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Gas Chromatographic Determination of Amino Acid Enantiomers in Tobacco and bottled Wines

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Dedicated to my wife Nermeen for her constant encouragement to perform and complete this work, to my father and my mother with thanks for their support and last not least to my daughter Haidy and my son Abdullah with best wishes for a prosperous and peaceful future.

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List of Abbreviation

Fig	Figure
AcOH	Acetic acid
A_D	Peak area of D-enantiomer
A_L	Peak area of L-enantiomer
Ala	Alanine
allo-lle	*allo*-Isoleucine
Arg	Arginine
AAs	Amino acids
Asn	Asparagine
Asp	Aspartic acid
Asx	Asn + Asp
BHT	Di-*tert*-butyl-hydroxy-toluene
CLASS-5000	Chemical Laboratory Analysis System and Software QP-5000
Cys	Cystein
DAAO	D-amino acids oxidase
DCM	Dichloromethan
Dap	Diaminopimelic acid
Fru	Fructose
f_R	Response factors
GABA	γ-Aminobutyric acid
GC	Gas chromatography
GC/MS	Gas chromatograph / Mass spectrometry
Glc	Glucose
$G L^{-1}$	Gram / Liter
Gln	Glutamine
Glu	Glutamic acid
Glx	Gln + Glu
Gly	Glycine
His	Histidine
HPLC	High-performance liquid chromatograph
Ile	Isoleucin
IS	Internal standard

Leu	leucine
Lys	Lysine
MeOH	Methanol
Met	Methionine
MO	Microorganism
MS	Mass Spectrometry
MW	Molecular weight
m/z	Mass-to-charge ratio
Nle	Norleucine
n.d.	not detected or not determined/determinable
nos	Numbers
Orn	Ornithin
PFPAA	Pentafluoropropionic acid anhydride
Phe	Phenylalanine
Pro	Proline
PrpOH	Propanol
Ser	Serine
SIM	Selected Ion Monitoring
St	Standard
Tab	Table
Thr	Threonine
TIC	Total Ion chromatogram
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
V	Volume

1 Introduction

1.1 Chirality and Stereoisomers

Optically active molecules have an asymmetry such that they are not superimposable on their mirror image in the same way that a left hand is not superimposable on its mirror image, a right hand. This situation is characteristic of substances that contain tetrahedral carbon atoms that have four different substituents. The central atoms in such atomic constellations are known as asymmetric centers or chiral centers and are said to have the property of chirality (Greek: *cheir*, hand). Chirality describes the handedness of a molecule to rotate is observable by the ability of a molecule to rotate the plane of linear polarized light either to the right or the left.

1.1.1 Chiral Molecules

Isomerism involves compounds whose molecules differ in the way their atoms are arranged in three-dimensional spaces. Such isomers are referred to as stereoisomers. However, there are two kinds of stereoisomers. Enantiomers, which are compounds whose molecules are mirror images of each other and whose mirror images do not superimpose when are laid on top of one another. Enantiomers differ only in the way they affect plane-polarized light as it passes through the isomers. Diastereoisomers, have different physical properties as they are not mirror images of one another (Mortimer 1996; Mislow 1972).

Each isomer of the pair is capable of rotating plane-polarized light. One isomer rotates light to the right, and the other isomer of the pair rotates light to the left for the same number of degrees. All other physical properties are exactly the same. One enantiomer will be configured right-handed (*R*) and the other will be configured left-handed (*L*).

Briefly, we could say that enantiomers are stereoisomers that exhibit a property known as chirality. Chiral molecules will have their mirror images non-superimposible, no internal plane of symmetry and have one stereogenic center carbon.

1.1.2 Chirality of Amino Acids

Amino acids are the "building blocks" or sub-units of proteins. About 20 different kinds of amino acids are found in proteins. Each amino acid has two chemical groups, an amino group (-NH_2) and a carboxyl group (-COOH), which are attached to the same tetrahedral carbon atom. This carbon is the α-carbon. Distinct side-chains (R-group), that distinguish one amino acid from another, are also attached to the α-carbon (except glycine). The amino group of one amino acid can combine with the carboxyl group of a second amino acid to form a "peptide" bond, and its carboxyl group can combine with the amino group of a third amino group, and the chain can thus be extended almost indefinitely. Proteins contain from 50 to several hundred amino acids.

All of the amino acids which occur in proteins, except for achiral glycine, which is the simplest amino acid, have at least one asymmetric carbon atom, and can exist as one of two possible stereoisomers. A tetrahedral atom with distinct constituents is said to be chiral. The only amino acid which is not exhibiting chirality is glycine since its "R-group" is a hydrogen atom.

Chemical groups attached to this particular carbon atom are all different and can be arranged in space in two different ways. When there is only a single asymmetric carbon atom, these two different forms are known as *optical isomers*. The two forms are known as L-amino acids and D-amino acids. The chemical and physical properties of these enantiomers are identical, with the exception that they rotate plane-polarized light in equal but opposite directions. They are mirror images of each other and cannot be superimposed on the other, just as is true of left and right hands. It is now well known that the amino acids, which are present in the proteins of living organisms, consist almost entirely of the L-enantiomers.

1.1.3 Nomenclature of Stereoisomers

There are three commonly used systems of nomenclature whereby a particular stereoisomer of an optically active molecule can be classified.

1.1.3.1 An Operational Classification

Molecules are classified as dextrorotatory (Greek: *dextro*, right) or laevorotatory (Greek: *laevo*, left) depending on whether they rotate the plane of plane-polarized light clockwise or counterclockwise from the point of view of the observer. An instrument known as a polarimeter can determine this. A quantitative measurement of the optical activity of the molecule is known as its specific rotation.

Dextrorotatory and laevorotatory molecules are assigned positive and negative values of specific rotation. Dextrorotatory molecules are therefore designated by the prefix (+) and their laevorotatory enantiomers have the prefix (-). In an equivalent but archaic nomenclature, the lower case letters d (*dextro*) and l (*laevo*) are used (Voet and Voet, 1995).

The sign and magnitude of a molecule's specific rotation depend on the structure of the molecule in a complicated and poorly understood manner.

1.1.3.2 The Fischer Convention

In this system, the configuration of the groups about an asymmetric center is related to that of glyceraldehyde, a molecule with one asymmetric center. By a convention introduced by Emil Fischer, the (+)- and (-)-stereoisomers of glyceraldehyde are designated D-glyceraldehyde and L-glyceraldehyde, respectively (note the use of upper case letters). Fischer proposed a convenient shorthand notation for these molecules, known as Fischer projections. In the Fischer convention horizontal bonds extend above the plane of the paper and vertical bonds extend below the plane of the paper as is explicitly indicated by the accompanying geometrical formulas in Figure 1-1.

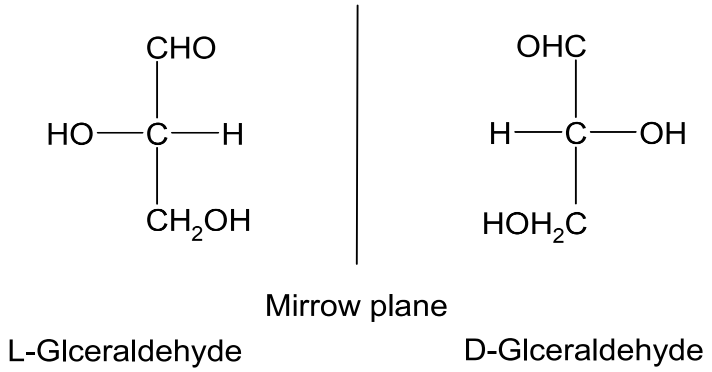


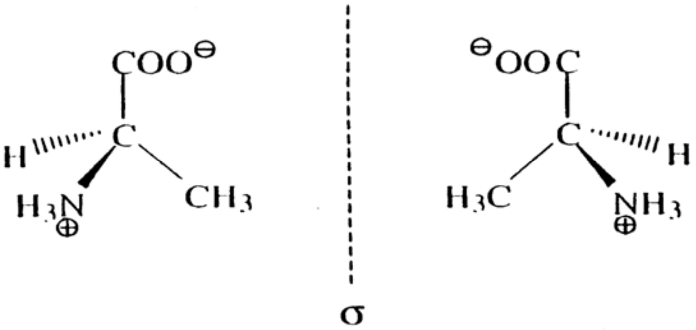
Figure 1-1 The Fischer convention for naming glyceraldehyde

For an amino acid the arrangement of the amino, carboxyl, R, and H groups around the C^{α} atom is related to that of the hydroxyl, aldehyde, CH_2OH, and H groups of glyceraldehydes, respectively. In this way, L-glyceraldehyde and L-amino acids are said to have the same relative configurations (Figure 1-1and 1-2). Through the use of this method, the configurations of the α-amino acids can be described without reference to their specific rotations (Mortimer 1996; Vollhardt 1995).

All α-amino acids derived from proteins have the L stereochemical configuration; that is, they all have the same relative configuration about their C-atoms. A molecule may have multiple asymmetric centers. For such molecules, the terms "stereoisomers"` and "optical isomers" refer to molecules with different configurations in at least one of their chiral centers, but otherwise are they identical. The term enantiomer still refers to a molecule that is the mirror image of the one under consideration, that is, different in all its chiral centers. Since each asymmetric center in a chiral molecule can have two possible configurations, a molecule with *n* chiral centers has 2^n different possible stereoisomers and 2^{n-1} enantiomeric pairs. Threonine and isoleucine each has two chiral centers and hence $2^2 = 4$ possible stereoisomers. The mirror images of the L-forms are the D- forms. Their other two optical isomers are said to be diastereomers (or *allo* forms) of the enantiomeric D- and L-forms. It could be noted that the D-*allo* and L-*allo* forms are mirror images of each other, as are the D and L forms. Neither *allo* form is symmetrically related to either of the D- or L- forms.

1.1.3.3 The Cahn-Ingold-Prelog or *(R,S)*-System

Despite its usefulness, the Fischer scheme is awkward and sometimes ambiguous for molecules with more than one asymmetric center. For this reason, the following absolute nomenclature scheme was formulated by Cahn et al. (1956). In this system the four groups surrounding a chiral center are ranked according to a specific although arbitrary priority scheme: atoms of higher atomic number bonded to a chiral center are ranked above those of lower atomic number. The order of priority of some common functional groups is SH > OH > NH_2 > COOH > CHO > CH_2OH > C_6H_5 > CH_3 > H. Note that each of the group's substituents to a chiral center must have a different priority rating; otherwise the center could not be asymmetric (Fig. 1-2) (Voet and Voet, 1995).



D-alanine
(*R*)-2-amino acid

L-alanine
(*S*)-2-amino acid

Mirror plane

Figure 1-2 Structural formula of L-D-alanine

To establish the configuration of the chiral center, it is viewed from the asymmetric center towards the lowest priority group. If the order of the groups as seen from this direction is clockwise, then the configuration of the asymmetric center is designated (*R*) (Latin: *rectus*, right). If the order of the group as is counterclockwise, the asymmetric center is designated (*S*) (Latin: *sinisterus*, left). L-Glyceraldehyde is therefore designated (*S*)-glyceraldehyde and similarly, L-alanine is (*S*)-alanine (Figure. 1-3). In fact, all the L-amino acids from proteins are (*S*) amino acids, with the exception of L-cysteine, which is (*R*)-cysteine. So, each enantiomeric pair would have the same name and then attach at the beginning of the name of the letter " *R*" or "*S*".

1.2 Occurrence of D-Amino Acids

1.2.1 D-Amino Acids in Microorganisms

The terrestrial organisms are grouped into three types, bacteria, yeast, and moulds. Bacteria are classified as Gram-positive or Gram-negative depending on whether or not they take up Gram stain. Gram-positive bacteria have a thick (~25 Å) cell wall surrounding their plasma membrane, whereas Gram-negative bacteria have a thin (~3Å) cell wall covered by a complex outer membrane (Voet and Voet, 1995).

It is well known that the cell walls of bacteria are structurally diverse, yet most of Gram-positive and Gram-negative bacteria species contain peptidoglycan, which consists of polysaccharides strands cross-linked through short peptides (oligopeptides). The numbers and types of amino acids that form these so-called stem peptides can vary, but it is commonly four, with alternating D-and L-isomers (Schleifer and Kandler, 1972). It could be detected that peptidoglycan of the cell walls of bacteria is composed of D-Ala, D-Asx (As = aspartic acid, asparagine or isoasparagin), and D-Glx (Glx = glutamic acid, glutamine or isoglutamine) as most frequent D-amino acid (Tipper and Wright, 1979). These D-amino acids are also those found in fermented foods and beverages. Other D-amino acids such as D-Lys, D-Orn, D-Ser, and D-Pro have are less frequently found in bacterial peptidoglycans. Further, the respective D-enantiomers of most protein L-AA have been found in the free state in cell pool of certain bacteria (Bhattacharyya and Banerjee, 1974). D-Ala occurs also in bacteria membrane teichoic acids (Lamert et al., 1977).

Besides Gram-positive and Gram-negative bacteria, D-amino acids in the free conjugated form have been detected in some species of moulds for the first time by Nagata et al., 1998, by which they established that D-Asp and D-Ser concentrations in some types of moulds (*P. Islandicum* and *H. salinarium*), were much higher than those of D-Ala and D-Glu which are generally found in high concentration in bacteria.

Furthermore, the results showed that the D-amino acid concentration was low in mould cells like those in yeasts and most bacteria (Nagata et al., 1998).

The cell walls of moulds do not contain peptidoglycan. Consequently, other moulds have also been thought to contain no D-amino acids (Nagata et al., 1998).

D-Amino acids have also been detected in various yeasts and yeast autolysates (Bhattacharyya and Banerjee 1969; Brückner et al., 1995a). Consequently, D-amino acids were detected in different beers (Brückner et al., 1995b) as well as in other microbial fermented foods. In Gram-positive bacteria usually 30-70% of the cell walls consist of peptidoglycan, whereas in Gram-negative bacteria the corresponding figure is less than 10% (Schleifer and Kandler, 1972). A high content of D-alanine (11.67%) and D-glutamine (22.32%) was found in Gram-positive bacteria, in compared to their relative amounts in Gram-negative bacteria (Nagata et al., 1998).

The muramic acid and diaminopimelic acid contents, which are specific components of the bacterial cell walls can vary with growth rate and physiological status of the cells (Tunlid and Odham, 1983).

The presence of free amino acids enantiomers in individual species of rumen bacteria was determined by GC-SIM-MS. The D-amino acids were predominately Ala, Asp, and Glu showing considerable variation between the species (Schieber et al., 1997). The factors which have major effects on cell pool amino acids composition of bacterial cells are physical condition of culture, medium composition, presences of metabolic inhibitors, and age of culture (Holden, 1962). Therefore, the concentrations of D-amino acids in cell pool of microorganisms varied due to differences in strains, cultur, and experimental techniques applied (Bhattacharyya and Banerjee1974; Brückner et al., 1992).

Bacteria from the oral and intestinal flora and rumen microorganisms are also potential sources of D-amino acids present in body fluids and tissues (Brückner et al., 1992). It is known that free D-amino acids in fermented food originate from microorganisms and are released as results of microbial metabolism, autolysis of cell walls, and action of microbial racemases (Johnston and Diven, 1969). This might also explain that samples of raw milk contained relative amounts of 1.9% D-Ala, 7.3% D-Asp, and 4.8%D-Glu (Brückner and Hausch, 1989b).

