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Formation of Flavor Components in Asparagus. 1. Biosynthesis of Sulfur-Containing Acids in Asparagus

Roland Tressl,* Maria Holzer, and Martin Apetz

Sulfur-containing acids and esters of white asparagus (*Asparagus officinalis*) were enriched by liquid–liquid extraction with pentane–methylene chloride (2:1), separated by means of LSC on silica gel and preparative GLC and investigated by capillary gas chromatography–mass spectrometry. Besides 1,2-dithiolane-4-carboxylic acid (asparagusic acid), its methyl and ethyl esters, 3-mercaptoisobutyric acid, 3-methylthioisobutyric acid, diisobutyric acid disulfide, and 3-*S*-acetylthiomethacrylic acid were identified as intracellular constituents. The biosynthesis of asparagusic acid was assayed with ^{14}C-labeled precursors and asparagus tissue discs. [^{14}C]-L-Valine was transformed via oxo acid, isobutyrate, methacrylate into 3-methylthioisobutyrate and to a lesser extent into asparagusic acid.

In 1948, Jansen isolated 32 g of a sulfur-containing component from 40 kg of asparagus aroma concentrate. By means of chemical reactions, Jansen determined the structure as 3,3′-dimercaptoisobutyric acid. Jansen suggested that this compound may also be present as a disulfide. Yanagawa et al. (1972) identified three sulfur-containing acids by thin-layer chromatography, mass spectrometry, and NMR spectroscopy. They called 1,2-dithiolane-4-carboxylic acid "asparagusic acid" and 3,3′-dimercaptoisobutyric acid "dihydroasparagusic acid". The third constituent was characterized as *S*-acetyldihydroasparagusic acid. The authors mentioned that all three acids act as growth inhibitors. No comment was made on the flavor characteristics of these components. Investigation of an enzyme-inhibited aroma extract of raw asparagus by means of adsorption chromatography, gas chromatography, and mass spectrometry revealed that asparagusic acid, its methyl and ethyl esters, and seven other sulfur-containing acids are synthesized in the intact plant cells. This is an exceptional case of formation of sulfur-containing flavor components. Normally sulfur compounds in vegetable are formed by enzymatic or chemical splitting of nonvolatile precursors, like *S*-alkylcysteine sulfoxides and glucosinolates during crushing of the plant material. We investigated two possible biosynthetic pathways into which some of the identified acids might fit as intermediates. L-Valine seems to be a possible precursor at least for some of the sulfur-containing acids. Valine might be transformed via isobutyric acid and methacrylic acid into mercaptoisobutyric acid. The transformation of [U-^{14}C]valine into 3-mercaptoisobutyric acid is nicely performed by asparagus tissue discs, but asparagusic acid showed only a small amount of radioactivity.

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MATERIAL AND METHODS

Sample Preparation. White asparagus of the region of Braunschweig, Germany, was prepared for analysis immediately after harvesting: (a) 2.5 kg were homogenized with 2.5 L of methanol in a mixer for 5 min in order to achieve enzyme inhibition; (b) 2.5 kg were homogenized with 2.5 L of phosphate buffer solution of pH 6.8 in a mixer for 5 min. Both of the homogenates were cleared by filtration with a "Hafico" tincture press at 400 atm. The filtrates were acidified to pH 2.5 with HCl and sulfur-containing acids were isolated and enriched by liquid–liquid extraction with pentane–methylene chloride (2:1). After drying over anhydrous Na_2SO_4, the extracts were methylated with CH_2N_2 dissolved in ether and concentrated to defined volumes (e.g., 500 μL).

Adsorption Chromatography. A separation according to polarity of components was carried out by liquid–solid chromatography. Extract (125 μL) was given on cooled columns (200 × 9 mm i.d.), filled with silica gel 60 (Merck 7734) of activity II–III, and seven fractions (40 mL each) with solvents of increasing polarity were eluted with: (I) pentane, (II) pentane (P)/methylene chloride (MC) (9:1), (III) P/MC (2:1), (IV) P/MC (1:2), (V) P/ether (E) (9:1), (VI) P/E (1:1), (VII) ether. Fractions were concentrated to 250 μL and then analyzed by gas chromatography.

