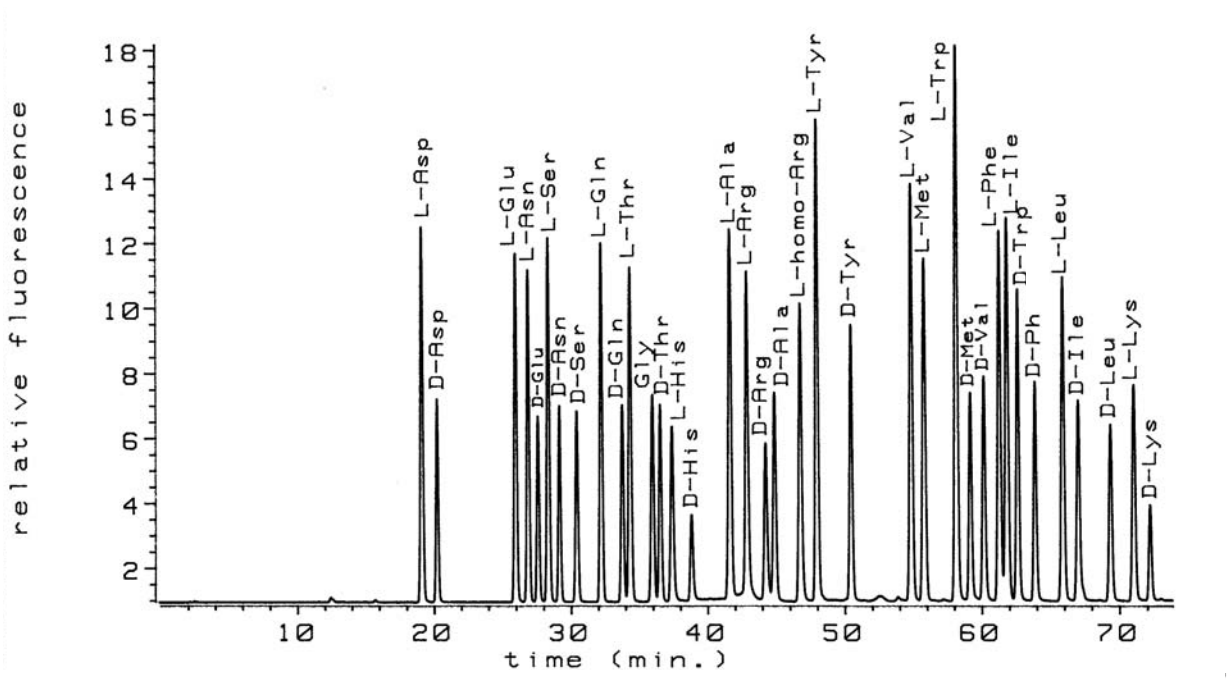

Fig. 1. HPLC of diastereomers of amino acids resulting from a representative standard composed of L-amino acids, Gly and the internal standard L-*homo*-arginine (L-*homo*-Arg) (each 100 pmol) and D-amino acids (each 50 pmol) derivatized with *o*-phthaldialdehyde/*N*-isobutyryl-L-cysteine (OPA/IBLC). Fluorescence detection at excitation 230 nm and emission 445 nm. For chromatographic conditions see Experimental

Table 4. Quantities of amino acid (AA) enantiomers in (A) seeds and (B) seedlings