1.2.2 D-Amino Acids in Plants

It is well known that plants contain free L-amino acids. It was also realized that several D-amino acids occur in higher plants (Robinson, 1976). Notably, most research on D-amino acids in plants focused on the metabolization and conjugation of selected D-enantiomers administered to plants under experimental conditions. It was assumed that D-amino acids are common in plants in the free state as well as the conjugated form. Zenk and Scherf (1963) recognized that *N*-acylation of D-amino acids, in particular *N*-malonylation of D-Trp, is common in mono- and dicotyledonous plants. 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). Recently Erbe and Brückner (2000a) pointed out that barley lings (*Hordeum sativum*) and hops blossoms (Humulus *lupulus*) also contained the same D-amino acids. Free D-amino acids were detected in gymnosperms as well as mono-and dicotyledonous angiosperms of major plant families. They were considered to be principle constituents of plants although in the low percentage range (Brückner and Westhauser, 2003). On the other hand, D-Ala is the most widely reported D-amino acids occurring in plants and is usually presented in the form of peptides. Frahn and Illman (1975) found D-Ala as well as D-Ala-D-Ala in pasture grass (*Phalaris tuberosa L.*), and (Manabe, 1985) in wild rice (*Oryzae australiensis* Domin.). Various amounts of free D-amino acid were found in cured tobacco leaves (Kullman et al., 1999) and conjugated D-amino acids such as γ-L-Glu-D-Ala have been detected in garden pea (*Pisum sativum L.*) and lentil (*Lens culinaris L.*) (Fukuda et al., 1973; Rozan et al., 2000) and D-Ala-D-Ala was isolated from tobacco leaves (Noma et al., 1973). D-alanylglycine, which is a normal constituent of leaf blades of rice plants grown on fields, was found in low concentrations in very young seedlings grown under the light or dark region and quantities seemed to increase with age (Manabe et al., 1981). The relative concentration of three dipeptides (D-Ala-Gly, D-Ala-D-Ala, and D-Ala-D-Ala) produced in rice plants (genus *Oryzae*) appear to be related to the nature of the rice strain (Manabe, 1992). The determination of D-amino acids in plants proved problematic in past because the separation of amino acids enantiomers has been technically difficult. Furthermore, D-amino acids in plants occurring in small amounts handly be distinguished from the prevalent L-isomers. However, advanced gas chromatographic and high-performance liquid chromatographic techniques together with chiral columns can separate D- and L- enantiomers of amino acids. The amounts of D-amino acids in unprocessed vegetables and fruits are in the range of about 0.5 - 3% relative to their L-enantiomers (Brückner and Westhauser, 1994). It was demonstrated that fruits (apples, grapes, oranges) and vegetables (cabbage, carrots, garlic, tomatoes) as well as the corresponding juices contain variable amounts of D-amino acids including D-Ala, D-Arg, D-Asp, and D-Glu (Brückner and Westhauser, 1994; Gandolfi et al., 1994). Microbial contamination or controlled microbial fermentation of edible plants or plant juices has been demonstrated to increase amounts and kinds of D-amino acids (Brückner, et al., 1995a). These amino acids could originate from plant sources, soil and microorganisms, and / or result from heat treatment used to pasteurize juices. Consequently, it has been suggested that quantities of certain D-amino acids in processed fruit juices exceeding significantly the natural level may serve as molecular markers for bacterial contamination (Brückner, and Lüpke, 1991; Gandolfi et al., 1992, 1994). Recently Brückner and Westhauser (2003) analyzed the D-amino acids in plants (leaves of coniferous and deciduous trees, fleshy fruits, leaf blades of fodder grasses, seeds and seedlings of edible legumes. They determined free D-amino acids in the range of about 0.2% (up to 8%) relative to the corresponding L-AA in plants. Notably, D-Asp, D-Glu, and D-Ala could be detected in all plants, D-Asn, D-Gln, D-Ser were found in many plants, and D-Ser in few plants. D-Val and D-Leu were found only in coconut milk. For detailed discussion of the origin of D-amino acids in plants we refer to the references cited above.

1.2.3 D-Amino Acids in Human Beings

Many studies have been performed from the 1980 till now to demonstrate the presence of D-amino acids in mammalian tissues (animal, human). Especially, D-Ser and D-Asp have been well investigated. The amounts of other D-amino acids in mammalian tissues are low in most cases and have been determined using sensitive and selective methods such as column switching HPLC. Many factors can affect the relative level of D-amino acid excretion in physiological fluids, including: diet, age, pregnancy, advanced cancer, and antibiotics (Armstrong et al., 1993a). The data showed that excreted D-amino acids ranged from the low percent levels to over 40 percent of the total specific amino acids level. It is also obvious that the relative amounts of D-enantiomers in blood serum are much lower as compared to urine samples. In general, the relative plasma levels of D-amino acids were about ten times lower than those detectable in urine samples (Armstrong et al., 1993b).

1.2.3.1 Distribution of D-Aspartic Acid

A number of investigations have been performed on the occurrence D-Asp in human tissue. It was demonstrated that D-aspartic acid has been found in various regions of the human brain frontal cortex (Neidle and Dunlop, 1990; Hashimoto et al., 1993a,b; Fisher et al., 1994).

It was recognized that the amounts of D-Asp in brain tissues increase after birth and reach their maximum values in cooperation with the tissue maturation, and to decrease with ageing.

D-Asp acid was found in human protein such as teeth (Helfman and Bada, 1975; Master 1983), dentine (Helfman and Bada, 1976), white matter (Man et al., 1983), myelin basic protein (Fisher et al., 1986a), aorta (Powell et al., 1992), bone (Ohtani et al., 1998) and human eye lenses (Fuji et al., 1994a). The localization of D-Asp in the lens and the mechanism of D-Asp formation have been investigated in detail (Fuji, 2002). D-Asp has been shown to accumulate with age in human lens. In general, the level of D-Asp increased with aging (Fisher et al., 1994). The proportion of the D-Asp, at the age of 20-45, 3-4% in the human brain, in contrast, is nearly twice as much as in brains of children up to 12 month (Man and Bada, 1987). The physiological function of D-Asp in human body was reported to regulate the hormonal release in endocrine glands such as testis, pituitary and pineal (D'Aniello et al., 2000; Wang et al., 2000).

1.2.3.2 Distribution of D-Serine

Many researchers have been interested in the presence of D-Ser in the mammalian brain. D-Ser has been reported to occur in the human prefrontal brain areas (such as the cerebrum, hippocampus, and hypothalamus), temporal cortex (cerebellum, medulla oblongata) (Morikawa et al., 2001; Hamase et al., 1997) and continues to be present throughout life. The concentration of D-Ser in temporal cortex areas was found to be low. In contrast, a large amount of D-Ser is present in the frontal brain areas. The distribution pattern of D-Ser in the brain is identical to the position of *N*-methyl-D-asparate (NMDA) receptor (Hashimoto et al., 1993a). The affinity to the strychnine insensitive glycine binding site of the NMDA receptor was the same as that of Gly (Danysz et al., 1990), suggesting the role of D-Ser as an intrinsic ligand of the receptor (allosteric effect) (Hashimoto et al., 1993a, 1993b).

Brückner and Hausch (1993) demonstrated that the relative amount of D-Ser in human serum is well correlated to that of blood urea nitrogen and creatinine, and that D-Ser was excreted in urine (Brückner et al., 1994; van de Merbel et al., 1995).

Enantiomers of 14 amino acids were separated and quantified in human urine and blood serum using Chirasil®-L-Val capillary column together with mass spectrometric detection in the selected ion monitoring mode. The authors demonstrated that D-Ala and D-Ser are most abundant both in urine and blood sera (Brückner and Schieber, 2001).

D-Ser is thought to have an important function role in the central nervous system of mammals to modulate NMDA subtype of the glutamate receptor, large numbers of reviews have been published on this topic (Hashimoto et al., 1993a,b; Schell et al., 1997, 1995; Mothet et al., 2000).

1.2.3.3 Distribution of other D-Amino Acids

The presence of D-amino acids other than D-Asp and D-Ser has been also demonstrated in vertebrate tissues and body fluids. Fisher et al. (1991) determined D-Ala in human brain. Until now, the amounts of D-Ala have been determined in various brains regions (Morikawa et al., 2001; Nagata et al., 1992), peripheral tissues (Nagata et al., 1994; Hamase et al., 1997), and physiological fluids (Brückner and Schieber 2001; Brückner et al., 1994).

Urinary excretion of large relative and absolute amounts of D-Ala and D-Ser were observed (Brückner and Schieber 2001). It was also demonstrated that the relative amounts of D-enantiomers were much lower in blood serum as compared to urine sample. The concentration of D-Ala in blood serum was correlated positively to serum creatinine (Morikawa et al., 2001). Trace amounts of the D-enantiomers of hydrophobic amino acids (D-Trp, D-Tyr, D-Phe, and D-Leu) were found and D-Ser and D-Ala have been determined in human urine using combined reversed-phase and chiral HPLC (Armstrong et al., 1993b, Hamase et al., 2001). The results obtained by GC-MS and HPLC showed that high amounts D-Ser, D-Ala, D-Thr, and D-Phe were found as the most abundant D-amino acids accompanied by lower amounts of other D-AA excreted in random urine samples of healthy volunteers, (Brückner et al., 1994). Occurrence of other D-amino acids such as D-Glu, D-Gln, D-Tyr, D-Trp, D-Lys, and D-Val had also been reported in previous studies but in small relative amounts.

Using GC together with Chirasil®-L-Val and a flame ionization detector was shown to be highly suitable for the resolution and determination of the relative amounts of AA enantiomers in human blood serum (Brückner and Hausch, 1993). The presence of high relative amounts of various D-amino acids in the blood serum of patients suffering from renal diseases was demonstrated. It appears that a positive correlation exists between the amounts of D-Asx and D-Ser and the serum creatinine level in patients with renal malfunction. Furthermore, it has been demonstrated that relative amounts of some D-amino acids (in particular D-Ala, D-Ser) in serum of patients with renal disorders are higher than those of normal persons (Nagata et al., 1987, 1992; Brückner and Hausch, 1993; Young et al., 1994). Also the alteration of the concentration D-amino acids with aging and renal diseases were show and it was found that the D-amino acids levels in the plasma of elderly people (age 76 years) are higher than those in younger people (age 42 years) (Nagata et al., 1987).

Fisher et al. (1991) first reported that free D-Asp and D-Ala were found in normal and Alzheimer human brain, and that free D-Asp amounts in the white matter of Alzheimer brains are found in less than 50% of normal brains, and absolute quantities on average was 10.5 nmol/g for Alzheimer brains and 22.46 nmol/g for normal brains.

1.2.4 D-Amino Acids in Animals

In his classical study Hoeprich (1965) was the first to provide convincing proof of the existence of free D-Ala in the blood serum of mice and guinea pigs. Since he could not detect D-Ala in the pooled blood sera of germ-free animals (guinea pigs and mice), he supposed already that the enteric microflora is the source of the D-amino acids in rodents (mice and rats). On the other hand Hoeprich and coworkers, under the same experimental conditions, did not detect D-Ala in human blood serum, although there are convincing experimental proofs that D-Ala, among other D-amino acids, occurs in human blood sera (Nagata et al., 1987; Brückner and Hausch, 1993).

Free D-Ala is one of the D-amino acids naturally occurring in mammalian tissues and has been found in the brain (Morikawa et al., 2001), liver and kidney (Nagata et al., 1994), blood and urine (Brückner and Schieber, 2001; Brückner et al., 1994; Morikawa et al., 2001). Recently, Morikawa et al. (2003) have investigated the distribution of free D-Ala in the rat central nervous system. The highest amount has been observed in the anterior pituitary gland (86.4 nmol/g wet tissue), and the second highest amount has been detected in the pancreas (29.2 nmol/g wet tissue).

D-Ser may modulate brain function by serving as ligand for the NMDA receptor in the forebrain. High concentrations of D-Ser are present in the forebrains of mice, rats, and bulls (Armstrong et al., 1993a; Nagata et al., 1992, 1995). It was concluded that the amount of D-Ser in food proteins that have not been exposed to alkaline conditions is too low to account for the relatively high amounts detectable in the brain.

Brückner and Schieber (2001) determined free D-amino acids in the blood serum, urine and aqueous ethanolic extracts of faeces of germ-free laboratory rats and in a rat made gnotobiotic by inoculation with three species of bacteria (*Streptococcus, Lactobacillus and Clostridium*). It was found that germ-free rats contain significant amounts of free D-amino acids in the sera and relatively large amounts in their urines and faeces. Quantities of D-Ala in sera of germ-free rats are lower in comparison to the normal rats investigated by Brückner and Schieber (2001). Since intestinal microorganisms are excluded in germ-free rats, endogenous mechanisms or intake with feed (or both together) have to be considered as sources for D-amino acids.

Nagata and Akino (1990) reported that there is no difference in the neutral free D-amino acid contents in germ-free and SPF mice and that, consequently, the majority of D-amino acids in tissues are not of microbial origin. In contrast, Konno et al. (1993) investigated conventional germ-free and gnotobiotic mice of a mutant strain lacking D-amino acid oxidase (DAO^-). They found that the urine of DAO^- mice contained 13 times more D-Ala than that of DAO^+ mice. It was concluded that most of the urinary D-Ala of conventionally reared mice of this strain is of gastrointestinal bacterial origin.

Free D-Asp was detected in the blood, brain, and certain tissues of rats and mice. Subsequently, several groups have also reported about high concentrations of free D-Asp in the cortex and retina of chickens, rats, and humans during an early development stage (Dunlop et al., 1986; Hashimoto et al., 1993b, 1995; Neidle and Dunlop, 1990). Surprisingly, the free D-Asp content rapidly decreased until it was present only in trace amounts in adult tissue. The level of free D-Asp was highest in the darnel, testis, spleen and pituitary, followed by the thymus, lung, ovary, pancreases and heart, and below the detection limit in the kidney, liver, brain, muscle and serum (Hashimoto et al., 1993a). These date prove that this D-amino acid might be an endogenous substrata for D-aspartate oxidase.

Hamase et al. (2001) reported that the relative small amounts of D-Pro and D-Leu in seven brain regions of mice, showing a higher concentration in the pineal gland and pituitary gland. Urinary excretion of D-Phe has been also reported (Brückner and Schieber, 2001, 2002; Pätzold et al. 2005, Armstrong et al., 1993a).

In terrestrial animals, free D-Asp has been found in nervous tissues of chicks (Neidle and Dunlop, 1990), rodents (Dunlop et al., 1986), rats (Hashimoto et al., 1993a,b). It has been demonstrated that during the fetal life of the rat D-Asp occurs in large amounts in the central nervous system. In adult animals it nearly disappears from brain but increases in endocrine glands, especially in the pituitary and testes. Recently, it was found that D-Asp is present not only in the brain, but also in the sexual glands such as ovaries and testes (D'Aniello et al., 1996). These results led to hypothesize that endogenous D-Asp plays both a nervous and endocrine role.

Insects are also reported to contain D-amino acids (Anand and Anand, 1994; Corrigan and Srinivasan, 1966). Insects can therefore serve as another source of D-amino acids for animals and humans. D-amino acid may play a role in animal metabolism and cell differentiation in insects. D-Serine is present in the body fluid of silkworms and in lombricine of the earthworm, and D-cysteine is a component of luciferine of the glowworm. These early results have been summarized in the review by Corrigan (1969). However, dietary D-Ala and D-Ser elicited adverse nutritional effects in insects and silkworms (Ito and Inokuchi, 1981).

Although it was demonstrated many years ago that D-amino acids are to be found in the cell wall of unicellular organisms such as bacteria and yeast, the widespread occurrence of peptides containing D-amino acids in multicultural organisms has not been readily accepted. However, in recent years, small peptides containing one D-amino acid have been found in both vertebrates and invertebrates (Fuji, 2002). Dermorphin is the first example of a D-amino acid-containing peptide in vertebrates. It was isolated from the skin of a frog (*Phyllomedusinae* from South and Middle America) and is an opioid peptide with the sequence Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (Montecucchi et al., 1981). Another two D-amino acid-containing peptides were isolated from the same skin of frog. The first peptide was *deltorphine I*, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (Mor et al., 1989) and the second peptide was *deltorphine II*, Tyr-D-Ala-Phe-Glu-Val-Gly-NH_2. In addition, these peptides have only been detected in the skin secretions of *Phyllomedusinae* from South and Middle America but not in other frogs. The small peptides which contain one D-amino acid at position 2 in animal cells are discussed in a recent review (Kreil, 1994).

Stereoisomers of diaminopimelic acid (Dap) serving as bacterial markers, were also detected in the urine of bovine and sheep (Schieber et al., 1999; Brückner and Schieber, 2000); relative amounts of 86.7% *meso*-Dap, 7.2% LL-Dpm, and 6.2% DD-Dap were determined. Although *meso*-Dap is most abundant in the peptidoglycan of bacteria it is occasionally replaced with LL-Dap or, rarely, DD-Dap (Schleifer and

Kandler, 1972). Stereoisomers of Dap were also detected in the urine of pig. This is of interest since pigs are monogastric animals in contrast to the ruminants bovine and sheep. These findings support the hypothesis that parts of the D-amino acids detectable in Mammalia are of microbial origin (Hoeprich 1965; Konno et al., 1993). The mechanisms leading to the formation of D-amino acids, which are not related to feed or micro organisms, have also to be considered as demonstrated with the occurrence of D-Ser in the brain of germ-free as well as normal rats (Brückner and Schieber, 2001). It was recently reported that a serine racemase occurs in the glial cells of rat brain (Wolosker et al., 1999). This also demonstrated the presence of the endogenous amino acids racemase in mammals.