Gas Chromatography. Investigations were performed with a Varian Aerograph 2740-1 with two FID, a linear temperature program, and an effluent splitter (10:1), with a Tracor 550 of Techmation, equipped with a FPD for sulfur-selective analysis and with a Perkin-Elmer Multifract F 40 with linear temperature programs and FPD.

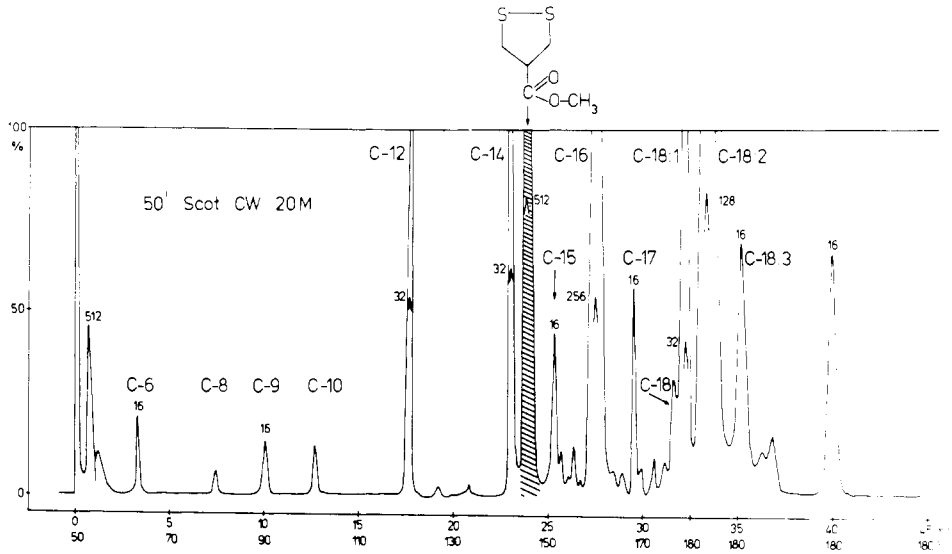

Figure 1. Gas chromatogram of an enzyme-inhibited aroma extract of raw asparagus.

Three packed glass columns and one S.C.O.T. column were used: column 1: 3 m (2 mm i.d.) 8% OV-17 on Chromosorb W-AW/DMCS, 80–90 mesh, temperature program 60–240 °C, 4 °C/min; column 2: 3 m (2 mm i.d.) 5% CW20M on Chromosorb WAW/DMCS, 80–90 mesh, temperature program 60–210 °C, 4 °C/min; column 3: 4 m (4 mm i.d.) 8% OV-17 on Chromosorb WAW/DMCS, 60–80 mesh, temperature program 60–240 °C, 4 °C/min for preparative GC; column 4: 50 ft S.C.O.T. CW20M stainless steel column, temperature program 50–180 °C, 2 °C/min.

Capillary Gas Chromatography–Mass Spectrometry. A 50-m glass capillary column (0.31 mm i.d.) coated with UCON HB 5100 (Jaeggi, Switzerland), temperature program 70–180 °C, 2 °C/min, in a Carlo Erba Fractovap 2101 AC and a 100-m stainless steel column (0.75 mm i.d.) coated with CW20M, temperature program 70–180 °C, 2 °C/min, in a Varian 2740-1 were connected to a Varian double-focusing mass spectrometer CH5-DF. The glass capillary (UCON) column (3 mL of He/min) directly lead to the ionization chamber via a platinum–glass capillary connection. The steel column (12 mL of He/min) was connected to MS via a one-stage helium separator. The ionization voltage was at 70 eV and the ion source temperature was at 200 °C. Mass spectra were recorded with Oscillofil of Siemens, Germany. Separated compounds were registered by the pressure-measuring ion source device.