AA	Soy bean			Runner bean			Garden cress			Alfalfa (lucerne)			Water cress		
	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D	L-AA μmol/kg	D-AA μmol/kg	% D
(A)															
Asp	1,947	20.0	1.0	1,969	18.9	1.0	2,810	15.0	0.5	1,061	20.4	1.9	1,947	18.3	0.9
Glu	4,473	79.8	1.8	4,120	112.7	2.7	1,549	27.2	1.7	2,422	30.9	1.3	4,952	147.7	2.9
Asn	1,759	34.0	1.9	3,699	61.9	1.6	4,631	52.2	1.1	2,564	20.6	0.8	2,481	40.6	1.6
Ser	160	–	–	252	–	–	394	–	–	308	–	–	951	–	–
Gln	246	–	–	8,642	–	–	169	–	–	2,478	–	–	1,837	–	–
Thr	262	–	–	192	–	–	227	–	–	573	–	–	764	–	–
Gly	411	–	–	371	–	–	308	–	–	719	–	–	840	–	–
His	314	–	–	844	–	–	189	–	–	372	–	–	371	–	–
Ala	908	75.3	7.7	1,323	118.3	8.2	842	25.8	3.0	8,069	121.5	1.5	2,156	41.2	1.9
Arg	1,819	–	–	7,680	–	–	447	–	–	2,380	–	–	2,148	–	–
Tyr	118	–	–	1,702	–	–	188	–	–	584	–	–	159	–	–
Val	209	–	–	286	–	–	372	–	–	466	–	–	758	–	–
Met	112	–	–	175	–	–	113	–	–	120	–	–	132	–	–
Trp	628	–	–	582	–	–	482	–	–	188	–	–	–	–	–
Phe	182	–	–	290	–	–	280	–	–	407	–	–	357	–	–
Ile	85	–	–	86	–	–	212	–	–	214	–	–	271	–	–
Leu	52	–	–	282	–	–	194	–	–	269	–	–	314	–	–
Lys	96	–	–	203	–	–	186	–	–	200	–	–	258	–	–
(B)															
Asp	640	5.9	0.9	666	3.6	0.5	385	2.4	0.6	457	5.2	1.1			
Glu	1,114	12.2	1.1	1,749	16.8	1.0	1,114	8.9	0.8	1,094	31.4	2.8			
Asn	3,379	30.3	0.9	2,177	52.9	2.4	1,948	35.7	1.8	6,710	25.7	0.4			
Ser	778	–	–	1,035	–	–	1,170	–	–	1,157	–	–			
Gln	165	–	–	1,922	23.2	1.2	2,545	–	–	270	–	–			
Thr	268	–	–	538	–	–	599	–	–	492	–	–			
Gly	109	–	–	137	–	–	193	–	–	380	–	–			
His	532	–	–	526	–	–	399	–	–	918	–	–			
Ala	481	10.1	2.1	763	18.2	2.3	790	10.3	1.3	1,369	21.1	1.5		n.d.	
Arg	751	–	–	1,392	–	–	561	–	–	1,552	–	–			
Tyr	73	–	–	80	–	–	261	–	–	209	–	–			
Val	497	–	–	563	–	–	616	–	–	554	–	–			
Met	32	–	–	61	–	–	30	–	–	41	–	–			
Trp	37	–	–	44	–	–	164	–	–	58	–	–			
Phe	245	–	–	284	–	–	423	–	–	413	–	–			
Ile	162	–	–	250	–	–	541	–	–	243	–	–			
Leu	115	–	–	218	–	–	543	–	–	241	–	–			
Lys	89	–	–	107	–	–	438	–	–	327	–	–			