1.2.5 D-Amino Acids in Foodstuff

Amino acids in proteins are generally assumed to occur as L-enantiomers. However certain mirror images (enantiomers, or epimers if several chiral centers are concerned) of the protein L-amino acids, named D-amino acids, do also occur in foods. Conversion of L-amino acids (L-AA) into the respective D-enantiomers (D-AA) in food protein might occur in food processing such as heating and roasting, especially under alkaline or acidic conditions; this would drastically alter the enzymic degradation in the digestive tract and lead to a significant loss of nutritional value.

A variety of evidence studies have demonstrated that there are two main sources of D-amino acid enrichment in diverse food. First, racemization by special treatment during food processing and, second, D-amino acid formation due to bacterial activity during fermentation processes (Friedman, 1999). As a result of exposure of food proteins to heat and or strongly alkaline or acid condition will lead to two major chemical changes: considerable D-amino acids formation of food proteins (racemization or epimerization), and concurrent formation of cross-linked amino acid. The possible consequence of amino acid racemization has been reviewed in detail (Friedman, 1999).

Such processing conditions are widespread in the commercial isolation and concentration of proteins. Extrusion of plant and milk proteins (used for example in coffee creams, infant formula, sausages), peeling (fruit and vegetables), baking (sourdough, toast), deboning meat, and of liquid spices, soy paste, and soy sauce are carried out with the help of alkaline solutions (Zagon et al., 1994).

A variety of studies in the last decades have analyzed the presence of D-amino acids in many processed foods. From these studies it is assumed that D-amino acids are quite common especially in dairy products, fermented beverages and other products matured by the activity of bacteria. Raw milk is usually contaminated with microorganisms, such as anaerobic bacteria of the genera *Bacterioides*, *Ruminococcus* and *Butyrivibrio* (Brückner et al., 1992). It is also reasonable to assume that the low but significant amounts of free D-amino acids found in milk are the result of the digestion and autolysis of rumen bacteria.

The use of various microorganisms to ferment milk into a variety of dairy products significantly enhances their D-amino acid content and results in the appearance of additional D-amino acids (Palla et al., 1989). For example, relative high proportions of D-amino acid are contained in some dairy products and beer (Brückner and Hausch 1989a,b, 1990). High relatively concentrations of D-Ala, D-Asp, D-Glu, D-Val, D-Leu, D-Ile, and D-Ser were present in yogurt, kefir and curdled milk. These D-amino acids were also present in widely consumed ripened cheeses such as Gouda, Emmentaler, and Parmesan. The difference may arise from the use of different microorganisms in the fermentation process (Brückner et al., 1992, 1993). In addition to bacterial cell walls, D-amino acid transaminases liberated by lyses of cells may also contribute to L-amino acid conversion. This could be of importance in dairy products (cheese and yogurt) as well as in fermented food and beverages in general. Use of lactic acid bacteria and yeast in the fermentation of sourdough before baking results in the introduction of D-Ala and D-Glu into the dough (Gobetti et al., 1994). Baking of the dough into bread induces a 44% decrease in the total free D-amino acids content.

Brückner and Hausch (1989a,b, 1990) have concluded that all fermented foods contain significant amounts of D-amino acids and that the absolute amount is strongly dependent upon the type of fermented foods. In most food D-Ala, D-Asp, D-Gal are the most abundant D-amino acids. In lactic fermented juice of carrots, D-Ala, D-Asx, D-Glx are found, but also relative high amounts of D-*allo*-Ile, D-Leu, and D-Met as well as D-Val and D-Phe are detected. In comparison, in cabbage juice mainly D-Ala is found and minor amounts of D-Asx and D-Glx (Brückner and Hausch, 1989a,b). From the results it was concluded that D-amino acids are common in fermented foods and originate from microorganisms which are added as starter cultures in food processing or which are involved in traditional fermentation processes. This is supported by the fact that D-Ala, D-Asp, and D-Glu, in particular, which occur in all fermented foods, are among the constituents of the peptide moieties of the peptidoglycan from the bacterial cell walls (Schleifer and Kandler, 1972). It is also well known that bacterial AA racemazes and epimerazes are involved in the formation of D-amino acids in fermented foods (Adams, 1972). The ubiquitous presence of free D-amino acids in fermented foods has to be taken into account when the nutritional, sensorial, and physiological aspects of fermented foods are discussed. The presence of D-amino acids other than D-Ala, D-Asp, and D-Glu in juices, detected by chiral GC (Ooghe et al., 1984) and by HPLC with chiral reagents may be an indicator of adulteration fortification of food.

Abundant D-amino acids have been also detected in diverse raw and processed foods, such as semi- hard chess (Innocente and Palla, 1999), fish sauces and other fermented fish products (Abe et al., 1999), sourdough (Gobbetti et al., 1994), coca powder, tee leaves, and soy sauces (Brückner and Hausch, 1989a, b).

The enantiomer purity profiles of AA can provide important information on the adulteration and quality of orange juices. Thus, it has been shown that high quality orange juices contain L-amino acids exclusively (Gandolfi et al., 1994), whereas juices of inferior quality also contain some D-amino acids (especially D-Ala, D-Asp and D-Glu) (Robards and Antolovich, 1995). Similar, detection of D-amino acids in orange juices can be an indicative of microbial spoilage or related to juices of unsatisfactory quality (Brückner and Lüpke, 1991). On the other hand, Simo et al. (2002) confirmed that D-amino acids detected in fresh orange juice are naturally occurring and are not due to microbial spoilage or storage (Gandolfi et al., 1992). In addition, D-Ala could be used as a possible molecular marker of thermal treatment of orange concentrates (Simo et al., 2002; Del Castillo, 1999) because the amounts of D-Ala seem to correlate with the intensity of the thermal treatment (pasteurization at 92°C for 1 min). The presence of D-Ala at a percentage greater than 4% in milk can be considered as an indicator of milk contamination, and, therefore, can be used for monitoring the shelf life of a product (Brückner et al., 1992). Also the evolution of free D-amino acids in wines of a Portuguese *Vitis vinifera* variety could be used as markers for the biotechnological process used in vinification and the age of the wine (Chaves das Neves et al., 1995). Furthermore, Pawlowska and Armstrong (1994) have shown that the enantiomeric ratio of Leu and Pro could serve as indicators of age, processing, and storage histories of honeys. In addition, D-amino acids in cheeses are considered as molecular markers of aging and quality of ripening.

A notable finding was that in all fermented malt beverages (beers) and table wines or fortified wines, Pro is the most abundant amino acid. Since this AA tends to have the highest absolute concentration, the presence of D-Pro was used as tool for age dating of different Italian wines (Calabrese and Stancher, 1999) or as proof for the maturation process of balsamic vinegar, a special Italian vinegar (Erbe and Brückner, 1998). Indeed, the pattern and enantiomeric distribution of amino acids can be used as fingerprint for the origin of raw materials used in many products, kind of food processing, the country of origin or regional characterization.

Enantioselective analysis of amino acids is also a new and effective tool for assessing the quality of unfermented foods such as fruit juices and honeys (Pätzold and Brückner, 2002). Roasted coffee contained 10-40% of D-Asp, D-Glu, and D-Phe (Palla et al., 1989), and D-Ala, D-Glu, and D-Asp have been reported as widespread in cheese, yogurt, table wine and fortified wine, tobacco products and vinegars.

1.3 Mechanisms of the Formation of free D-Amino Acids

Alkali and heat treatments have been known for fifty years to racemize amino acids (Cavallini et al., 1958). As a result of food processing using these treatments D-amino acids are continuously consumed by animals and man. All amino acids residues in proteins undergo simultaneous racemization at differing rates (Friedman and Master, 1982). The process, in which the conversion of free or protein or peptide-bound physiological L-amino acid into their mirror images (enantiomers) takes place, is commonly referred to as racemization. Mechanisms of the formation of free amino acids will be briefly discussed below.

1.3.1 Chemical Racemization of free Amino Acids

1.3.1.1 Base-catalyzed Racemization

Base-catalyzed racemization of an amino acids is governed by removal of a proton from the asymmetric C-H bond of an amino acid, or amino acid reside in a peptide or protein, to form a negatively charged planar carbanion intermediate (see Figure 1-3). This carbanion can then be reprotonated on either side with a proton from the solvent to regenerate the original amino acid, which is now racemic (DL) (Friedman 1999; Liardon and Hurrell 1983). The reaction is written as:

$$\text{L-Amino Acid} \quad \underset{K'}{\overset{K}{\rightleftarrows}} \quad \text{D-Amino Acid}$$

Where k and k' are the first-order rate constants for the forward and reverse racemization rates, respectively. The product is racemic if recombination can take place equally well on either side of the carbanion, given an equimolar mixture of the L-and D-isomers. At the equilibrium point, the extent of inversion of L- to D-enantiomer equals the corresponding inversion of D- to L-enantiomer.
Racemization follows the rate expression:

$$\ln \frac{\{1+D/L\}}{\{1+D/L\}} = 2k_i t$$

The rate of racemization (2 k_i) depends on whether the amino acids are free or peptide bound and is also a function of temperature and pH. The extent of racemization is usually calculated as % D = (D/ D+L) x 100 and/or the absolute concentration of the D-amino acid in the sample usually in milligrams per gram. Base-catalyzed racemization of amino acids in alkali-treated casein, lactalbumin, soy protein and wheat gluten were investigated by Master and Friedman (1979) and Liardon and Hurrell (1983). The structural and electronic factors are specific for each amino acid and facilitate the formation and stabilization of the carbanion intermediate, (Fig. 1-3).

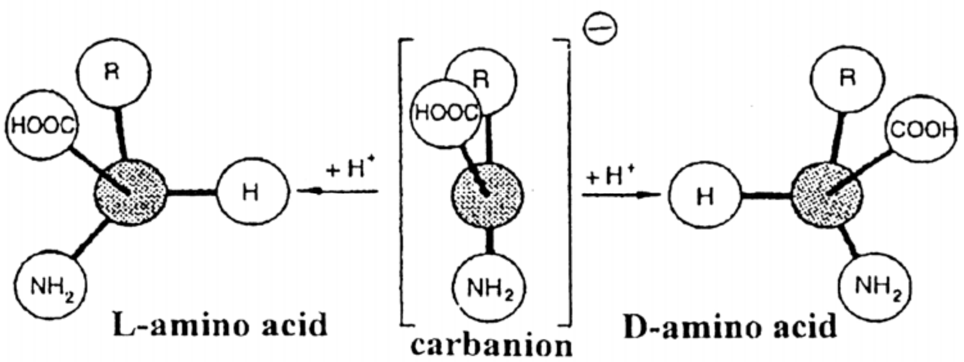


Figure 1-3 Mechanisms of racemization of an amino acid (modified from Bada, 1982)

Consequently the reaction rate for the isomerization of each amino acid is also unique (Friedman and Master, 1982). It has been established that the racemization of amino acids in food proteins increases with increasing pH, time, and temperature (Friedman and Liardon, 1985). At the initial stages of alkaline treatment, a higher degree of racemization resulted from higher protein denaturation rates (Friedman and

Master, 1982). Furthermore, racemization of free amino acids has been observed to be less significant than of amino acid residues in proteins.

1.3.1.2 Acid-catalyzed Racemization

Acid-catalyzed racemization of an amino acid involves protonation of the carboxyl group of the L-amino acid to form dehydroalanine. The latter then regenerates an equimolar mixture of D- and L-amino acid isomers by the addition of a proton to two sides (*R* and *S* faces) of the double bond (Liardon et al., 1991) (Figure 1-4). Acid-catalyzed racemization occurs at a much slower rate than the base-catalyzed process.

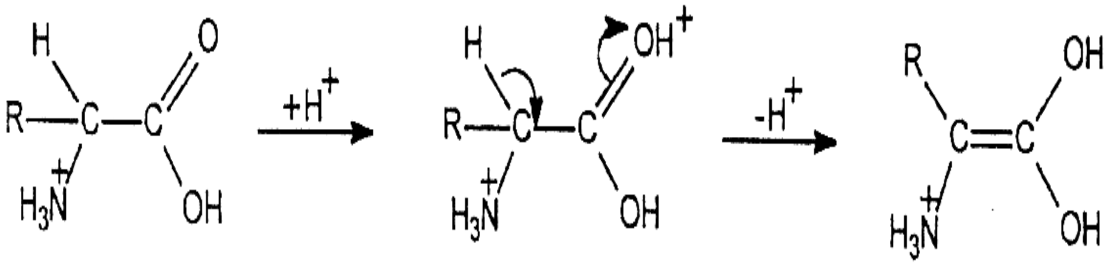


Figure 1-4 Acid-catalyzed racemization (modified from Friedman, 1999)

The maximum rate of racemization of Asp at pH 2.5 was explained by the formation of a planar intermediate which is stabilized by an intramolecular hydrogen bond between β-carboxylate group resulting in a seven-membered ring system (Bada, 1982). For Ser, a similar intermediate was postulated via formation of a six-membered cycle between the hydrogen atom of the hydroxyl group and the negative charged oxygen atom of the carboxylate group. Therefore, acid catalyzed racemization of Asp and, to a lower extent, Ser might contribute to the amounts of D-amino acids detected in acidic fermented foods.

1.3.2 Enzymic-catalyzed Racemization

The formation of free D-amino acids in bacteria cytoplasm can be attributed to the action of bacterial enzymes, in particular racemases and epimerase, which have been shown to convert almost all physiological L-amino acids into their D-amino acids (Adams, 1972). It assumed that microbial enzymes, in particular racemases and epimerases, are responsible for the formation of D-amino acids in fermented foods (Brückner et al., 1992; Brückner and Hausch, 1990). In a previous study Brückner et al. (1992) showed, by using chiral liquid chromatography, that significant amounts of D-Ala, D-Asp and D-Glu were present in rumen microorganisms of cows. The effects of the rumen microflora and bacteriae starter cultures on the occurrence of D-amino acids in cow's milk and fermented products were discussed. These Quantification of AA-enantiomers in beers and raw materials used for manufacturing revealed that raw materials contribute to a negligible (hops) or minor (grains, malts) extent to the D-amino acids content of beer (Erbe and Brückner, 2000a). Therefore, the amounts of D-amino acids detected in beers were mainly attributed to the activity of the racemases of the microorganisms used for brewing. Heat alone can also racemize amino acids in proteins. For example, Hayase et al. (1975, 1979) observed racemization of eight amino acids in roasted casein and bovine serum albumin.

1.3.3 New Racemization Mechanisms for Amino Acids

The Maillard reaction (nonenzymic browning), which occurs between reducing sugars and amino components (amino acids) can also explain the formation of D-amino acids in food. Brückner et al. (2001) have recently pointed out that D-amino acids are formed on heating aqueous solutions of L-amino acids (2.5 mM) together with an excess (278 mM) of saccharides (glucose, fructose, and saccharose) at 100 °C for 24-96 h in aqueous solutions of pH 2.5 (AcOH) or pH 7.0 (NaOAc). Thus, the formation of D-amino acids in many foods of plant and animal origin are the results of nonenzymic browning since the presence of amino acids together with saccharides is common. As for the racemization mechanism, it is postulated that the reaction of amino acids with glucose or fructose starts with the reversible formation of Schiff bases. Proton abstraction takes place from the C^{α}-atom of the L-amino acid and a more or less planar carbanion is formed, possibly stabilized by conjugative delocalization of the electron pair. Reattachment of the proton can take place at both sides of the carbanion resulting in its partial racemization.