Synthesis of Components. (*a*) *Asparagusic Acid and Its Methyl Ester.* With reference to the work of Claeson and Langsjoen (1959), Böhme et al. (1949), and du Vigneaud and Brown (1958), we synthesized dibenzyl thiomethyldiethylmalonate starting with diethyl malonate and benzylthiomethyl chloride. Dibenzylthiomethyl malonic acid in toluene was split in liquid ammonia with sodium into 2,2′-dimercaptomethylmalonic acid. Oxidation with O_2 and $FeCl_3$ as catalyst yielded 1,2-dithiolane-4-dicarboxylic acid which decarboxylated to 1,2-dithiolane-4-carboxylic acid at 150–170 °C. After purification by preparative GC, we obtained GC, MS, and IR data fully identical with the natural compound. The methyl ester of asparagusic acid was prepared by methylation with CH_2N_2.

(*b*) *Methyl 3-Mercaptoisobutyrate.* We saturated an ice-cooled solution of methyl methacrylate in ether with H_2S and added one drop of triethylamine as a catalyst. It was warmed to 60 °C in a vial for 4 h.

(*c*) *Diisobutyric Acid Disulfide.* Methyl 3-mercaptoisobutyrate was cooled and treated with O_2 and 5% methanolic potassium hydroxide to yield the disulfide.

(*d*) *Methyl 3-methylthioisobutyrate* was synthesized by adding methanethiol to a cooled solution of methyl methacrylate and ether; reaction time, 10 h; temperature, 25 °C.

Labeling Experiments with ^{14}C Compounds. For labeling experiments we used acetate, cysteine, valine, and isobutyrate, all uniformly labeled. Asparagus (50 g) (only 3 cm of the top of the asparagus) was cut into fine discs (about 3 mm thick) and incubated with 50 μCi of the actual compound in 70 mL of 0.4 M sucrose solution for 5 h at 25 °C. After filtration and washing, asparagus discs were homogenized under enzyme inhibition with methanol and extracted with pentane–methylene chloride (2:1). Methylation was performed with CH_2N_2, and after mild concentration, analysis was carried out with a preparative GC, collecting fractions via split in scintillation bottles. Scintillation counting was performed with a Hewlett Packard HP 3380 liquid scintillation counter (Tressl et al., 1970).

RESULTS AND DISCUSSION

Investigation of Sulfur-Containing Acids in Asparagus. Investigation of an enzyme-inhibited aroma extract of raw asparagus by means of gas chromatography in combination with a sulfur selective detector revealed only one strongly concentrated component. After methylation with diazomethane, the amount of this component increased. Moreover, some other sulfur-containing components could be traced for the first time. Figure 1 shows a chromatogram of the enzyme-inhibited extract. The strongly concentrated sulfur component was identified as methyl 1,2-dithiolane-4-carboxylate. The other components are fatty acids. Asparagusic acid (1,2-dithiolane-4-carboxylic acid) was synthesized according to the method described above. The methyl ester (100 mg) was isolated and purified by preparative gas chromatography. The MS, IR, and chromatographic data of the synthesized product and of the isolated natural component were identical. The purified methyl ester possessed an aroma character like raw asparagus. In certain varieties, the concentration of the ester amounts to 7 ppm, whereas the free asparagusic acid amounts to approximately 3 ppm,

Table I. Sulfur-Containing Acids and Esters (Acids Determined as Methyl Esters)