n.d.; not determined

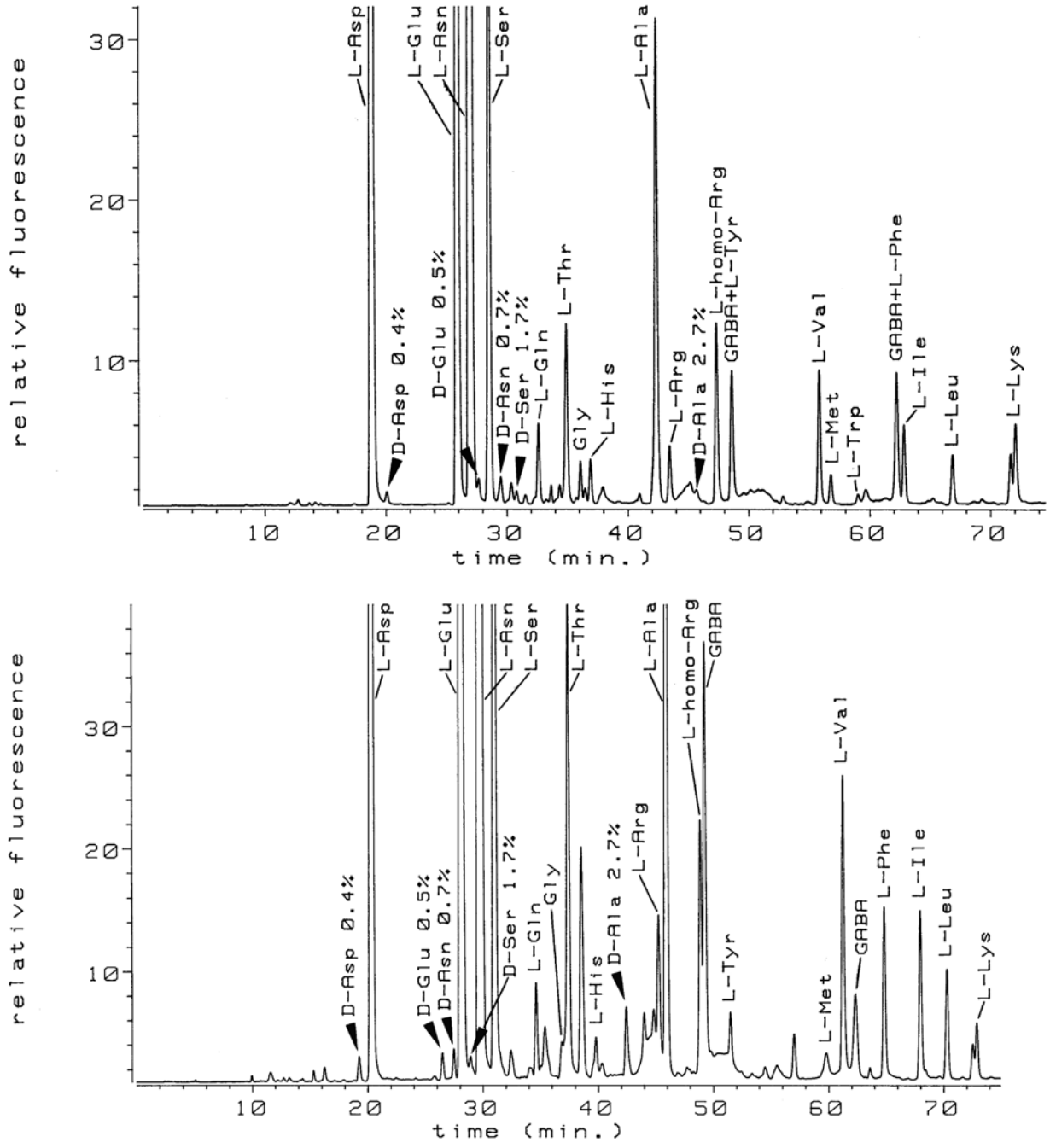

Fig. 2. HPLC of free amino acids of the juice of the apple cultivar "Golden Delicious" derivatized with (above) OPA/IBLC, and (below) OPA/IBDC. Note that GABA (γ-aminobutyric acid) forms two derivatives. Arrows in figures indicate positions of D-amino acids

mination of AA enantiomers in seawater and fossil mollusks (Fitznar et al., 1999).

In this study free D-AAs have been determined in gymnosperms of the botanical families Ginkgoaceae (ginkgo), Taxodiaceae (metasequoia), and Pinaceae (spruce tree). Notably, ginkgo and metasequoia are also referred to as ‚living fossils' as they belong to evolutionary very old gymnosperms. D-AAs occur also in monocotyledons of the families Arecaceae (coconut), Bromeliacea (pinapple) and Poaceae (grasses), and in dicotyledons of the families Aceraceae (maple tree), Anacardiaceae (mango), Brassicaceae (cress), Cactaceae (prickly pear), Caricaceae (papaya), Cucurbitaceae (water melon), Fabaceae