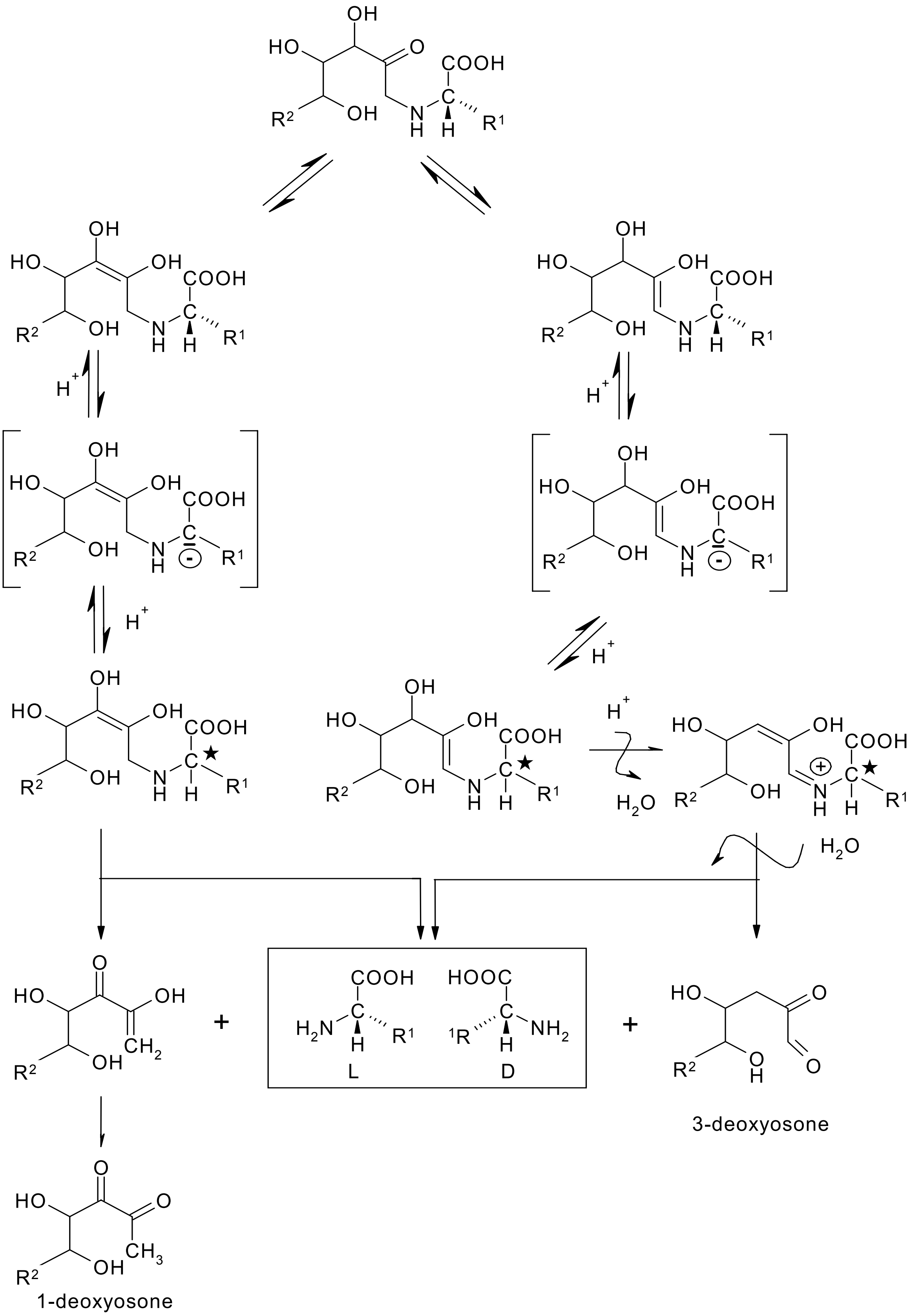


Figure 1-5 Possible ways for racemization of amino acids during the Maillard reaction via formation of Amadori compounds and carbanions (from Pätzold and Brückner, 2006a)

The degree of racemization depends in particular on steric and electronic properties of the amino acid side chains. It should be noted that the early stages of the Maillard reaction proceeds already under mild conditions (Brückner et al., 2001; Ledl and Schleicher, 1990) and do not require alkaline or acidic condition. For a possible racemization mechanism of D-amino acids see Figure 1-5. This new racemization mechanism based on the releatively stable Amadori compounds has been used to explain the generation of free D-amino acid in foods such as dried fruits, concentrated plant juices and fortified wines (Pätzold et al., 2003a). It might also explain the occurrence of D-amino acids in biological systems which is not depending on microorganisms or racemazes (Brückner and Schieber, 2000; Erbe and Brückner, 2000). Recently, heating experiments of synthetic Amadori compounds proved that they are sources of AA-enantiomers (Pätzold and Brückner, 2005; 2006a,b).

1.4 Metabolism of D-Amino Acids

Two metabolic pathways are available for biological utilization of D-amino acid. The first pathway involves epimerazes or racemazes which may convert D-amino acids directly to the L-isomers or enantiomers. The second pathway involves D-amino acid oxidases which may catalyze oxidative deamination of the α-amino acid to form α-keto acids, which can then specifically be reaminated to the L-Form (Konno and Yasumura, 1992).

It is well known that low amounts of amino acids entering the kidney with the blood stream are neither metabolized nor reabsorbed in the glomerular nephrons of the kidney (Blender, 1985). On average, quantities of 0.4 - 1.1 g of free amino acids are daily excreted in urine of healthy adults. Concerning the metabolism of D-amino acids it could be noted that most free D-amino acids in physiological fluids or the body are thought to be metabolized or oxidized by D-AAO or aspartic acid oxidase which are localized in the kidney, liver and tissues (Nagata et al., 1999; D'Aniello et al., 1993). Enzymic oxidation leads to the formation of the corresponding 2-oxo acids, which can be reaminated to yield L-AA. D-AAO activity is highest in the kidney followed by the liver and brain. It was shown that parts of the D-amino acids applied were renally excreted and parts were metabolized (Bansi et al., 1964). Younger rats have lower amounts of D-AAO and higher concentrations of free D-amino acids compared with adult animals. The activities of these enzymes are 50-150 times lower in the fetus than in the adult. So, from the previous results, the reaction sequence of metabolized D-amino acids via the D-AAO could be shown by the equation.

$$\mathbf{R{-}CH(NH_3^+){-}COO^- + O_2 + H_2O \rightarrow R\text{-}CO{-}COO^- + NH_4^+ + H_2O}$$

The α-keto acids can undergo stereospecific transamination yielding the L-enantiomer of the original amino acid, which is then metabolized by conventional pathways. Alternatively, they may be directly catabolized e.g. via oxidative decarboxylation.

The conversion of D-amino acid into α-keto acids takes place primarily in the kidney and liver. Thus, ingested D-amino acids must first diffuse through membranes before they can be metabolized via this pathway. Transport processes are themselves stereoselective and discriminate against D-amino acids (Schwass et al., 1983).

1.5 Nutritional Aspects

Amino acids in their L-forms are most important since they are the building blocks for the biosynthesis of proteins. The antipodes of L-AA, namely D-amino acids, are not used for protein synthesis. So, the occurrence of D-amino acid in foods is undesirable for nutritional reasons since they do not contribute to the biological value of food (Friedman, 1999). Biochemically, the uptake of large amounts of D-amino acids may lead to an overloading of the D-amino acid oxidase system.

It is reported that low levels of racemization caused an extensive decrease in protein digestibility from alkali treatment of proteins. The loss of digestibility is explained by the fact that a racemized amino acid residue, which itself is not a substrate for proteases, can affect the reactivity of its non-racemized neighbors. Thus, the racemization of any particular amino acid residue could cause a significant loss of a neighboring essential amino acid as well as a decrease in the proteolytic digestibility of the protein itself (Man and Bada, 1987). In other words, the nutritional value of both non racemized and racemized proteins could be adversely affected by competition in the digestive tract for active sites of proteinases.

Taking also into account other findings on the occurrence of D-amino acids in food, possible negative nutritional aspects of D-amino acids have to be discussed under consideration of a realistic food intake.

Some D-amino acids apparently have long-term toxicities. This has been extensively discussed by Master and Friedman (1979) and Friedman (1999). The authors noted that dietary D-Ser, lysinoalanine, and various alkali-treated proteins have been reported to cause kidney lesion in rats. Furthermore, it is also evident that human beings have a steady intake of D-amino acids via microorganisms occuring in foodstuffs and beverages. In addition, D-amino acids from the bacterial oral flora are swallowed permanently with saliva (Nagata et al., 1992). Intestinal bacteria with the representative genera *Lactobacilli* and *Streptococci* (Brückner et al., 1993) are also potential sources of D-amino acids. This might also explain that low but significant amounts of certain D-amino acids are detectable in the blood of healthy humans (Nagata et al., 1992; Brückner and Hausch, 1993; Brückner et al., 1994; Pätzold et al., 2003b) or in human urine (Huang et al., 1998; Brückner and Schieber, 2001). It is also obvious that the relative amounts of D-enantiomers were much lower in blood plasma compared to urine excreted.

It has also been demonstrated that D-amino acid administered parenterally to human beings in much higher doses than is to be expected by the consumption of foodstuffs are mainly excreted renally, oxidized to α-keto acids by D-amino acid oxidases, and, in part, reaminated to L-AA or otherwise metabolized (Heine et al., 1983).

Oral feeding of an aqueous solution of D-Pro (50 mg/kg of body weight) for 1 month to rats induced fibrosis and necrosis of kidney liver cells and elevation of serum enzymes (Kampel et al., 1990). No D-Pro was detected in the serum, liver, kidney, or urine of the dosed rats. However; these observations could not be confirmed by Schieber et al. (1997), who found no hepatotoxic or nephrotoxic manifestation in rats following oral administration of D-Pro or D-Asp solutions for 28 days. There was a 20-30 fold increased renal excretion of D-Pro in rats compared to controls. It was shown that D-Pro is common in blood plasma of man and animals (Brückner et al., 1993). It is partly renally excreted in its unmetabolized form and effectively oxidized by D-amino acid oxidase to α-keto-δ-aminovaleric acid which is further metabolized (Berg, 1953). Taking the occurrence of D-Ala, D-Glx, D-Ser, and D-Pro in the blood of healthy volunteers into account conclusions with respect to human beings drawn from reports of the nephrotoxicity of D-Ser (Ganote, 1974) and D-Pro (Kampel et al., 1990) to the proximal tubules of kidneys of rats have to be considered with reservation and certainly should not deter healthy individuals from the moderate consumption of fermented foods and drinks. It was realized that the mechanism for the toxicity of D-amino acids remains unclear. So no clear cut and general answer can be given about the possible toxic effects of common D-amino acids.

It has been pointed out that no toxic effects have been found by intravenous administration of chemically pure, racemic AA to adults and infants (Heine and Drescher, 1975; Heine et al., 1983). In conclusion, as no toxic effects attributable to D-amino acids have been reported by intravenous administration of decagramm amounts of racemic D-amino acids to adults and infants this is not to be expected for free D-amino acids consumed by food intake (Brückner et al., 1992).

On the other hand, presence of certain D-amino acids in dietary proteins may be useful in pain inhibition. For example, D-Phe and D-Leu have been shown to be analgesic (Cheng and Pomeranz, 1979) and have been used in the treatment of intractable pain (Budd, 1983). This analgesic effect is apparently due to the inhibition of enzymes such as carboxypeptidase A, which are involved in the degradation of the opioid pentapeptides present in the brain and spinal (Budd, 1983).

D-Ser also may be involved in neurotransmission in the brain (Kapoor and Kapoor, 1997), and was beneficial in the treatment of schizophrenia.

The occurrence of D-amino acids in starter cultures and, consequently, foods and beverages, in part in high amounts, is also of interest with respect to their flavor (Brückner and Hausch, 1990; Erbe and Brückner, 2000a,b; Pätzold et al., 2003a). D-enantiomers of many amino acids have a sweet taste in contrast to their L-enantiomers and, therefore, might contribute to the final texture and flavor of fermented food stuffs.

1.6 Analysis of D- and L-Amino Acids

Chiral separation is one of the most important themes in analytical chemistry, and a number of methods have been developed. Analyses of D-amino acids in biological samples are challenging, because large amounts of L-amino acids and a large numbers of biological substances such as peptides and amines interfere with the analyses. Therefore, sensitive and selective methods are needed for the determination of D-amino acids (Schurig, 2001).

The progress in analytical methods for D-amino acids analysis has been stimulated by the findings that some D-amino acids are present in the tissues of vertebrates. There are mainly two major methodologies for the enantiomeric separation of amino acids. One uses enzymes such as D-amino acids oxidases and the other employes chromatography such as GC and HPLC.

1.6.1 Thin-Layer Chromatographic and Enzymatic Methods

Optical isomers of amino acid are nowadys preferably determined by HPLC or GC. These methods require costly equipment and are sometimes time-consuming. In contrast, thin-layer chromatography (TLC) is a simple and inexpensive technique that requires no sophisticated instruments (Günter, 1988). A procedure was described for separating D-amino acids using reversed-phase TLC without using expensive impregnated plates or a chiral mobile phase (Nagata et al., 2001). Amino acids were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) and spotted on a reversed phase pre-coated TLC plate. Each FDAA amino acid could be separated from the others by two-dimensional TLC and then extracted from the plate with solvent and subsequently analyzed (Nagata et al. ,1992).

A simple and rapid technique for determination of D-amino acid was described using a spectrophotometer (Soda, 1968). D-amino acids are oxidized by D-amino acid oxidase followed by the reaction of the resultant α-keto acids with 3-methyl -2-benzothiazolone hydrazone hydrochloride (MBTH).

1.6.2 Advanced Chromatographic Methods

Determination of the optical antipodes of D-amino acids in the past was a difficult and time-consuming process. In the last 20 years, gas-liquid chromatography employing a chiral stationary phase and more recently, HPLC using the diastereomeric approach (Buck and Krummen, 1987) have enabled the rapid and precise determination of the enantiomer rations of protein and no protein amino acids.

1.6.2.1 Gas Chromatography

Gas chromatography is widely used to separate and determine enantiomers of L-and D-amino acid in biological samples. Diastereomer formation by the reaction with an optically pure reagent or separation on a chiral stationary phase has been adapted. Generally, chiral derivatization reagents are used to convert amino acids into diastereomers followed by their separation on non-chiral stationary phases.

Chiral stationary phases were also used for the separation of amino acid derivatives. The most widely used chiral stationary phase is Chirasil$^{®}$-L-Val (Frank et al., 1977). Prerequisites for the use of gas chromatography, however, are volatility, thermal stability, and resolvability of the chiral analyte.

Chirasil-Val enables the enantioseparation of most DL-amino acids as their (*N*,*O*)-pentafluoropropionyl isopropyl esters (Brückner and Schieber, 2000; 2001). Using this method Brückner and Schieber separated and quantified enantiomers of 14 amino acids in human urine and blood serum using Chirasil$^{®}$-L-Val and demonstrated that D-Ala and D-Ser are most abundant in urine and blood sera. This method was successfully applied to biological samples and food or beverages (Erbe and Brückner, 1998; 2000a,b).

Other types of stationary phases such as cyclodextrins and their derivatives or chiral metal coordination compounds were also used for the separation of derivatized amino acid enantiomers in GC (König, 1992; Schurig, 2001).

1.6.2.2 High-Performance Liquid Chromatography

High performance liquid chromatograph (HPLC) is the most widely used technique for the determination of D-amino acids in mammalian tissues, and a variety of methods have been reported (Buck and Krummen 1987; 1984). Most of the AA have low absorption of light and do not fluoresce; therefore, precolumn or postcolumn derivatization is needed for the sensitive determination of D-amino acids in biological samples.

Precolumn diastereomer formation using chiral derivatizing reagents followed by their separation on a non-chiral stationary phase, or enantiomer separation using a chiral stationary phase, or use of a chiral mobile phase or additive following the derivatization with non-chiral derivatizing reagents are employed.

Derivatizing reagents such as *o*-phthalaldehyde (OPA) in combination with chiral thiols are widely used to determine AA enantiomers. All primary AA enantiomers are derivatized with OPA to form highly fluorescent diastereomeric isoindole derivatives within a few minutes which can be separated on a reversed-phase column.

Other chiral derivatizing reagents used for the separation of AA enantiomers by HPLC are 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfeys reagent), (+)-1(9-fluorenyl)ethyl chloroformate (FLEC), and O-tetraacetyl-ß-D-glucopyranosyl isothiocyanate (GITC). Many enantiomers of D-amino acids could be separated using these reagents (Schurig, 2001).

Pirkle-type chiral stationary phases (CSPs) are widely used for the separation of amino acid enantiomers by HPLC. Hamase et al. (1997) determined D-amino acids in rat brain using a Pirkle-type CSP.

Enantiomer separation could be achieved on a non-chiral stationary phase using a mobile phase containing a chiral selector. Cyclodextrin, a useful chiral selector, was added to the mobile phase to determine D-amino acids. With this system, an assay for monitoring traces of D-Ala and D-Glu in protein and peptide hydrolysate was presented by Rizzi et al. (1992). Also L-aspartyl-L-phenylalanine methyl ester was used as a chiral selector added to the mobile phase in order to determine D-Asp (Dunlop et al., 1986).

A highly sensitive and selective method using a column-switching HPLC system has been described. It was applied for the precise determination of D-amino acids in biological samples (Hamase et al., 2001; van de Merbel et al., 1995).

1.6.3 Enzymatic Methods

Enzymatic methods are valuable because of their specificity and simplicity. D-amino acid oxidase (DAO, EC 1.4.3.3) and D-aspartic acid oxidase (DAspO, 1.4.3.1) are commonly used. DAO catalyzes the oxidation of most of the neutral D-amino acids to form α-keto acids and hydrogen peroxide. The α-keto acids react with hydrazine to form hydrazones; which could be determined by measuring the absorbance of the derivatives at 445 nm. Using this method Nagata et al. (1987) determined the total amounts of D-amino acids in human blood samples, but individual D-amino acid could not be determined by the procedure.