Compound	I_K (UCON)	Mol wt	Mass spectral data	Approx. concn, ppb
Acids				
(1) 2-Methylthioacetic	1102	120	61 74 120 35 47 121 122	<50
(2) 3-Methylthiopropanoic	1286	134	74 61 134 75 47 135 136	<50
(3) 3-Methylthioisobutyric	1317	148	61 41 88 148 89 39 59 45	50
(4) 3,3′-Dimethylthioisobutyric		194	41 61 178 39 111 45 55	<50
(5) 3-Mercaptoisobutyric	1240	134	41 42 39 47 69 75 59 134	200
(6) 3-*S*-Acetylthioisobutyric	1524	176	43 42 45 144 59 41 101 134 176 133	<50
(7) 3-*S*-Acetylthiomethacrylic	1586	174	43 39 100 40 45 72 59 142 71 132 131 174	<50
(8) Diisobutyric acid disulfide	1960	266	41 59 69 42 73 43 101 45 266	600
(9) 1,2-Dithiolane-4-carboxylic	1742	164	164 41 104 45 59 105 86 55	3000-5000
(10) 1,2,3-Trithiane-5-carboxylic		196	41 45 164 131 196 59 39 99 55	300
(11) 1,2-Dithiane-4-carboxylic[a]	1898	178	45 41 39 73 85 103	60
(12) 1,2-Dithiane-5-methyl-4-carboxylic[a]	1967	192	145 91 61 45 192 85 39 59 71	<50
(13) Thiacyclopropanoic		118	86 59 118 55 58 87 44 27 119 120	100
Esters				
(14) Methyl 1,2-dithiolane-4-carboxylate	1742	164	164 41 104 45 59 105 86 55	up to 7000
(15) Ethyl 1,2-dithiolane-4-carboxylate	1778	178	29 41 104 45 39 105 178 55 86 59	50
(16) Methyl 3-*S*-acetylthiomethacrylate	1586	174	43 39 100 40 45 72 59 142 71 132 131 174	<50

[a] Postulated from mass spectrometric data.

but these amounts varied considerably.

It is interesting to theorize over the biosynthesis of asparagusic acid and the pathways leading to this unique sulfur compound in asparagus. To solve these problems we tried to identify further sulfur-containing trace components which might be precursors of asparagusic acid. This investigation is rendered extremely difficult because these compounds are masked by the more strongly concentrated fatty acids and by other constituents. After liquid–liquid extraction, aroma concentrates of raw and cooked asparagus were separated by the method of adsorption chromatography into fractions of graduated polarity. This procedure was followed by further separation of the components by means of gas chromatography, preparative gas chromatography, and capillary gas chromatography–mass spectrometry. The organic acids were extracted with pentane–methylene chloride (2:1) at pH 2.5, transformed into the methyl esters and separated by solid–liquid chromatography on silica gel. Fraction 3 contained asparagusic acid and other sulfur-containing acids. The trace components were isolated, enriched by preparative gas chromatography, and investigated by GC–MS. Fraction 5 contained mercapto acids, besides dicarboxylic acids and lactones. These separations occur strictly according to functional groups. In our opinion, these simple methods are most effective in tracing essential components in complex mixtures.

In Figure 2 certain sulfur-containing acids are presented. Asparagusic acid (I), its methyl (II), and ethyl ester (III) are intracellular components; 1,2,3-trithiane-4-carboxylic acid (IV) could be determined only in cooked asparagus. Components V and VI are formed intracellularly, like asparagusic acid. The structures of components V and VI were postulated only from mass spectrometric data. The synthesis of these compounds is in progress. In Table I the mass spectra and approximate concentrations of the sulfur compounds are summarized. The structures of components I to IV are close to isobutyric acid and they might be derived from valine. On the other hand, the structures of the postulated components are related to 2-methylbutyric acid, therefore isoleucine might be a possible precursor.

Figure 3 illustrates certain aliphatic sulfur-containing acids which were identified in the trace range. 3-Mercaptoisobutyric acid (I), diisobutyric acid disulfide (IV), and 3-methylthioisobutyric acid (V) were confirmed by synthesis. The structures of the acids II, III, and VI

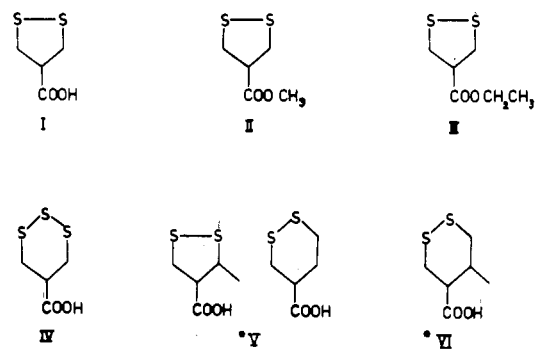

Figure 2. Cyclic sulfur-containing acids in asparagus: (I) 1,2-dithiolane-4-carboxylic acid (asparagusic acid), (II and III) methyl and ethyl 1,2-dithiolane-4-carboxylate, (IV) 1,2,3-trithiane-5-carboxylic acid, (*V and *VI) structures postulated from mass spectrometry.