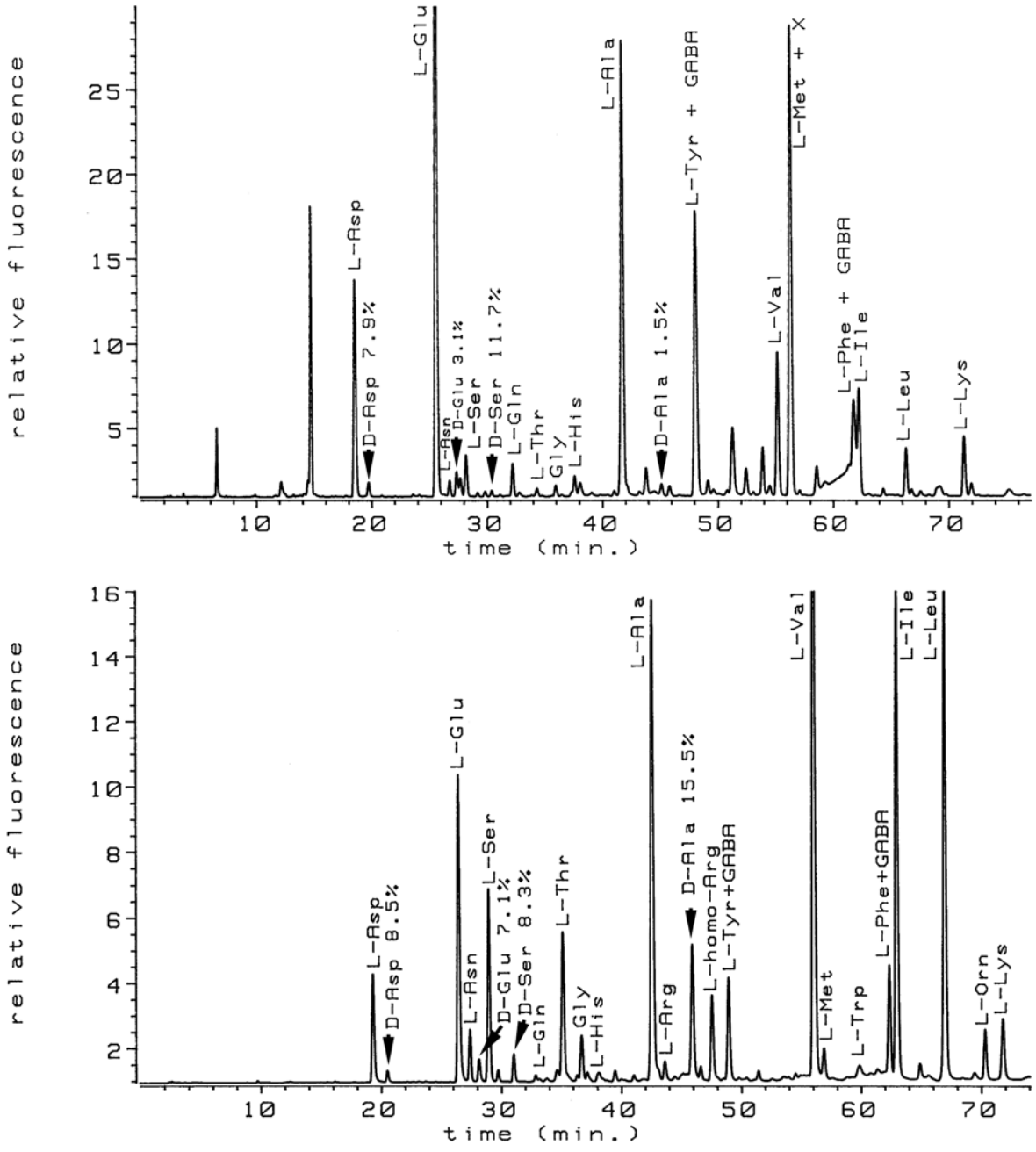

Fig. 3. HPLC of free amino acids derivatized with OPA/IBLC from (above) leaves of apple tree, cultivar "Golden Delicious", and (below) from a soil sample taken at this apple tree

(beans, lucerne), Passifloraceae (passion fruit), and Rosaceae (apple). With respect to common biochemical pathways of plants, D-AAs were detected in C3 plants as well as C4 plants such as grasses and maize, and CAM plants like cacti, which show the crassulacean acid metabolism.