DAspO catalyzes the oxidation of acidic D-Asp and D-Glu which form oxaloacetate and α-ketoglutarate. These α-keto acids are converted to L-malate and L-Glu in combination with NADH by malate dehydrogenase and glutamate dehydrogenase, respectively. The amount of each D-amino acid is determined by alteration of NADH (Fisher et al., 1991). D'Aniello et al., (2000) determined D-Asp in the rat brain and endocrine glands using DAspO in combination wit HPLC. D-amino acid transminase (DAT) has also been used to determine D-amino acids (Jones et al., 1985). D-enantiomers were determined by measuring the decrease of the contents of the corresponding amino acids after treatment with DAT.

1.7 Tobacco

1.7.1 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. 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.

1.7.2 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.

1.7.3 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 granulates.

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 dipping 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).

1.7.4 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 et al. (1970) for estimation of the concentration of proteins and amino acids in burley tobacco during air curing (Hamilton and 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 et al. (1971). Conjugated 1-deoxy-1-L-prolino-D-fructose (fructose-L-Pro) from cured tobacco leaves was characterized by Tomita et al. (1965) and conjugated D-Ala-D-Ala was isolated from tobacco leaves by Noma et al. (1973).

The enantiomeric composition of amino acids in processed American tobacco standards was analyzed by Kullman et al. (1999). The authors established a sophisticated HPLC column switching systems composed of non-chiral C-18 and modified chiral β-cyclodextrin phases followed by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbaminate.

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.

1.8 Wines

Wines are defined as the alcoholic fermented juices or musts of cultivars and crosses of the genus *Vitis* in the botanical family *Vitaceae*. Grape juice is rich in glucose and fructose as well as amino acids. Alcoholic fermentation is performed by strains of the yeast *Saccharomyces cerevisiae* which might be added as starter cultures or grow naturally on grapes together with other yeasts. However, an abundance of other genera and species of yeasts is also involved in the winemaking process (see also discussion). For their growth, i.e. formation of biomass, the yeasts use amino acids as nitrogen sources and convert the sugars into alcohol and carbon dioxide. Amino acids are also precursors of flavor compounds and biogenic amines. Owing to their abundance and importance many attempts have been undertaken to use the pattern of amino acids for the identification, classification and proof of authenticity of grape juices and wines. Consequently, the majority of reports on amino acids in wines deal with methods for their quantitative analysis (Herbert et al., 2000; Hernández-Orte et al., 2003; Moreno-Arribas et al., 2001; Nouadje et al., 1997]. Few reports, however, are concerned with the chirality of wine amino acids, *i.e*. the possible occurrence of the mirror images of the common L-amino acids, named D-amino acids or D-enantiomers [Nouadje et al. 1997; Brückner and Hausch 1989a; Chaves das Neves and Noronhaq 1995; Calabrese et al. 1995; Kato et al. 1995; Brückner et al. 1995; Calabrese and Stancher 1999; Jin et al. 1999; Tsunoda et al. 1999; Pätzold et al. 2003a]. In particular the finding that L-amino acids in wines appear to be subjects of partial conversion into D-amino acids, a process commonly named isomerization or racemization, attracted attention. Since the time-dependent racemization of bonded or free amino acids under constant environmental conditions is treated as first order kinetic, bonded D-amino acids released on acidic hydrolysis acids are used for the age dating of fossils and recent organisms (Bada and Schroer, 1975). This method is also referred to as amino acid age dating. In analogy it was intriguing to correlate the presence, quantities and kinds of D-amino acids in foodstuffs with their age, in particular when commercial value and sensorial properties are correlated with age. This is the case, for example, for various types of wine matured and/or aged for long periods of time, balsamic vinegar that might be aged for decades, or long-ripened cheeses (Nouadje et al., 1997; Palla et al., 1989; Marchelli et al., 1996; Brückner et al., 1992). Amino acid racemization, however, is very much depending on temperature, pH, and presence of catalysts (Bada, 1972).

The situation in foodstuffs, however, is very complex, since it had been realized in recent years that microbial fermented foods are rich on D-amino acids owing to the presence of bacteria and their racemases (Friedman, 1999). Furthermore, recently convincing evidence has been established that D-amino acids are formed in the course of the Maillard reaction, i.e. reaction of reducing sugars and amino compounds such as amino acids (Brückner et al.; 2001; Pätzold and Brückner 2006a; Pätzold and Brückner 2005).

In continuation of previous research on the use of D-amino acids as stereochemical markers in wines (Pätzold et al., 2003a), and since in particular quantities of D-Pro in wines had been considered as potential markers for age dating (Calabrese and Stancher, 1999), we investigated wines stored for up to 36 years for their amounts and pattern of D-amino acids by GC-SIM-MS.

2 Materials and Methods

2.1 Instrumental

Enantioselective separation of derivatized amino acids was performed on a chiral fused silica capillary column Chirasil®-L-Val (*N*-propionyl-L-valine *tert.* butyl amide polysiloxane) (Pätzold and 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 2-1).

English snuffs (nos. 17-23) (‘Smith’s Snuffs’; no manufacturer given) represented light to dark brown fine powders and were purchased from a tobacconist at 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.

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 AAs 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 gram amounts of tobacco (or 2 g of leaves) 70% MeOH (2.5 mL) were added and the mixture was ultrasonified for 25 min at room temperature. Then the mixture was centrifuged at 3500 x *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 x 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 (50µL) was added and amino acids were converted into *N*(*O*)-pentafluoropropionylamino acid-(2)-propyl esters by treatment with AcCl in 2-PrOH for 1h 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 Sources and Storage of Wine

Bottled wines were commercial products; the names, vintages and analytical data are compiled in Table 2-1. Wines were analyzed in 2003. Bottles were corked and stored horizontally in the cellar of the senior author at an average temperature of about 15°C. Contents of alcohol (% by volume) are given as indicated on bottle labels. The pH was measured using a calibrated glass electrode and quantities of glucose and fructose were determined photometrically using an enzyme assay (catalogue no.0716260; from R-Biopharm, Darmstadt, Germany).

Table 2-1 Characterization of wines

No		vintage[a]	alcohol (%vol)	pH	Glc g L^{-1}	Fru g L^{-1}
1	Spätlese [b], Rheinhessen, 'Erben', Germany	2003	10.0	3.10	9.1	12.5
2	Riesling, Qualitätswein, Mosel-Saar-Ruwer, 'Erben', Germany	2003	11.0	2.80	8.6	11.2
3	Riesling, Qualitätswein, 'Grünes Land', Germany	2003	11.5	2.70	7.3	11.2
4	Riesling, Kabinett, Mosel-Saar-Ruwer, 'Klüsserather Bruderschaft', Germany	2003	10.5	2.83	5.6	6.5
5	Riesling, Spätlese, Mosel-Saar-Ruwer, 'Bernkastler Kürfürstlay', Germany	2002	8.0	2.74	7.7	8.3
6	Riesling, Qualitätswein, Mosel-Saar-Ruwer, 'Bernkastler Kurfürstlay', Germany	2001	9.0	3.10	3.0	3.7
7	Müller-Thurgau, Qualitätswein, Mosel-Saar-Ruwer, 'Moselland Akzente', Germany	2001	9.5	3.17	2.6	5.1
8	Riesling, Spätlese, 'Erdener Treppchen', Germany	2000	7.5	3.16	2.6	2.8
9	Riesling, Kabinett, 'Zeltinger Himmelreich', Germany	1999	8.5	2.64	0.8	1.0
10	Riesling, Spätlese, 'Reiler Goldlay', Germany	1997	11.0	3.10	0.9	2.8
11	Riesling, Kabinett, 'Bopparder Hamm-Ohlenberg', Germany	1986	12.0	3.31	0.8	1.0
12	Riesling, Spätlese, 'Reiler vom heißen Stein', Germany	1983	9.0	3.33	1.1	1.9
13	Kerner, Spätlese, Rheinhessen, 'Sprendlinger Hölle', Germany	1981	8.5	3.47	0.4	0.5
14	Silvaner, Kabinett, 'Birkweiler Kastanienbusch', Germany	1981	9.5	3.41	1.0	0.6
15	Ruländer, Baden, 'Leiselheimer Vulkanfelsen', Germany	1979	9.0	4.10	0.5	0.1
16	Beerenauslese, 'Erdener Treppchen', Germany	1976	8.5	3.70	0.2	0.3
17	Tempranillo, Cabernet Sauvignon, 'Osborne Solaz', Spain	2001	13.0	3.35	10.4	13.0
18	'Chateau Smith Haut Lafitte', France	1994	-	3.60	1.8	2.3
19	Tempranillo, Cabernet Sauvignon, Grand Reserva 'Tarragona', Spain	1994	14.0	3.63	0.4	1.0
20	'Caves de Sarragan', AOC, France	1984	13.0	3.80	0.2	0.3
21	Eiswein, Silvaner, Riesling, Rheinhessen, 'Mainzer Domherr', Germany	2001	7.5	3.44	9.9	10.2
22	Eiswein, Riesling, Württemberg, 'Reiner Altenberg', Germany	1979	-	3.50	2.6	2.8
23	'Napoleon, blanc de blanc', France	2002	10.5	3.00	15.4	17.7
24	'Napoleon, muscat rose', France	2001	11.0	3.20	12.9	14.4
25	'Kupferberg Gold', Germany	2002	11.0	2.71	12.5	15.3
26	'Henkel Trocken', Germany	2002	11.5	2.67	11.7	13.0

- : not given, [a] wines were analyzed in 2003; nos. 1-16 represent white wines; nos. 17-20 red wines; nos. 21 and 22 ice wines and nos. 23-26 sparkling wines.

[b] German quality wines are classified as table wines (Tafelwein), quality wines with vintage (with all the required characteristics of the growing region), and the special high quality wines, denoted according to increasing quality as Kabinett, Spätlese, Auslese, Beerenauslese and for the top quality as Trockenbeerenauslese. Eisweine (ice-wines) are prepared from frozen grapes harvested at a temperature below –7°C. The sparkling wines were produced by the tank fermentation process where a second fermentation of base wines is carried out with addition of sugar and yeast at a pressure of about 7 bars over a 3 to 4.

2.6 Treatment of Wine Samples

Aliquots of wines (5 mL) were adjusted to pH 2.3 by the addition of 0.01 M HCl and passed through a Pasteur pipette packed with activated Dowex 50W X 8 ion exchanger (H^+-form) having a bed volume of 3.5 cm length and 0.5 cm diameter. The resin was washed with water (about 5 mL) until the effluent was almost neutral and amino acids were eluted with 4 M aqueous ammonia (4 mL). The effluent was evaporated to dryness using a rotatory evaporator at 1 mbar and a bath temperature of 40°C. To the remaining residue water (1 ml) was added, the solute transferred to a vial and the solvent removed in a stream of nitrogen at 40°C. For esterification a mixture of AcCl in 2-PrOH (1:9, v/v) (500 µL) was added and the tightly closed vial heated at 100°C for 1 h. Reagents were removed at ambient in a stream of nitrogen, DCM (300 µL), PFPAA (50 µL) and 5% BHT in 2-PrOH (10 µL) were added and the mixture was heated in the closed vial at 100°C for 20 min. Reagents were removed at ambient in a stream of nitrogen, DCM (100 µL) was added and the solvent removed in a stream of nitrogen. This procedure was carried out twice. Then the residue was dissolved in DCM (100 µL) and aliquots of 0.5-1 µL injected onto the GC column at a splitting-ratio of about 1:30.

2.7 Preparation of Amino Acid Standards for Calibration

For testing the efficiency of the capillary column and optimization of chiral resolution appropriate quantities of D- and L-amino acids (ratio approx. 1:2) was prepared and the internal standard L-Nle and achiral Gly and GABA were added. This standard was derivatized and analyzed on a Chirasil®-L-Val column (for a chromatogram of this mixture see Figure 4-1). For quantitative analyses and determination of response factors an L-amino acid standard solution for calibrating amino acid analyzers was used, comprising 2.50 µM (+/-4%) of each L-amino acid and Gly per ml 0.1 M HCl. To 1 ml of this standard each 250 µL of 10 mM L-Nle, L-Orn and Gaba were added. This standard mixture was passed through a column filled with Dowex 50W-X8 cation exchanger and amino acids were eluted and derivatized as described. Response factors of AA and quantities of amino acids in all samples examined were calculated as described in 3.8. AAs are abbreviated according to three letter nomenclature (see list of abbreviations). Chromatograms (GC-SIM-MS) of a standard mixture of DL-AAs is presented in Figure 2-1.

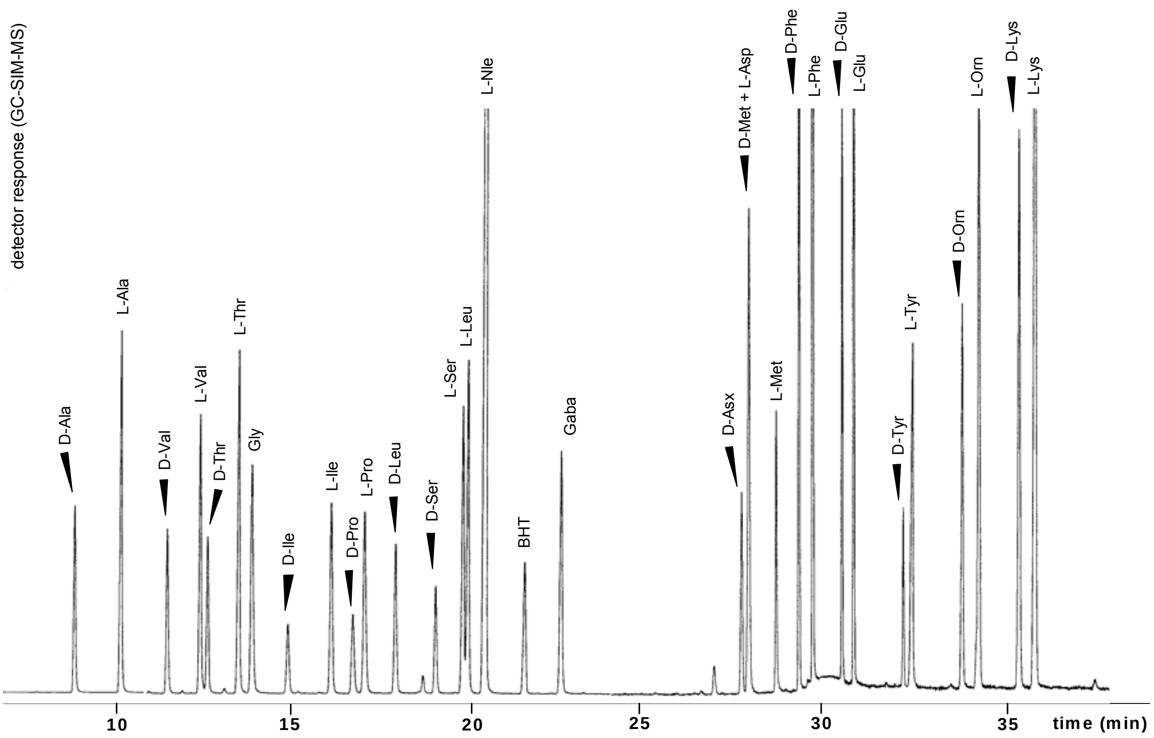


Figure 2-1 GC-SIM-MS of PFP-amino acid-(2)-Prp esters resolved on Chirasil®-L-Val of standard of DL-amino acids (ratio D:L ca. 1:2); for chromatographic conditions, see experimental

2.8 GC-MS Quantification of Amino Acid Enantiomers

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

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

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 equation (2).

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

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

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

Where c_{LAA} is the amount of L-amino acid, f_R the response factor, A_{LAA} the peak area of the L-amino acid in the sample, A_{IS} the peak area of the IS added to the sample and c_{IS} the amount of the IS added to the sample.

From the relative quantities of D-amino acids (%D) and the quantities of L-amino acids c_{LAA} presented in Table 1-4, the absolute quantities of D-amino acid c_{DAA} can be calculated, if required, according to equation (4).

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

2.9 Determination of Sucrose, Glucose and Fructose in Wine Samples

Sugars were determinated according to the manufactures data sheet, r-Biopharm AG, Darmstadt, Germany, Article. Nr 10716260035.

3 Results

3.1 Tobacco

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 3-1 to 3-4 and chromatograms (GC-SIM-MS) of a standard and of selected tobacco samples are presented in Figure 3-1. 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 3-4).