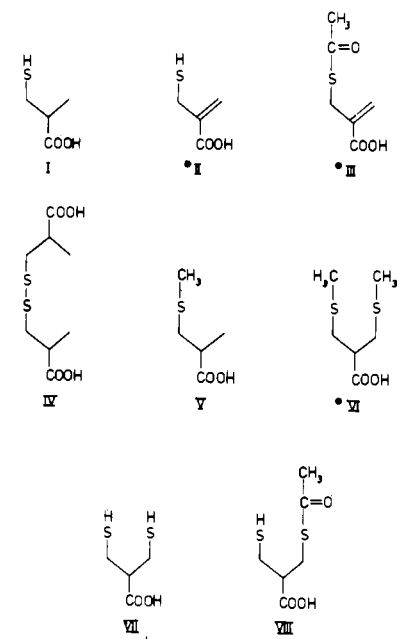

Figure 3. Aliphatic sulfur-containing acids in asparagus: (I) 3-mercaptoisobutyric acid, (II) 3-mercaptomethacrylic acid, (III) 3-*S*-acetylthiomethacrylic acid, (IV) diisobutyric acid disulfide, (V) 3-methylthioisobutyric acid, (VI) 3,3′-dimethylthioisobutyric acid, (VII) 3,3′-dimercaptoisobutyric acid (dihydroasparagusic acid), (VIII) *S*-acetyldihydroasparagusic acid; (*) postulated from mass spectrometric data and chemical reactions.

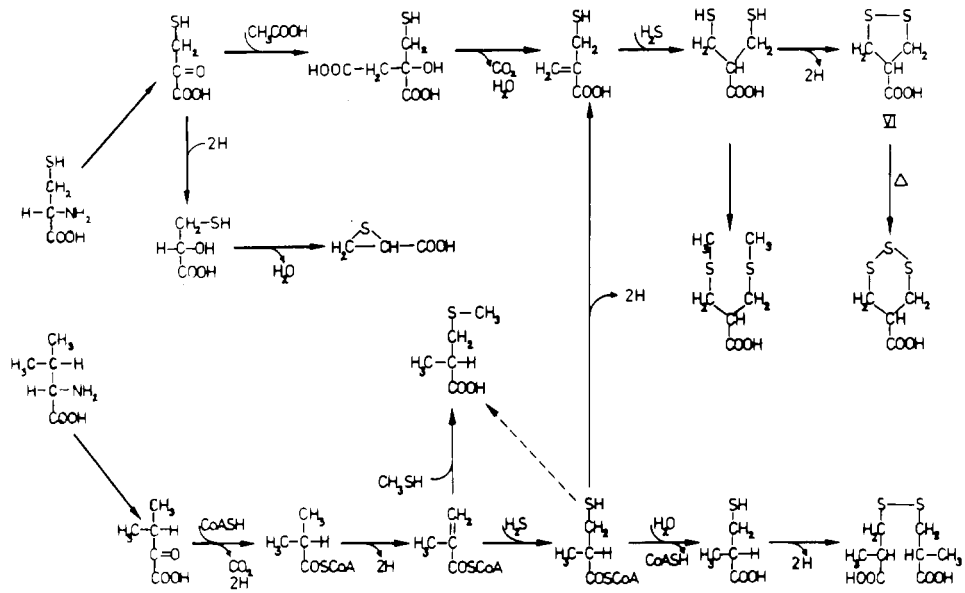

Figure 4. Possible biosynthetic pathways leading to asparagusic acid (VI).