Since enantiomers of Pro are not determinable using the HPLC method, and in order to confirm presence of D-AAs in plants by an independent analytical method, several plants have also been analysed by GC-MS using the chiral phase "Chirasil-Val". For detailed discussions of these approaches for the determination AA enantiomers we refer to previous papers (Brückner et al., 1995; Brückner and Schieber, 2000).

As free D-amino acids have been detected in all plants investigated, and taking the literature and our

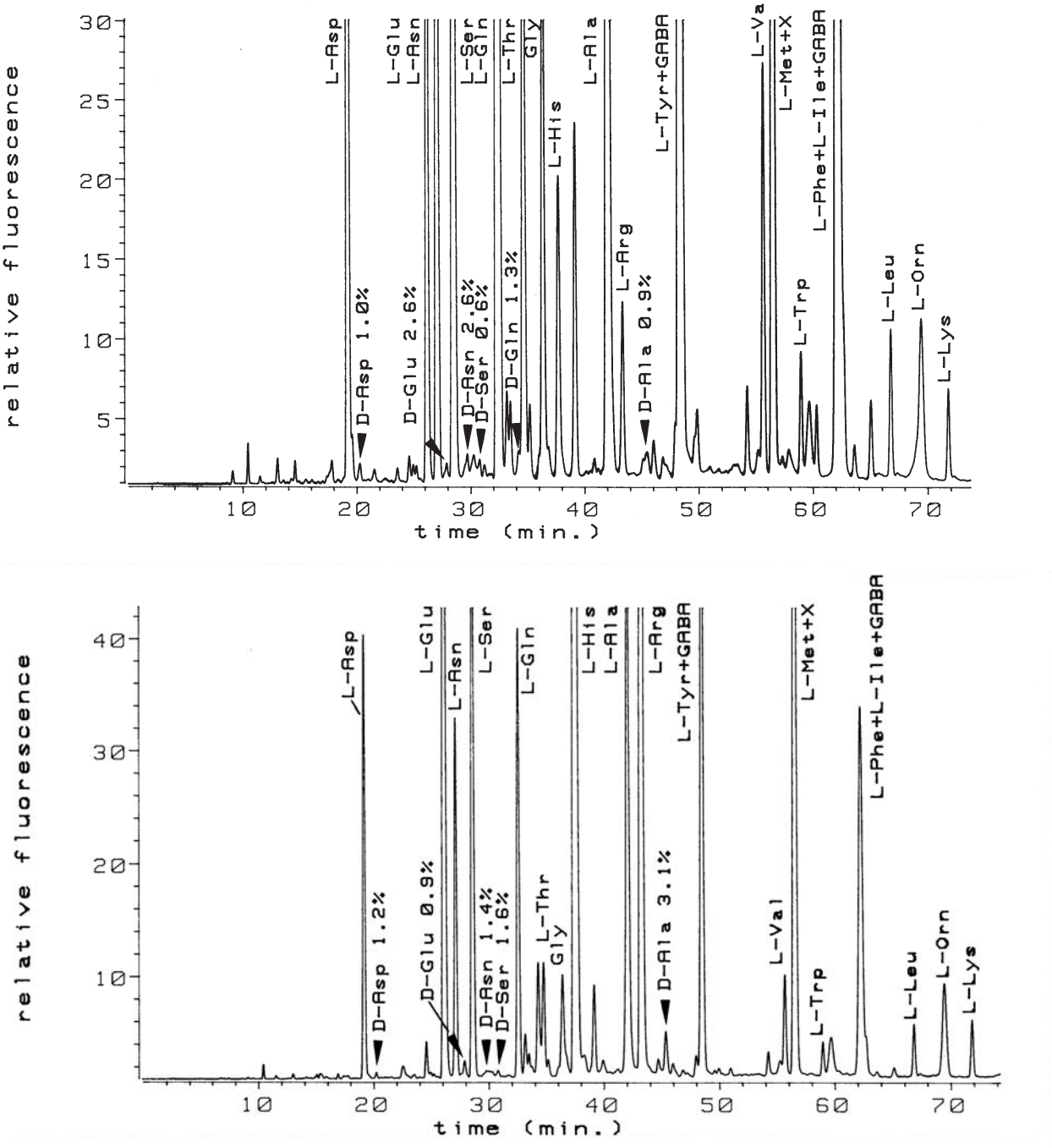

Fig. 4. HPLC of free amino acids derivatized with OPA/IBLC from leaves of (above) ginkgo tree, and (below) metasequoia tree

previous reports (Brückner and Westhauser, 1994) into account, it is reasonable to assume that D-AAs are common in plants in the free (subject of this report) as well as conjugated form (see literature cited).