3.1 German Snuffs

In the German snuffs (nos. 1 - 16) L-Pro ranged from 0.63 - 6.31 mg/g, L-Asx from 0.64 - 7.95 mg/g, and L-Glx from 0.39 - 2.26 mg/g. Quantities of the other L-amino acids in all snuffs were much lower (see Table 3-1). Varying patterns of D-amino acids could be detected in all snuff tobaccos. D-Pro, ranging from relative 0.1 - 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 %) was detected in two snuff and D-Thr (10.6 - 18.0 %) in three snuffs. Whereas quantities of D-Ser ranged from 0.0 - 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 Figure 3-1b. 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 3-1.

Table 3-1 Quantities of L-AAs (mg/g tobacco) and relative amounts of D-AAs (%D) in German snuffs (nos. 1-16)

	1		2		3		4		5		6		7		8		9		10	
	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
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
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
Gly[b]	0.31	--	0.06	--	0.04	--	0.12	--	0.14	--	0.08	--	0.02	--	0.07	--	0.02	--	0.04	--
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
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
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
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
GABA[b]	0.18	--	0.10	--	0.06	--	0.13	--	0.13	--	0.02	--	0.02	--	0.10	--	0.04	--	0.03	--
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
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
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
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
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
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
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

[a] AAs in Tables 3-1 to 3-4 are listed according to their order of elution from GC-column [b] non-chiral AA

Table 3-1 continued

	11		12		13		14		15		16	
	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D
Ala	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	0.01	0.0	0.01	3.9	0.01	0.0	0.00	0.0	0.00	0.0	0.04	0.9
Thr	0.01	0.0	0.01	0.0	0.01	0.0	0.00	0.0	0.00	0.0	0.02	12.3
Gly[b]	0.04	--	0.04	--	0.04	--	0.04	--	0.06	--	0.08	--
Ile	0.01	11.5	0.01	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.03	8.7
Pro	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	0.01	11.3	0.00	0.0	0.01	0.0	0.01	0.0	0.01	0.0	0.23	0.0
Ser	0.00	0.0	0.07	0.0	0.03	11.1	0.01	0.0	0.00	0.0	0.20	8.6
GABA[b]	0.05	--	0.03	--	0.02	0.0	0.02	--	0.02	0.0	0.07	--
Asx	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	0.02	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.01	0.0
Phe	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	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	0.00	0.0	0.02	0.0	0.00	0.0	0.03	0.0	0.00	0.0	0.12	0.0
Orn	0.05	0.0	0.03	0.0	0.03	0.0	0.01	0.0	0.00	0.0	0.00	0.0
Lys	0.06	0.0	0.00	0.0	0.03	0.0	0.19	0.0	0.07	0.0	0.02	0.0

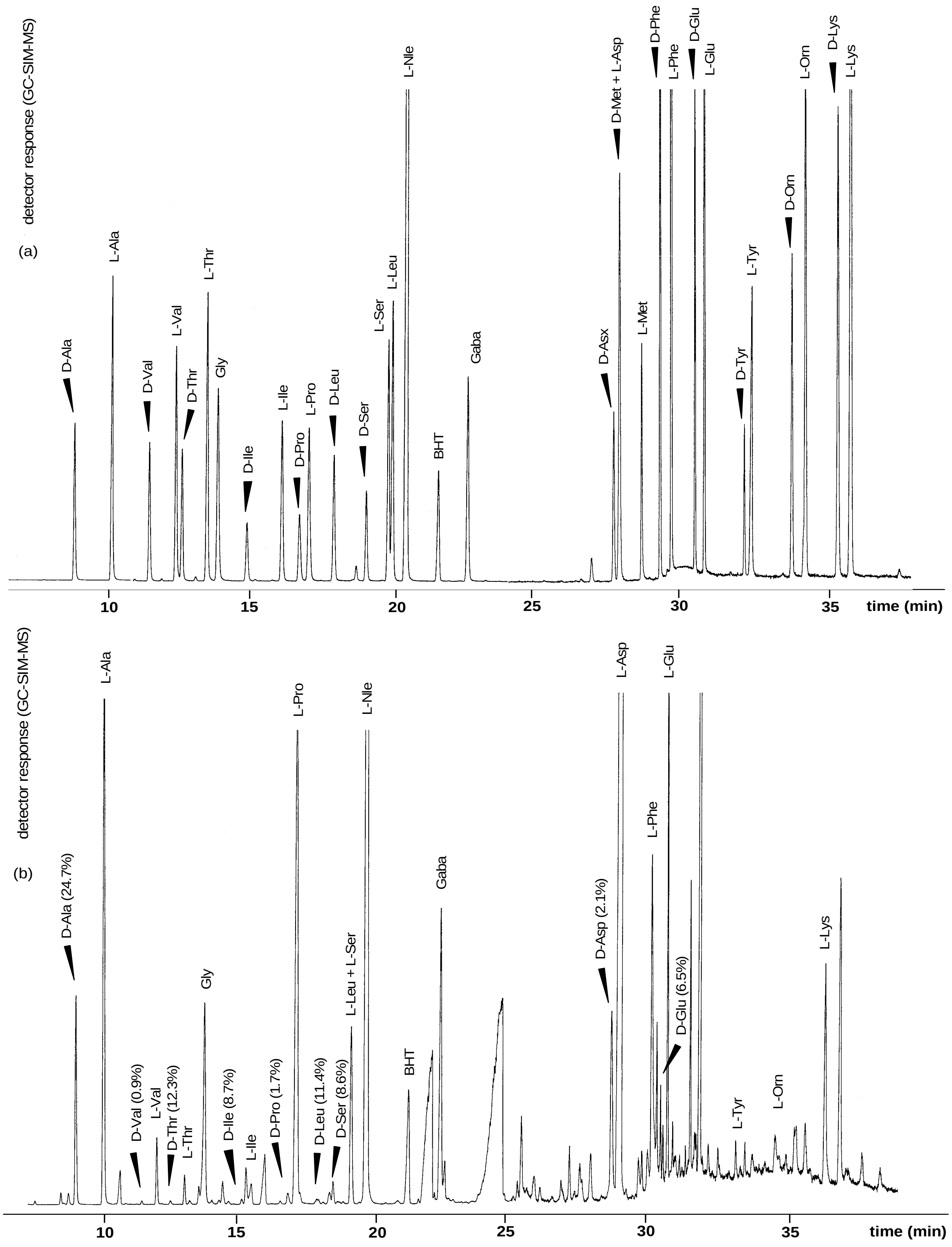


Figure 3-1 GC-SIM-MS of PFP-amino acid-(2)-Prp esters resolved on Chirasil-L-Val of (a) standard of DL-amino acids (ratio D:L ca. 1:2), and amino acids extracted from (b) German snuff (no. 16).

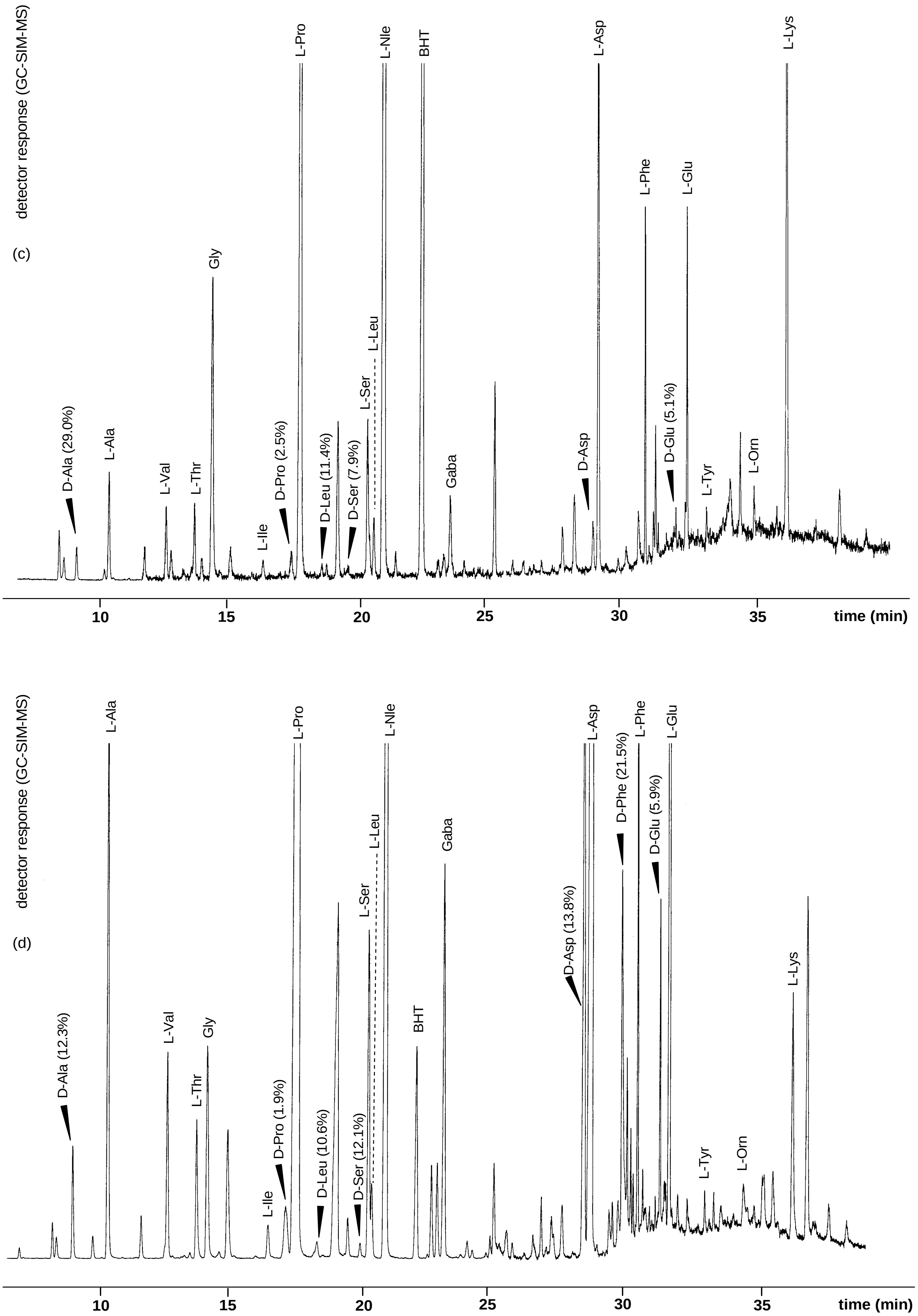


Figure 3-1 continued (c) English snuff (no. 22), (d) Philippine chewing tobacco (no. 25).

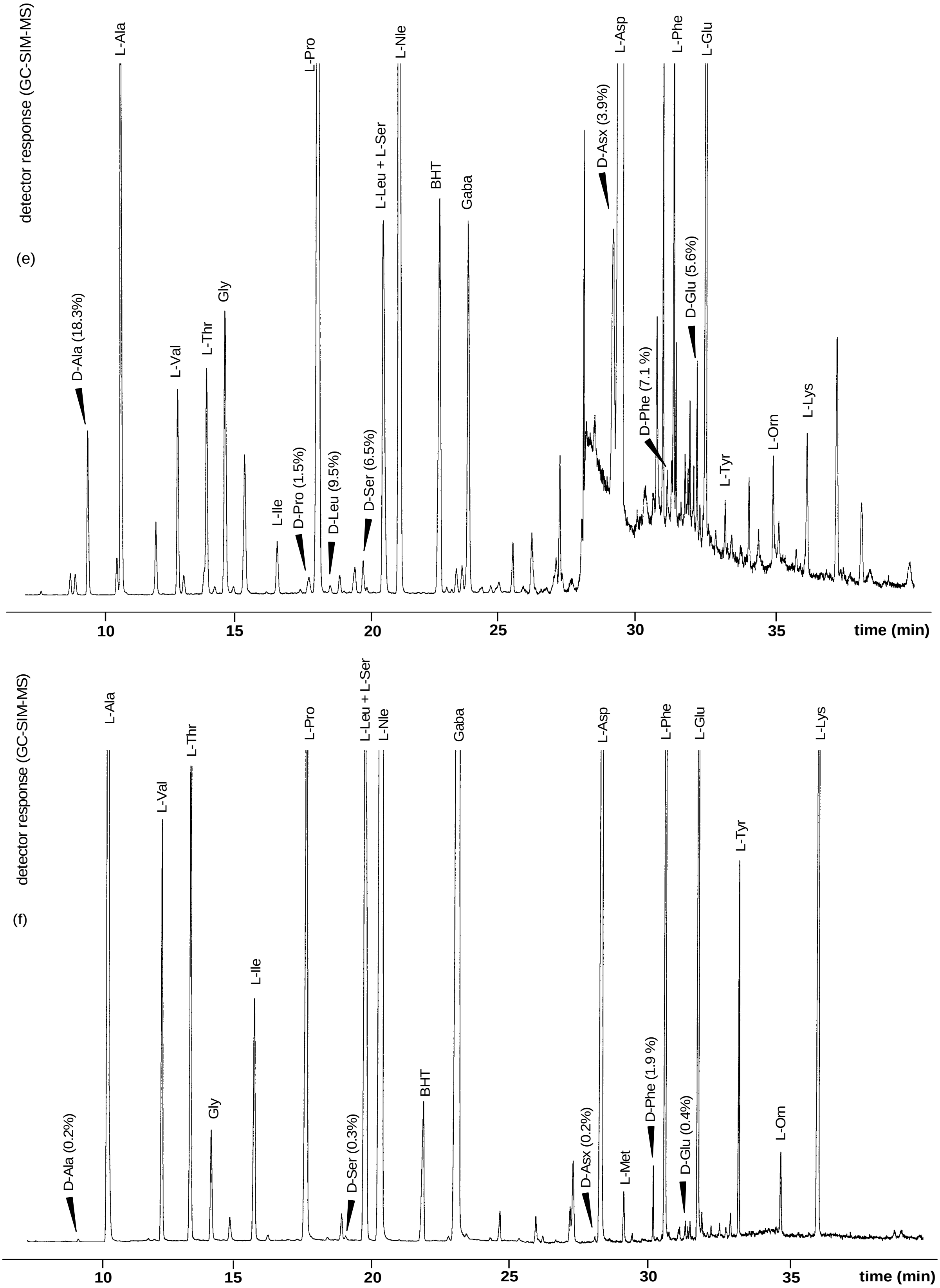


Figure 3-1 continued (e) cigar (no. 33), (f) fresh tobacco leaf (no. 35).

3.2 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 3-2.

Amounts of L-Pro ranged from 0.30 - 3.33 mg/g, L-Asx from 0.58 - 6.78 mg/g and Glx from 0.32 - 4.74 mg/g in the snuffs from Great Britain and Sweden D-Ala was most abundant, ranging from 10.5 - 30.6 %. D-Leu ranged from 0.0 - 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 a British snuff (no. 22), see Figure 3-1c.

3.3 Chewing Tobaccos

Quantities of L-amino acids and relative quantities of the corresponding D-amino acids determined in chewing tobaccos are presented in Table 3-3. 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 Figure 3-1d.

3.4 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 3-4. L- Asx, L-Glx and L- Pro ranging from 0.17 - 5.39 mg/g were the most abundant among L-amino acids. Highest amounts among D-amino acids were analyzed 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 %.