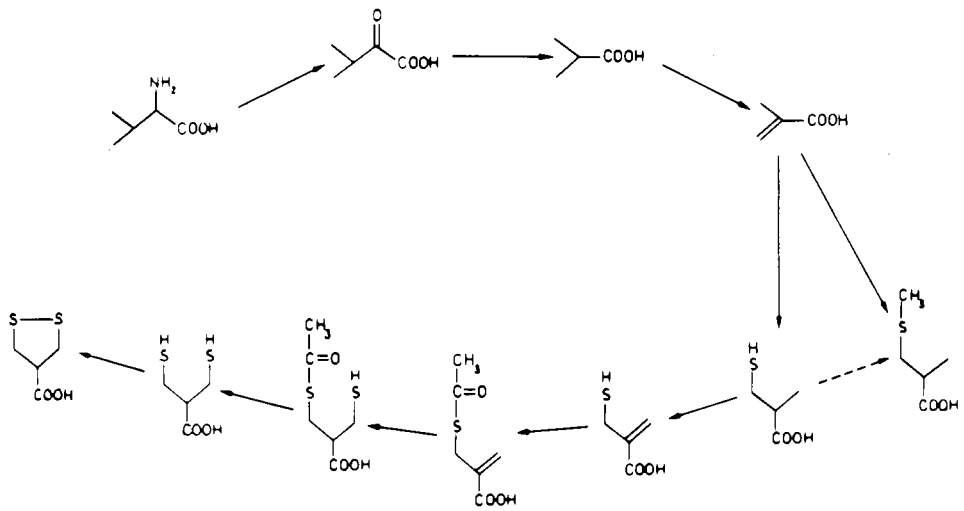

Figure 5. Possible biosynthetic pathway for formation of asparagusic acid in asparagus tissue discs.

were postulated from mass spectrometric data and chemical reactions. Dihydroasparagusic acid (VII) and *S*-acetyldihydroasparagusic acid (VIII) were not detected in our experiments. We suggest that these mercaptoacids are decomposed during gas chromatographic separations. The acids are found in the enzyme-inhibited aroma extracts. This means that they are formed in the intact plant cells and might be possible intermediates in the biosynthesis of asparagusic acid.

Biosynthesis of Sulfur-Containing Acids in Asparagus. Figure 4 illustrates our biosynthetic approach to asparagusic acid. The experiments are very time consuming. In Germany, fresh asparagus is only available during 2 months per year. We investigated two possible biosynthetic pathways into which some of the identified acids might fit as intermediates. In labeling experiments, [U-^{14}C]cysteine and [U-^{14}C]acetate were not transformed into asparagusic acid. [U-^{14}C]acetate was converted to some extent into caprylic, decanoic, and palmitic acids by asparagus tissue discs. Therefore, this route recently proposed by Metzner (1973) is obviously inoperative. Valine seems to be a possible precursor at least for some of the sulfur-containing acids. Valine might be transformed via isobutyric acid and methacrylic acid into 3-mercaptoisobutyric acid. This is a known pathway leading to 3-hydroxyisobutyric acid and 3-aminoisobutyrate, respectively.

In Table II, the results of the conversion of [U-^{14}C]valine and of [U-^{14}C]isobutyrate into volatiles are presented. Asparagus discs were incubated with the labeled precursors, homogenized, and extracted. The distribution of the radioactivity among the volatile components was performed by gas chromatography in combination with liquid scintillation (Tressl, 1970). It can be seen that valine is converted to some extent into 2-oxo-3-methylbutyrate, isobutyrate, and methacrylate. 3-Mercaptoisobutyric acid and 3-methylthioisobutyric acid are strongly labeled components. On the other hand, asparagusic acid shows only a small amount of radioactivity. Isobutyric acid is better transformed into volatiles by asparagus discs than is valine. The results show that obviously in asparagus a pathway is operative, transforming valine via the corresponding oxo acid, isobutyric acid, methacrylic acid into 3-mercaptoisobutyric acid and into 3-methylthioisobutyric acid. The 2-oxo acid, isobutyric acid, and methacrylic acid were identified as intracellular constituents of asparagus.