As to the origin of D-AAs in higher plants it has been shown that plant amino acid racemases are capable of generating certain D-amino acids from L-amino acids (Ogawa et al., 1978). Further, aminotransferases, detected e.g. in germinating pea seedlings (*Pisum sativum*), are capable of transferring amino groups from various D-AA to pyruvate or 2-ketoglutarate with formation of D-Ala and D-Glu (Ogawa and Fukuda, 1973). Aliquots of D-Ala applied to rice sus-

Table 5. Relative amounts (%) of D-amino acids (D-AAs) in plants determined comparatively by GC-MS

D-AAs	Ginkgo	Meta-sequoia	Spruce tree	Maple tree	Cactus	Big Bent grass	Meadow Foxtail	Maize	Soy bean		Runner bean		Garden cress		Alfalfa		Water cress
									(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)
D-Asx	1.8	2.5	0.4	2.3	1.6	5.4	3.8	2.1	1.2	1.8	3.0	2.6	2.4	1.7	1.9	0.9	1.9
D-Glx	1.8	0.8	0.6	1.1	0.7	3.9	2.1	2.4	0.7	0.6	1.2	1.0	2.0	0.5	1.3	0.5	1.8
D-Ser	0.4	0.8	0.3	0.3	1.0	0.7	0.5	0.3	–	–	–	–	–	–	–	–	–
D-Ala	2.0	2.5	1.8	1.0	5.1	3.4	3.5	1.9	2.2	1.4	2.3	1.4	2.4	1.0	2.1	0.7	1.5
D-Lys	–	–	–	–	–	1.9	1.4	3.1	–	–	–	–	–	–	–	–	–
D-Pro	0.7	1.0	–	–	–	–	–	–	–	–	–	0.9	–	0.6	–	0.4	–

(a), seed; (b), seedlings; *Asx* = Asp + Asn; *Glx* = Glu + Gln; % *D* = 100 D/(D + L); –, not detected
For data (% D) of leaves of 'Golden Delicious' and soil of orchard see Fig. 3

pension cultures were converted to D-Asp and D-Glu in the cells of these plants (Manabe, 1984). From feeding experiments on plants it was confirmed that *N*-malonylation is common for all D-AAs, whereas γ-L-glutaminylation was assumed to be specific for D-Ala (Kawasaki et al., 1982). Notably, endogenous *N*-malonyl-D-Trp was reported to have been isolated from the apple "Golden Delicious" (Zenk and Scherf, 1964). However, the apparently established D-configuration of Trp in plant's endogenous *N*-malonyltryptophan became recently a matter of discussion (Markova and Gamburg, 1997; Rekoslavskaya et al., 1999). Conjugated D-AAs such as γ-L-Glu-D-Ala have been detected in garden pea (*Pisum sativum*) and lentil (*Lens culinaris*) (Fukuda et al., 1973; Rozan et al., 2000), D-Ala-Gly and D-Ala-D-Ala in wild species and cultivars of *Oryza* (rice) (Manabe, 1990), and 1-[(N-γ-L-glutamyl)amino]-D-proline in *Linum utisatissimum* (flaxseed) (Klostermann et al., 1967). Further, the tetrapeptide L-Val-γ-L-Glu-D-Arg-Gly was detected in ginseng (*Panax ginseng* C. A. Meyer) (Yagi et al., 1996). Thus enzymic *de novo* design of free and conjugated D-AAs in plants is established.