Table 3-2 Quantities of L-AAs (mg/g tobacco) and relative amounts of D-AAs (%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[b]	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[b]	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 3-3 Quantities of L-AAs (mg/g tobacco) and relative amounts of D-AAs (%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[b]	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[b]	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

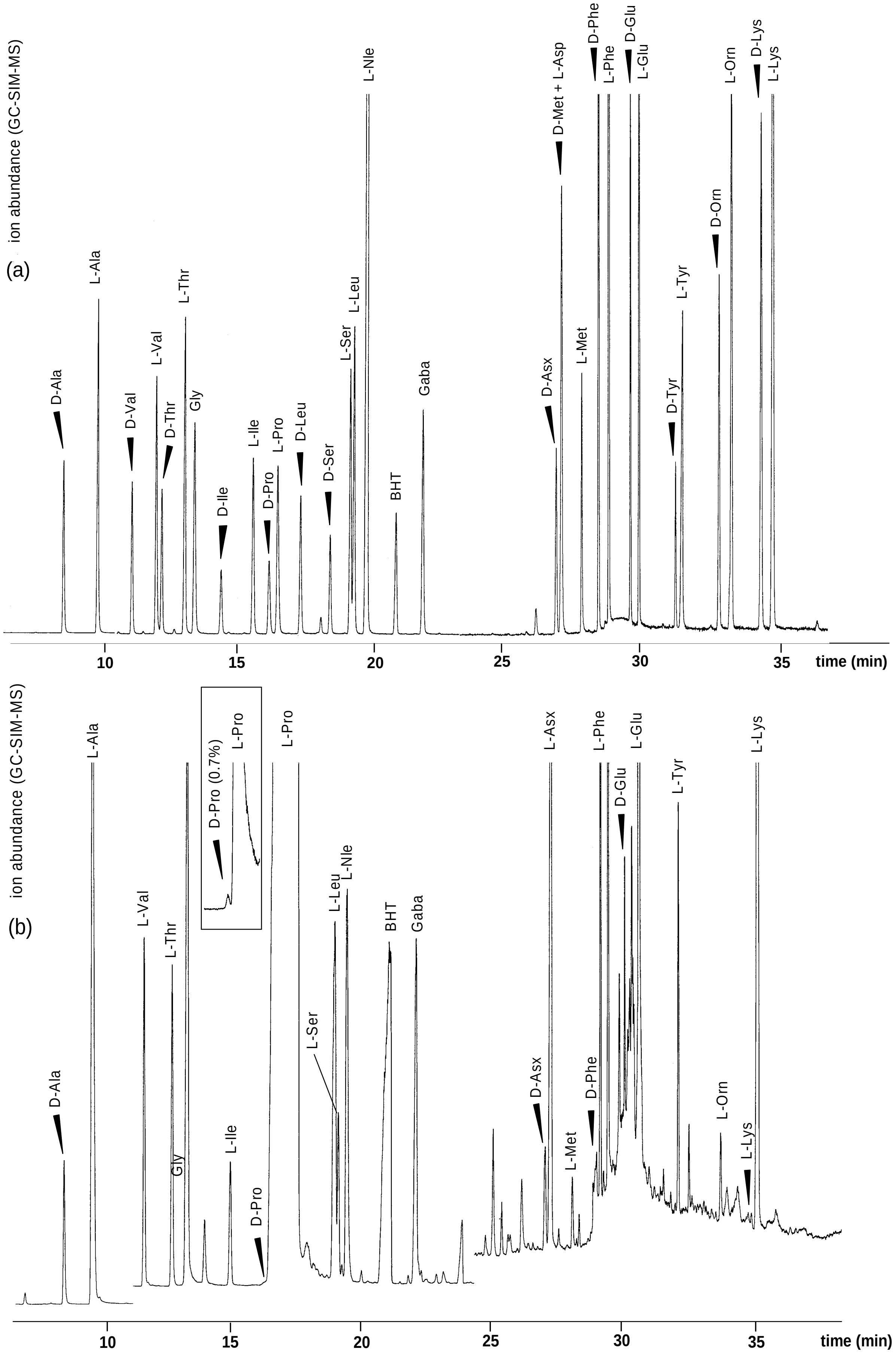
Table 3-4 Quantities of L-AAs (mg/g tobacco) and relative amounts of D-AAs (%D) in cigar (no.33), cigarillo (no. 34) and freshly harvested tobacco leaves (nos. 35 and 36)

	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[b]	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[b]	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

In the tobacco leaves no or low amounts of D-amino could be detected (see Table 3-4). 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 presented in Figure 3-1e and Figure 3-1f, respectively.

3.2 Wines

Wines compiled in Table 3-5 are characterized with regard to type, geographic and regional origin, vintage and contents of alcohol as well as measured pH and contents of glucose (Glc) and fructose (Fru). Contents of ethanol ranged from 7.5 - 14.0 % by volume, pH ranged from 2.64 - 4.10, and quantities of reducing sugars in wines aged up to ten years ranged from 0.8 - 10.4 g L^{-1} Glc and 1.0 - 13.0 g L^{-1} Fru. Quantities of Glc and Fru were higher in sparkling wines, approaching 15.4 g L^{-1} Glc and 17.7g L^{-1} Fru in a French wine (no. 23). Total quantities of L-amino acids (including non-chiral Gly and the typical plant amino acid Gaba) and relative quantities of the corresponding D-amino acids determined by GC-SIM-MS are presented in Table 3-1 to 3-4. The GC of a DL-amino acid standard and of two wines aged for 19 years (no. 20) and 27 years (no.16), respectively, are shown in Figure 3-2.


(a)
ion abundance (GC-SIM-MS)
D-Ala
L-Ala
D-Val
L-Val
D-Thr
L-Thr
Gly
D-Ile
L-Ile
D-Pro
L-Pro
D-Leu
D-Ser
L-Ser
L-Leu
L-Nle
BHT
Gaba
D-Asx
D-Met + L-Asp
L-Met
D-Phe
L-Phe
D-Glu
L-Glu
D-Tyr
L-Tyr
D-Orn
L-Orn
D-Lys
L-Lys
10
15
20
25
30
35
time (min)
(b)
ion abundance (GC-SIM-MS)
D-Ala
L-Ala
L-Val
L-Thr
Gly
D-Pro (0.7%)
L-Pro
L-Ile
D-Pro
L-Pro
L-Ser
L-Leu
L-Nle
BHT
Gaba
D-Asx
L-Asx
L-Met
D-Phe
L-Phe
D-Glu
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L-Tyr
L-Orn
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25
30
35
time (min)

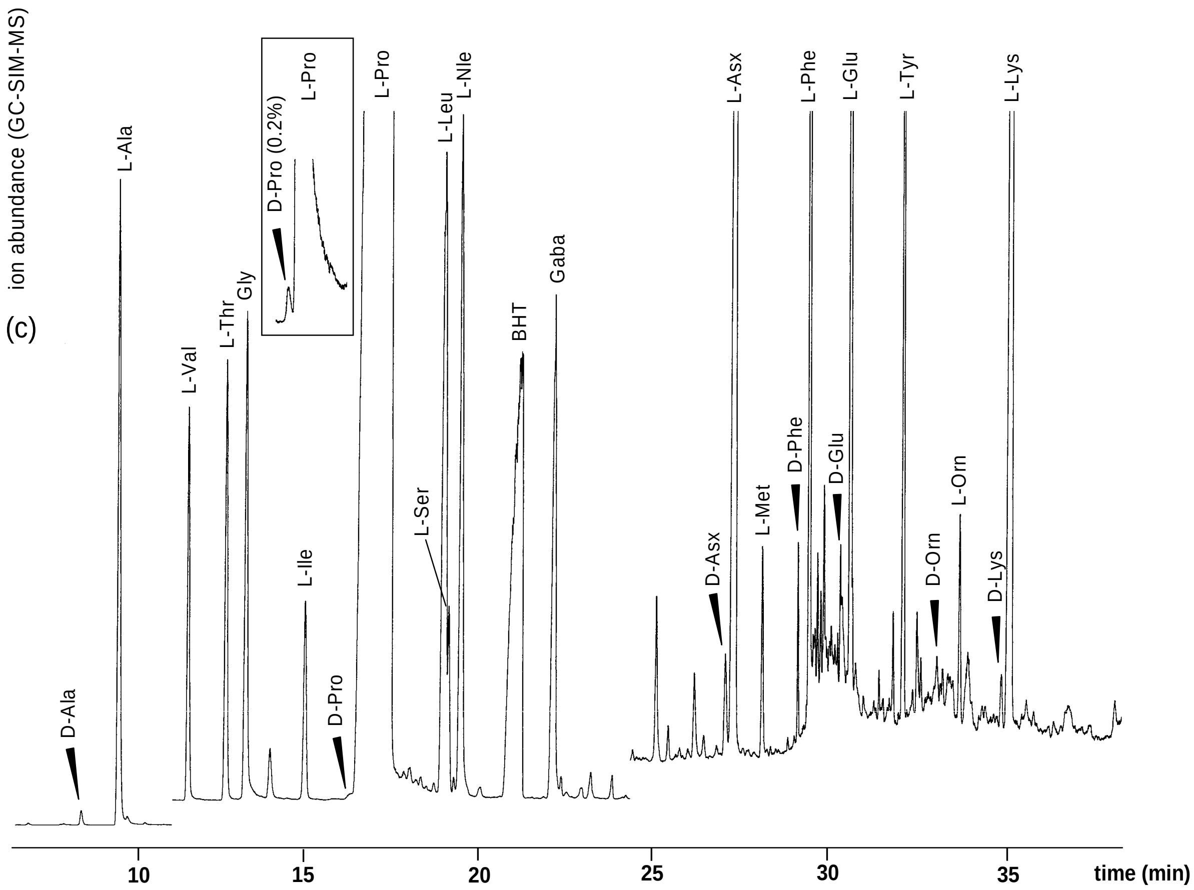

Figure 3-2 GC-SIM-MS of (a) a standard of PFP/2-Prp esters of DL-amino acids (ratio D:L ~ 1:2), (b) of derivatized amino acids of a German white wine (‘Erdener Treppchen, vintage 1976) stored for 27 years, and (c) of a French red wine (‘Caves de Sarragan’, vintage 1984) stored for 19 years (analyses were performed in 2003). Inserts in (b) and (c) represent sections of chromatograms of analytes diluted about 1:30 in order to resolve proline enantiomers. Note that varying baseline levels (offsets) in (b) and (c) are the result of varying amplifications of the ion sets selected for SIM-MS.

Table 3-5 Quantities of amino acids (L) (mg L^{-1}) and relative amounts (D) (%) in white wines (nos. 1-16)

	1		2		3		4		5		6		7		8	
	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D
Ala[a]	134	2.9	30.4	1.6	88.0	1.9	94.1	0.5	132	0.4	39.1	0.5	142	0.7	72.2	2.4
Val	38.1	0.0	2.4	0.0	27.2	0.0	19.5	0.0	59.0	0.0	14.1	0.0	32.8	0.0	27.8	0.0
Thr	72.6	0.0	4.7	0.0	40.1	0.0	17.0	0.0	74.3	0.0	31.6	0.0	41.0	0.0	28.1	0.0
Gly[b]	53.3	--	9.3	--	50.7	--	27.5	--	59.1	--	35.2	--	24.6	--	85.6	--
Ile	1.0	0.0	1.1	0.0	22.4	0.0	15.8	0.0	39.8	0.0	27.5	0.0	22.6	0.0	17.8	0.0
Pro	890	0.0	489	0.0	904	0.0	914	0.0	701	0.0	1269	0.0	631	0.0	967	0.2
Leu	9.6	0.0	1.0	0.0	14.4	0.0	9.1	0.0	16.7	0.0	14.2	0.0	8.9	0.0	9.3	0.0
Ser	22.3	0.0	2.4	0.0	18.6	0.0	4.4	0.0	23.1	0.0	15.6	0.0	6.3	0.0	11.0	0.0
GABA[b]	232	--	2.7	--	65.4	--	17.0	--	92.3	--	170.1	--	19.4	--	10.1	--
Asx	120	1.0	12.0	5.8	122	1.4	64.6	1.9	109	0.9	239	1.2	28.8	1.1	87.5	2.2
Met	12.0	0.0	2.0	0.0	15.2	0.0	7.0	0.0	12.8	0.0	26.1	0.0	3.0	0.0	6.2	0.0
Phe	53.7	0.0	6.2	0.0	57.1	0.0	33.6	0.0	83.4	0.0	129	0.3	21.1	0.0	46.2	0.0
Glx	166	1.5	25.2	3.1	142	8.9	68.5	4.3	134	0.5	304	1.2	24.5	1.6	114	1.6
Tyr	71.1	0.0	7.1	0.0	51.4	0.0	19.7	0.0	65.0	0.0	67.3	0.0	5.4	0.0	36.1	0.0
Orn	97.3	0.0	2.4	0.0	44.7	0.0	3.5	0.0	34.0	0.0	33.0	0.3	2.8	0.0	4.2	0.0
Lys	75.2	0.0	4.0	0.0	91.8	0.0	27.2	0.0	63.8	0.0	112	0.9	3.5	0.0	118	0.0

[a] Amino acids in Tables 3-5 to 3-7 are listed according to their order of elution from GC column; [b] non-chiral amino acids; data presented in tables are average of two sample work ups and each two injections; detection limit about 0.05 - 0.1% D-enantiomer

Table 3-5, continued

	9		10		11		12		13		14		15		16	
	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D
Ala[a]	11.7	3.3	46.2	3.9	337	0.6	87.0	0.5	318	0.7	119	1.0	232	3.9	61.3	2.1
Val	3.1	0.0	13.6	0.0	54.7	0.0	24.2	0.0	143	0.0	38.0	0.0	34.3	0.0	33.6	0.0
Thr	2.2	0.0	17.4	0.0	199	0.0	60.0	0.0	256	0.0	54.5	0.0	48.1	0.0	40.5	0.0
Gly[b]	17.3	--	35.8	--	129	--	43.4	--	14.0	--	67.2	--	74.1	--	64.2	--
Ile	2.8	0.0	15.7	0.0	34.7	0.0	25.1	0.0	109	0.0	26.3	0.0	24.6	0.0	27.0	0.0
Pro	1237	0.2	1017	0.2	928	0.1	1595	0.2	1732	0.2	1019	0.3	1745	0.3	1308	0.7
Leu	1.7	0.0	10.0	0.0	20.1	0.0	5.5	0.0	0.0	0.0	65.0	0.0	14.2	0.0	59.0	0.0
Ser	6.1	0.0	8.1	0.0	39.3	0.0	140	0.7	364	0.0	19.1	11.5	82.8	0.0	8.4	0.0
GABA[b]	2.5	--	62.6	--	0.0	--	169	--	0.0	--	77.6	--	0.0	--	64.2	--
Asx	69.3	8.3	120	3.3	72.5	3.9	191	2.7	633	2.1	127	2.5	262	3.8	94.0	2.1
Met	6.6	0.0	19.2	0.0	26.3	0.0	22.8	0.0	61.5	0.0	20.6	0.0	12.9	0.0	16.1	0.0
Phe	39.0	4.3	101	2.0	88.7	1.4	105	0.2	343	1.0	58.0	0.2	45.8	0.6	56.4	0.5
Glx	113	3.8	123	4.3	419	1.1	200	0.7	573	0.9	123	0.8	73.2	3.1	94.0	2.1
Tyr	20.4	0.0	57.0	0.0	139	0.0	96.1	0.0	273	0.0	60.7	0.0	39.0	0.0	88.0	0.0
Orn	4.3	0.0	21.5	0.0	66.6	3.6	28.0	1.8	154	0.7	30.0	1.3	199	1.8	8.4	0.0
Lys	28.2	0.0	80.6	2.1	117	0.0	146	0.7	368	0.3	92.9	0.3	27.5	1.9	68.0	0.3

Table 3-6 Quantities of amino acids (L; mg L^{-1}) and relative amounts (D; %) in red wines (nos. 17-20) and ice wines (nos. 21-22)

	17		18		19		20		21		22	
	L	% D	L	% D	L	% D	L	% D	L	% D	L	% D
Ala [a]	150	2.9	213	5.5	185	5.4	161	10.6	369	1.1	390	1.5
Val	9.5	0.0	28.1	0.0	34.0	0.0	50.0	0.0	156	0.0	190	0.0
Thr	23.2	0.0	48.3	0.0	20.0	0.0	48.1	0.0	139	0.0	180	0.0
Gly[b]	61.1	--	106	--	90.7	--	140	--	98.3	--	144	--
Ile	5.4	0.0	24.6	0.0	27.0	0.0	28.4	0.0	88.1	0.0	134	0.0
Pro	1605	0.1	1515	0.1	1689	0.2	1533	0.2	680	0.1	1084	0.9
Leu	6.2	0.0	49.1	0.0	91.6	0.0	54.1	0.0	28.5	0.0	66.1	0.0
Ser	8.1	0.0	17.7	0.0	0.0	0.0	22.8	0.0	28.4	0.0	121	0.0
GABA[b]	166	--	70.0	--	101	--	72.3	--	154	--	356	--
Asx	89.0	2.6	84.2	7.7	276	2.2	110	10.9	78.3	0.9	190	3.0
Met	12.0	0.0	9.0	0.0	21.5	0.0	10.3	0.0	14.0	0.0	33.0	0.0
Phe	32.2	0.9	42.4	0.2	128	0.5	36.7	1.0	69.1	0.0	182	1.3
Glx	135	3.9	130	4.6	475	5.0	135	7.4	84.0	1.4	199	6.4
Tyr	15.5	0.0	61.2	0.0	51.5	0.0	46.4	0.0	21.7	0.0	137	0.0
Orn	45.1	1.4	68.7	1.5	95.2	1.8	4.2	7.1	12.9	0.0	70.5	2.8
Lys	38.9	0.0	87.4	0.0	319	0.6	60.0	0.5	6.5	0.0	72.0	0.6

Among L-amino acids Pro is the most abundant in wines (0.49 – 2.00 g L^{-1}) followed by L-Ala (0.01 g - 0.39 g L^{-1}), Glx (0.05 - 0.48 g L^{-1}), and Asx (0.01 - 0.28 g L^{-1}). Lower quantities of other L-amino acids are also detected (*cf*. Table 3-4). The data on quantities of amino acids in wines agree with those reported in the literature, with the restriction that Arg, representing together with Pro a major amino acid, could not be determined in this study. With regard to the aim of the work emphasis in the following is put on relative quantities of D-amino acids in relation to bottling (storage) age and type of the wines. Analyses of white wines (nos. 1-16) are presented in Table 3-6. Despite the fact that L-Pro is the most abundant amino acid in wines no D-Pro was detected in white wines nos. 1-7, in wines nos. 8 – 15 relative quantities did not exceed 0.3 %, and in wine no. 16 quantities of just 0.7 % D-Pro could be detected. The GC-SIM-MS of this wine (no. 16) stored for 36 years is presented in Figure 3-2b. The insert shows the section of a chromatogram resulting from an about thirtyfold dilution of derivatized analytes in order to quantify Pro enantiomers.