Figure 5 illustrates a reaction scheme which may explain the biogenesis of the identified sulfur-containing acids. The transformation of [U-^{14}C]valine into 3-mercaptoisobutyric acid is nicely performed by asparagus tissue

Table II. Conversion of [U-^{14}C]-L-Valine and [U-^{14}C]Isobutyric Acid into Volatiles by Asparagus Tissue Slices

Precursor (50 μCi):	[U-^{14}C]-L-Valine		[U-^{14}C]Isobutyric acid
Incubation time, h	5	13	3
Radioactivity in the aroma extracts, %	1.9	2.3	15.9
Distribution of Radioactivity among Volatile Components, %			
2-Oxo-3-methyl-butyric acid	3.0	17.5	
Isobutyric acid	7.0	2.1	42.5
Methacrylic acid	0.5		3.2
3-Mercapto-isobutyric acid	65.5	61.6	1.3
3-(Methylthio)-isobutyric acid	15.1	1.5	28.5
Asparagusic acid	0.3	0.7	0.3

discs, but 3-*S*-acetylthiomethacrylic acid and asparagusic acid showed only small amount of radioactivity. The experiments will be continued. The discussed biosynthetic route is an analogous reaction to the formation of *S*-1-propenylcysteine sulfoxide in onion (Schwimmer and Friedmann, 1972; Schwimmer and Guadagni, 1968).

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Formation of Flavor Components in Asparagus. 2. Formation of Flavor Components in Cooked Asparagus

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Aroma components of cooked white asparagus were enriched by liquid–liquid extractions at different pH values and separated by the method of adsorption chromatography into fractions of graduated polarity. Individual components or fractions were isolated and enriched by means of preparative gas chromatography for capillary gas chromatography–mass spectrometry. More than 100 constituents (among them: thiophenes, thiazoles, pyrroles, pyrazines, aldehydes, ketones, alcohols, and phenols) have been identified. Most of the aroma components are formed during heating processes: by thermal degradation of precursors like *S*-methylmethionine, asparagusic acid, *p*-coumaric acid, and ferulic acid, by lipid oxidation of linoleic acid and linolenic acid, and via Maillard reactions.

The typical flavor components of asparagus are formed during cooking of the plant material. According to Freytag and Ney (1968, 1972), dimethyl sulfide is formed as a principal aroma constituent in cooked asparagus. *S*-Methylmethionine is supposed to be the corresponding precursor. This amino acid had been identified by Challenger and Hayward (1954) in uncooked asparagus. Casey et al. (1963) showed that dimethyl sulfide (Me_2S) is formed during the heating of methionine with pectin. The latter reacts to give *S*-methylmethionine which in turn decomposes to afford dimethyl sulfide. Therefore, Me_2S is known as a common constituent in most cooked vegetable. Besides Me_2S, more than 100 constituents (among them: aldehydes, ketones, alcohols, phenols, and heterocyclics) have been identified by means of liquid–liquid extraction at different pH values, LSC, GLC, and MS. These aroma constituents are formed during cooking of asparagus: (1) by thermal fragmentation of precursors like *S*-methylmethionine, asparagusic acid, *p*-coumaric acid, and ferulic acid, (2) by oxidative degradation of unsaturated fatty acids, (3) via Maillard reactions.

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MATERIAL AND METHODS

Sample Preparation. White asparagus (20 kg) was homogenized with 20 L of phosphate buffer (0.1 M, pH 6.8), refluxed for 30 min, and pressed at 400 atm to give 36 L of filtrate.

Liquid–Liquid Extraction. Eighteen liters of the obtained filtrate was directly extracted with pentane–ether (1:1) for 24 h at pH 6.8 to isolate neutral compounds. The other part of the filtrate was adjusted to pH 8.5, and basic compounds were isolated in the same way. Both extracts were dried over Na_2SO_4, concentrated to a definite volume (3 mL), and separated by adsorption chromatography.

Adsorption Chromatography. A separation according to polarity of components was carried out by liquid–solid chromatography. Ten 300-μL fractions of the extracts were separated on two different columns. Column A (250 × 12 mm i.d.) was filled with two parts of Al_2O_3 90 (acidic,