However, other pathways contributing to the pool of plant's D-AAs have also to be considered. Thus release from conjugated D-AAs as results of deacylation or peptide bond cleavage is to be expected. Further, nonenzymic formation of D-AAs in living organisms resulting from the reaction of L-amino acids and reactive carbonyl compounds has also to be considered (Brückner et al., 2001). Exogenous sources of D-AAs occurring in plants have also to be discussed as rationalized in the following. Legumes (soy bean, runner bean, and lucerne) as well as other host plants are associated with symbiotic root bacteria such as *Rhizobium* spp. and *Bradyrhizobium* spp. which are capable of converting nitrogen from the atmosphere to ammonia. Bacteria are abundant sources of peptidoglycan-bound as well as free D-amino acids (Schleifer and Kandler, 1972; Brückner et al., 1993). Further, mycorhizal associations between the roots of plants and fungi are common in plants. As D-AAs are readily taken up by plants, a contribution of microbial D-AAs to the pool of plant D-AA has also to be taken into consideration (Aldag et al., 1971). Indicatives are that certain D-AAs which have been detected in the fruits and leaves of the apple cultivar "Golden Delicious" have also been determined in the soil in which the tree grew.

Besides Gram-positive and Gram-negative bacteria, D-AAs in the free and conjugated form have also been detected in hyperthermophilic archaea of species of *Thermococcus* and *Pyrococcus* (Matsumoto et al., 1999), in eucaryotes such as in cyclopeptide toxins (microcystins) of blue-green algae (*Microcystis viridis*), in marine macroalgae, in particular brown algae of the genus *Phaeophyta* (Nagahisa et al., 1992), as well as ascomycetous fungi such as the yeast *Saccharomyces* (Brückner et al., 1995). As D-AAs occur in living organisms of the domains Bacteria, Archaea, and Eucarya (using the terminology proposed by Woese et al., 1990), there is much evidence that D-AAs fulfill specific roles in living organisms which are not covered by L-AAs and which in the cases of plants, in agreement with Gamburg and Rekoslavskaya (1992), still have to be established.

References

Aldag RW, Young JL, Yamamoto M (1971) An enzymic chromatographic procedure for the determination of D-amino acids in plant and soil extracts. Phytochemistry 10: 267–274

Brückner H, Lüpke M (1991) Determination of amino acid enantiomers in orange juices by chiral phase capillary gas chromatography. Chromatographia 31: 123–128

Brückner H, Schieber A (2000) Determination of free D-amino acids in mammalia by chiral gas chromatography – mass spectrometry. J High Resol Chromatogr 23: 576–582

Brückner H, Westhauser T (1994) Chromatographic determination of D-amino acids as native constitutents of vegetables and fruits. Chromatographia 39: 419–426

Brückner H, Jaek P, Langer M, Godel H (1992) Liquid chromatographic determination of D-amino acids in cheese and cow milk. Implication of starter cultures, amino acid racemases, and rumen microorganisms on formation, and nutritional considerations. Amino Acids 2: 271–284

Brückner H, Becker D, Lüpke M (1993) Chirality of amino acids of microorganisms used in food biotechnology. Chirality 5: 385–392

Brückner H, Langer M, Lüpke M, Westhauser T, Godel H (1995) Liquid chromatographic determination of amino acid enantiomers by derivatization with *o*-phthaldialdehyde and chiral thiols. Applications with reference to food science. J Chromatogr A 697: 229–245

Brückner H, Justus J, Kirschbaum J (2001) Saccharide induced racemization of amino acids in the course of the Maillard reaction. Amino Acids 21: 429–433

Davies JS (1977) Occurrence and biosynthesis of D-amino acids. In: Weinstein B (ed) Chemistry and biochemistry of amino acids, peptides and proteins, vol 4. Marcel Dekker Inc, New York Basel, pp 1–27

Erbe T, Brückner H (2000) Chromatographic determination of amino acid enantiomers in beers and raw materials used for their manufacture. J Chromatogr A 881: 81–91

Fitznar HP, Lobbes JM, Kattner G (1999) Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatization with *o*-phthaldialdehyde and *N*-isobutyrylcysteine in seawater and fossil samples (mollusks). J Chromatogr A 832: 123–132

Frahn JL, Illman RJ (1975) The occurrence of D-alanine and D-alanyl-D-alanine in *Phalaris tuberosa*. Phytochemistry 14: 1464–1465

Friedman M (1999) Chemistry, nutrition, and microbiology of D-amino acids. J Agric Food Chem 47: 3457–3479