Table 3-7 Quantities of L-amino acids (mg L^{-1}) and relative amounts (%D) in sparkling wines (nos. 23-26)

	23		24		25		26	
	L	% D	L	% D	L	% D	L	% D
Ala [a]	10.2	9.8	44.5	9.0	107	5.4	64.8	2.2
Val	2.3	0.0	6.3	0.0	39.2	0.0	19.0	0.0
Thr	5.0	0.0	7.2	0.0	31.9	0.0	23.4	0.0
Gly [b]	25.1	--	40.4	--	80.3	--	84.5	--
Ile	4.9	0.0	3.3	0.0	11.8	0.0	9.0	0.0
Pro	1203	0.0	2000	0.1	1906	0.1	1245	0.1
Leu	3.2	0.0	1.3	0.0	15.3	0.0	4.7	0.0
Ser	0.0	0.0	3.5	0.0	0.0	0.0	12.6	0.0
GABA [b]	46.2	--	42.4	--	87.4	--	87.4	--
Asx	91.4	4.4	27.5	4.4	96.4	2.3	72.3	2.1
Met	5.1	0.0	1.5	0.0	8.4	0.0	7.5	0.0
Phe	34.3	0.3	7.0	1.4	51.6	0.0	31.5	0.0
Glx	108	6.1	49.4	6.1	93.1	2.2	176	1.3
Tyr	20.3	0.0	23.1	0.0	68.1	0.0	65.5	0.0
Orn	55.0	1.8	68.4	1.5	44.1	2.5	38.4	0.0
Lys	83.4	1.6	26.6	1.1	89.1	0.0	80.2	0.5

Analyses of red wines and ice-wines are presented in Table 3-6. In red wines relative quantities of D-Pro (0.1 - 0.2 %), D-Ala (2.9 - 10.6 %), D-Asx (2.0 - 10.9 %), and D-Glx (3.9 - 7.4 %), could be determined. The GC-SIM-MS of French red wine (no. 20) stored for 19 years is shown in Figure 3-2c. The insert shows a section of a chromatogram resulting from the analysis of a sample of analytes diluted about thirtyfold in order to calculate relative quantities of D-Pro.

Among the two ice-wines investigated, the wine stored for 24 years (no. 22) contained relative quantities of 0.9 % D-Pro, 1.5% D-Ala, 3.0 % D-Asx and 6.4 % D-Glu together with few other D-amino acids. Just 0.1 % D-Pro was detected in the ice-wine (no. 21) stored for 2 years and quantities of few other D-amino acids in the low percentage range. Data of sparkling wines are compiled in Table 3-7. No or very low relative quantities of D-Pro (0.1 %) were detected together with D-Ala (2.2 - 9.8 %), D-Asx (2.1 - 4.4 %), and D-Glx (1.3 - 6.1 %) and low amounts of few other D-amino acids. Quantities of L-amino acids are much lower in sparkling wines in comparison to white and red table wines.

4 Discussion

4.1 Tobaccos

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 and 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 and 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 and 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 4-1. As can be seen the data agree favourably.

Table 4-1 Range of relative quantities of D-amino acids (%D) determined in (a) twenty seven 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); (+) indicates most abundant fructose amino acids.

	(a)	(b)	(c)
D-amino acid	%D	%D	
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

Since native tobacco leaves contain very low amounts of D-amino acids (see Table 3-4 and Figure 3-1f) 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, or 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, et al., 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 and Reynolds, 1956; Cremer and Eichner, 2000; Sanz et al., 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 4-1.

Quantities and types of D-amino acids released from fructose amino acids are expected to dependent 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 in 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 et al. 2002, Pätzold and Brückner 2004). This general route for the generation of D-amino has been extended on other foods rich in reducing sugars and amino acids. Examples are fortified wines (Pätzold et al., 2003a), balsamic vinegars (Erbe and Brückner, 2000b), and fruit juice and dietary plant sap concentrates (Pätzold and 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 et al. 2005; Kutz et al., 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 et al., 2005).

4.2 Wines

Chiral analyses of white and red wines, special ice wines and, for comparison, sparkling wines, were performed with the aim to determine whether or not there is a correlation between type of wine and storage time following bottling of wine. It is understood that the time period prior to bottling of wine is named maturation and the storage time following bottling is called ageing. Reports on the enantioselective analysis of wine amino acids are rather scare in the literature and are briefly discussed in the following. Chaves das Neves and Noronha (1995) investigated elementary (base) wines made from the Portuguese grape cultivar ‘Roupeiro’ under controlled conditions. The authors determined the ratio of amino acid enantiomers by GC of PFP/2-Prp esters on Chirasil®-L-Val. Following one year maturation in concrete, wines were bottled, stored and analyzed regularly for an ageing period of eleven years. Very high amounts of D-Ala, approaching or exceeding 60 % were already detected in wines aged for few years together with high relative amounts of the D-enantiomers of Val, Leu, Ser, Asx, Met, Phe and Glx exceeding 20% in some cases. Notably, no D-Pro was detected. Authors state that it was not possible to correlate bottling age and quantities of D-amino acids (Calabrese et al. 1995). determined quantities of L- and D-Pro exclusively in 23 well characterized Italian red and 7 white wines using HPLC following pre-column derivatization with chiral Marfey's reagent (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (Bushan and Brückner, 2004. Authors report that young wines up to 2 years did not show detectable amounts of D-Pro and wines of two to five years of age contained traces of about 0.2 % D-Pro, whereas quantities of D-Pro in older wines increased and amounted to about 15 % D-Pro in a red wine aged for 15 years. Kato et al. (1995) investigated several white, red, and rose wines for the presence of D-amino acids by chiral phase HPLC using pre-column derivatization with 4-fluoro-7-nitro-2,1,3 benzoxadiazole. The authors detected the presence of D-Ala in all wines and of D-Leu and D-Ile in certain wines amounting relative to the L-enantiomers to 25.5 %, 7.5

% and 5.0 %, respectively. They did not report, however, on the detection of D-Pro. Using capillary electrophoresis together with chiral cyclodextrin additives Tsunoda et al. (1999) analyzed D-Asp in a red and white wine and detected 2.19 % and 3.21 % D-Asp, respectively. Using a fluorescent chiral derivatizing reagent and HPLC Jin et al. (1999) determined 4.0 % D-Ala and 10.1 % D-Glu in a wine sample but no D-Pro was detected. Since these latter authors focused on the analytical methodology of amino acid enantioseparation the wines investigated were not further characterized. In a recent work Calabrese and Stancher (1999) studied again the isomerization of Pro in Italian 'Merlot' and 'Cabernet' wines using Marfey's reagent and found an increase of D/L ratio of this amino acid with age. From the data they deduced a first order kinetic process considering an ageing period of about 10 years and postulated an increasingly overlaying microbial (enzymatic) activity in bottled wines aged for more than 10 years. In previous work we had analyzed table wines and fortified wines for D- and L-amino acids using HPLC and pre-column derivations with *o*-phthaldialdehyde together with chiral thiols (Brückner et al., 1995) or GC on various chiral stationary phases using flame ionization detectors Nouadje et al., 1997) or mass spectrometry in the selected ion monitoring mode. It was found that in particular fortified wines (desert or liquor wines) such as Sherry, Port, Madeira or Marsala contain high relative amounts of D-amino acids (Pätzold et al., 2003a). No or relatively low quantities of D-Pro could be detected in the fortified wines investigated. As the authors had postulated that the enantioratios of amino acids are indicatives for the respective vinification technology (Chaves das Neves and Noronhaq, 1995) we analyzed also two ice-wines. However, no clear difference among D-amino acids and no D-Pro could be observed among the ice-wine stored for a short and the wine stored for a long period of time (cf. Table 3-7). Four sparkling wines, characterized by a second fermentation of the base wines were also analyzed for comparison with white and red wines. A variety of D-amino acids could be detected and the most abundant D-Ala approached 9.0 % and 10.2 % in French sparkling wines. However, quantities of D-Pro did no exceed 0.1 % in the sparkling wines. In summary, from the data of wines compiled in Table 3-5 no correlation between storage time of bottled wines, pH, quantities of Glc or Fru and relative quantities of D-amino acids presented in Tables 3-6 to 3-8 could be deduced. In particular the quantities of D-Pro do not correlate with the age of wines. Thus it appears that enological and vinification procedures and in particular the conditions of maturation together with the microorganisms involved play a major role (Brückner et al., 1993). As soon as bacteria are involved in the winemaking process D-amino acids will be formed. This has been shown for example for the malolactic fermenting *Leuconostoc oenos* in wine. Further, it should be stressed that amino acids are important nitrogen sources for the growth of yeasts being responsible for the alcoholic fermentation of grape most. Consequently, total quantities of amino acids decrease on fermentation but faster metabolization of L-amino acids in comparison to D-amino acids should increase relative amounts of the latter. Indeed, it was reported (LaRue and Spencer, 1967) that strains of yeasts involved in winemaking such as *Saccharomyces cerevisiae*, *Saccharomyces uvarum* or *Metschnikowia kamienski* metabolize L-amino acids but not D-amino acids, including D-Ala, D-Asp, D-Glu and D-Pro. In contrast, *Hansenula anomala* metabolizes D-Ala and D-Glu but not D-Asp and D-.Pro. Notably, genera of yeasts and their ratios change in the course of the winemaking and maturation process (Jackson, 2000). Furthermore, some yeasts contain a D-amino acid oxidase or D-amino acid acetylase the action of which will affect the ratio of free D- and L-amino acids. These facts make the interpretation of analytical data resulting from the enantioanalysis of wine amino acids rather difficult. They might explain, however, the relative high abundance of D-Ala, D-Asp and D-Glu in sparkling wines (see Table 3-8). These special wines are produced by varying methods from base wines by performing a second fermentation. Invert sugar is legally added together with pure strains of special yeasts such as *Saccharomyces bayanusa* (*cf.* legend of Table 3-5). Finally, we have previously shown that intermediates of the Maillard reaction, in particular the fructose-amino acids resulting from the Amadori rearrangement of *N*-glycosyl amino acids, are potential intermediates of amino acid racemization (Brückner et al., 2001). Indeed the Maillard reaction proceeds in the course of maturation and ageing of wines (Jackson, 2000) and might contribute to the formation of D-amino acids in wines. However, proceeding of the Maillard reaction in wine or must is very dependent on structures and concentrations of reducing sugars and amino acids, the temperature, pH, and the presence catalysts or inhibitors such as hydrogen sulfite. Since kinetics of the individual reactions of this very complex reaction are different a simple first order kinetic as used for the amino acid age dating based on racemization of free amino acids cannot be applied to wines. Consequently, and taking all data together, quantities and kinds of D-amino acids are not considered good and robust markers for general age dating of wines.

5 Summary

5.1 Tobacco

Quantities of free L- and D-amino acids were determined by GC-SIM-MS in twenty five 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 gas chromatography selected ion monitoring mass spectrometry 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 to 1.9 %), all processed tobacco samples had D-amino acids in varying amounts and pattern.

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.

5.2 Wines

Quantities of free L- and D-amino acids were determined in twenty six wines, comprising eighteen white wines, two ice wines, four red wines and four sparkling wines. Aim of the study was to investigate whether quantities and pattern of D-amino acids, in particular D-proline, correlates with the time of storage of bottled wines.

Summary

Amino acids were isolated from wines by treatment with Dowex 50W X8 cation exchanger. After elution and evaporation to dryness amino acid enantiomers were converted into their *N(O)*-pentafluoropropionyl-(2)-propyl esters and analyzed on a chiral capillary column (Chirasil®-L-Val) together with selected ion monitoring mass spectrometry. Besides common wine L-amino acids, varying kinds and quantities of D-amino acids were detected. The most abundant D-amino acids, detectable in all wines, were D-Ala (alanine), D-Asx (sum of aspartic acid and asparagine) and D-Glx (sum of glutamic acid and glutamine). The relative quantities with respect to the corresponding L-enantiomers in white wines ranged from 0.4 - 3.9 % D-Ala, 0.9 - 8.3 % D-Asx, and 0.5 - 8.9 % D-Glx, in red wines from 2.9 - 10.6 % D-Ala, 2.2 - 10.9 % D-Asx, and 3.9 - 7.4 % D-Glx, and in sparkling wines from 2.2 - 9.8 % D-Ala, 2.1 - 4.4 % D-Asx 1.3 - 6.1 % D-Glx. Low relative quantities of 0.3 % - 0.7 % D-Pro were detected in three white wines stored for more than twenty years and did not exceed 0.2 % D-Pro in two red wines stored for ten and twenty years, respectively. An ice-wine stored for 24 years contained 0.9% D-Pro, 6.4% D-Glx, 3.0% D-Asp and 1.5% D-Ala. The data do not provide evidence for a correlation between the age of the bottled wines and quantities of D-amino acids, in particular of D-Pro, determined therein.

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7 Appendix

Table 7-1 Response factors (f_R) (n = 5) of PFP-(2)-prpyl esters of an equimolar AA standard; RSD is relative standard deviation.

Amino acid	f_R (A)	f_R (B)	f_R (C)	f_R (D)	f_R (E)	Average	RSD %
Ala	1.06	1.00	1.06	0.87	1.22	1.04	12.5
Val	1.03	0.99	1.00	0.76	1.08	0.97	12.2
Thr	0.51	0.16	0.54	0.63	0.44	0.46	17.6
Gly	0.78	0.75	0.76	0.75	0.84	0.78	3.6
Pro	1.21	1.24	1.13	1.11	1.20	1.18	5.6
Ile	0.95	0.78	0.93	0.83	0.91	0.88	7.3
Ser	0.06	0.04	0.07	0.10	0.04	0.06	2.3
Leu	0.66	0.30	0.31	0.33	0.30	0.38	15.8
GABA	0.57	0.53	0.43	0.66	0.59	0.57	0.57
Asx	1.13	1.11	1.07	0.90	1.08	1.06	1.06
Met	0.64	0.60	0.46	0.49	0.47	0.53	8.2
Phe	0.84	0.85	0.78	0.69	0.80	0.79	6.5
Glx	1.61	1.76	1.58	1.35	1.59	1.58	14.8
Tyr	0.01	0.01	0.14	0.37	0.01	0.11	15.7
Orn	1.06	1.00	1.06	0.87	1.22	1.04	12.5
Lys	0.72	1.01	0.89	0.79	0.86	0.85	11.2

Table 7-2 Retention times and characteristic mass fragments (*m/z*) of the PFP-(2)-Prp esters

Retention time (min)	**Amino acid**	**Mass fragment (*m/z*)**
6.0 to 10.50	DL- Ala	191, 191, 235
10.50 to 14.70	DL- Val	203, 218, 219
	DL-Thr	202, 203
	Gly	176, 177
14.70 to 17.90	DL-Ile	203,221,232
	DL-*allo*-Ile	203, 232
	DL-Pro	216
17.90 to 22.00	DL-Leu	190, 232, 233
	DL-Ser	188, 189
	L-Nle	176, 190, 232
22.00 to 25.50	GABA	176, 204, 232, 249
25.50 to 27.90	DL-Asp	189, 234, 235, 262
27.90 to 30.70	DL-Met	203, 221, 263
30.70 to 33.00	DL-Phe	91, 148, 190, 266
	DL-Glu	202, 230, 248, 276
33.00 to 37.50	DL-Tyr	253, 266
37.50 to 39.50	DL-Orn	216, 217, 261
39.50 to 42.50	DL-Lys	176, 230

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