Fukuda M, Tokumura A, Ogawa T (1973) D-alanine in germinating *Pisum sativum* seedlings. Phytochem 12: 2593–2595

Gamburg KZ, Rekoslavskaya NI (1992) Formation and functions of D-amino acids in plants. Soviet Plant Physiology 38: 904–912

Gandolfi I, Palla G, Marchelli A, Decennia A, Puelli S, Salvador C (1994) D-Alanine in fruit juices: a molecular marker of bacterial activity, heat treatments and shelf life. J Food Sci 59: 152–154

Kawasaki Y, Ogawa T, Sasaoka K (1982) Two pathways for formation of D-amino acid conjugates in pea seedlings. Agric Biol Chem 46: 1–5

Klosterman HJ, Lamoureux GL, Parsons JL (1967) Isolation, characterization, and synthesis of Linatine. A vitamin B_6 antagonist from faxseed (*Linum usitatissimum*). Biochemistry 6: 170–177

Kullman JP, Chen X, Armstrong DW (1999) Evaluation of the enantiomeric composition of amino acids in tobacco. Chirality 11: 669–673

Manabe H (1985) Occurrence of D-alanyl-D-alanine in *Oryza australiensis.* Agric Biol Chem 49: 1203–1204

Markova TA, Gamburg KZ (1997) Stereoconfiguration of endogenous *N*-malonyltryptophan in plants. Plant Sci 122: 119–124

Matsumoto M, Homma H, Long Z, Imai K, Iida T, Maruyama T, Aikawa Y, Endo I, Yohda M (1999) Occurrence of free D-amino acids and aspartate racemases in hyperthermophilic archae. J Bacteriol 181: 6560–6563

Nagahisa E, Kanno N, Sato M, Sato Y (1992) Occurrence of free D-aspartic acid in marine macroalgae. Biochem Int 28: 11–19

Ogawa T, Fukuda M (1973) Occurrence of D-amino acid aminotransferase in pea seedlings. Biochem Biophys Res Commun 52: 998–1002

Ogawa T, Bando N, Sasaoka K (1976) Occurrence of D-α-amino-*n*-butyric acid in legume seedlings. Agr Biol Chem 40: 1661–1662

Ogawa T, Kimoto M, Sasaoka K (1977) Identification of D-aspartic acid and D-glutamic acid in pea seedlings. Agric Biol Chem 41: 1811–1812

Ogawa T, Kawasaki Y, Saaoka K (1978) *De novo* synthesis of D-alanine in germinating *Pisum sativum* seedlings. Phytochemistry 17: 1275–1276

Rekoslavskaya NI, Yurjeva OV, Salyaev RK, Mapelli S, Kopytina TV (1999) D-Tryptophan as IAA source during wheat germination. Bulg J Plant Physiol 25: 39–49

Robinson T (1976) D-amino acids in higher plants. Life Sci 19: 1097–1102

Rozan P, Kuo YH, Lambein F (2001) Nonprotein amino acids in edible lentil and garden pea seedlings. Amino Acids 20: 319–324

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36: 407–477

Singh BK (ed) (1999) Plant amino acids: biochemistry and biotechnology. Marcel Dekker Inc, New York Basel

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87: 4576–4579

Yagi A, Ishizu T, Okamura N, Noguchi S, Itoh H (1995) Growth of cultured human bronchiogenic epithelioid CCD-14 Br cells and dermal fibroblasts, NB1 RGB treated with ginseng tetrapeptide and its isomer. Planta Med 62: 115–118

Zenk MH, Scherf H (1963) D-Tryptophan in höheren Pflanzen. Biochim Biophys Acta 71: 737–738

Zenk MH, Scherf H (1964) Verbreitung der D-Tryptophan-Konjugationsmechanismen im Pflanzenreich. Planta 62: 350–354

Authors' address: Prof. Dr. Hans Brückner, Interdisciplinary Research Center, Institute of Nutritional Science, Department of Food Sciences, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26, D-35392 Giessen, Germany, Fax: +49-641-9939149, E-mail: hans.brueckner@ernaehrung.uni-giessen.de