Odor and Flavor Contribution of 2-Pentyl Pyridine to Soy Protein Isolates

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ABSTRACT: Total lipid extracts from two commercial soy protein isolates (SPI) were subjected to analyses by gas chromatography/olfaction by using capillary columns with stationary phases of three different polarities. With Kovats indices to identify the location of the odorous compounds on chromatograms produced with the same lipid extracts by gas chromatography/mass spectroscopy, the corresponding mass spectra of clearly distinguishable odors were examined. Compounds, tentatively identified by their mass spectra, were confirmed by retention times (on all three stationary phases), odor intensity, and odor description when compared with standards. Major odorous compounds identified included 2-methyl butyric acid methyl ester, hexanal, butyric acid, and 2-pentyl pyridine. Subsequent sensory analyses demonstrated that the mean levels of 2-pentyl pyridine found in two SPI (0.28 and 1.01 ppm), combined with its repulsive flavor profile (throat-catching and grassy in water) and extremely low flavor threshold level (0.000012 ppm), make it a major contributor to the undesirable flavor of SPI. Its mean contents in SPI were 23,333 and 84,167 times greater, respectively, than its flavor threshold. The level of hexanal was 112 and 201 times, respectively, above its flavor threshold, and the level of 2-pentyl furan in each SPI was 433 and 592 times, respectively, above its flavor threshold, which indicates that these two compounds make relatively minor contributions to the flavor of SPI.

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The flavor of soy protein products (often described as beany, intensely bitter, throat-catching, and biting) has been the most difficult problem limiting expanded use of soy proteins in human foods (1,2,8). Aliphatic carbonyls, volatile fatty acids, amines, and alcohols (3), oxidized phosphatidylcholine (PC) (4), phenolic acids (5), isoflavones (6), and saponins (7) have been implicated as contributing to the undesirable flavor associated with soybean products.

Headspace analyses of unheated and heated soy protein isolates (SPI) by gas chromatography/mass spectroscopy (GC/MS) have been used to examine volatile components associated with soy proteins (9,10). This method provides qualitative information about the broad range of volatile components of soy proteins. The components responsible for the undesirable flavor of soybeans were not identified. Similarly, the volatile compounds associated with hydrolyzed soy proteins were investigated without conclusive evidence regarding the contribution of the identified compounds to the flavor of soy proteins (11).

Investigation of the nonvolatile components that contribute to the bitter/astringent flavor of soy proteins has focused on the compounds extracted with aqueous ethanol or hexane/ethanol mixtures (6,7,12,13). Honig *et al.* (13) extracted and isolated components from hexane-defatted soybean flakes with hot 95% ethanol and an ethanol/hexane azeotrope. Subsequent fractionation produced a mixture of components that contributed to the intensely bitter aftertaste, but the only compounds identified in this fraction (including isoflavones) did not contribute such flavors. The investigators concluded that minor, unidentified components were mainly responsible for the undesirable flavors.

Arai *et al.* (12) found seven phenolic acids associated with soy flour. Subsequent investigations by Rackis *et al.* (14) and Maga and Lorenz (15) indicated that the level of combined phenolic acids in soybean flour is close to their synergistic flavor threshold level of 40 to 90 parts per million (ppm). However, processing modifications that substantially reduced the level of phenolic acids in SPI resulted in no significant improvement in the bitter/astringent flavor properties of SPI (16).

An investigation of the undesirable flavor components extracted from whole soybean meal with 70% ethanol (7) concluded that the soy saponins and isoflavones contributed to the bitterness and astringency of whole soybeans, of which soy saponin A group made the strongest contribution. This investigation provided flavor thresholds and profiles in water for many soy saponins and isoflavones. The level of total saponins in SPI was previously determined to be 0.76% (17), with approximately one-sixth of it being the soy saponin A (18). Because the level of saponin A in soy protein products (including SPI) is approximately 1000 times greater than its flavor threshold, this compound likely contributes to the bitterness/astringency of soy protein products.

Oxidized PC in aqueous suspensions was reported to develop a bitter taste (19) with a flavor threshold value of

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0.006% (20). Because defatted soy flakes contain approximately 0.08% oxidized PC (13 times above threshold), this was proposed to contribute to the bitterness in soy protein products. However, Honig *et al.* (13) found no such flavors associated with the phospholipids isolated from defatted soy flakes.

In addition to the bitterness/astringency of soy proteins, the components that contribute to the characteristic "beany" flavor have been thought to be derived from the action of soybean lipoxygenase and subsequent formation of lipid oxidation products, including low molecular weight acids, alcohols, aldehydes, ketones, and furans (3,20). The levels of volatile aldehydes, ketones, etc. associated with soy protein products are usually reported as parts per billion in the headspace over a sample. Among the volatile aldehydes detected in unheated and heated SPI, hexanal and ethanal were present in the greatest amount (9,10). Sessa and Rackis (21) concluded that the beany flavor of soybean was likely due to 2-pentyl furan, 3-*cis*-hexenal, and 1-penten-3-one. Of these compounds, the only one subsequently detected by headspace sampling was 2-pentyl furan (9,10).

This current investigation was undertaken to examine the total lipid extracts from SPI and to determine which compounds contributed significant odors. Quantifying these odorous compounds from SPI and determining their flavor thresholds in water were accomplished to determine their relative importance to the flavor of SPI.

MATERIALS AND METHODS

Materials. SPI, designated as Pro Fam 970 (referred to as Samples A and B to distinguish the different code dates), were obtained from the Archer Daniels Midland Co. (Decatur, IL). Butyric acid, 2-methyl butyric acid methyl ester, and hexanal were obtained from Sigma Chemical Co. (St. Louis, MO). Lancaster Synthesis Inc. (Windam, NH) provided 2-pentyl pyridine. Bedoukian Research, Inc. (Danbury, CT) donated 2-pentyl furan.

Lipid extraction. Lipid extraction from SPI was accomplished by a modification of the method of Bligh and Dyer (22) as previously described (23,24). Approximately 2 g SPI was extracted twice, each time with 20 mL chloroform/methanol/water (5:10:4, vol/vol/vol).

The lipids obtained from each extraction were combined and brought to near dryness with a rotary evaporator at 50°C at 0.7 kg/cm^2 vacuum, followed by removal of the last few mL of solvent with a stream of dry nitrogen. Then, samples were suspended in 300 µL methylene chloride and stored in a freezer at −15°C. Throughout the extraction procedure, the samples came into contact only with glass and Teflon that had been rinsed with methanol/chloroform (2:1, vol/vol) and chloroform. To examine the stability of certain compounds during the extraction process, the process was repeated with butylated hydroxyanisole and butylated hydroxytoluene added to the extraction solvent at a concentration at which the final lipid extract would contain approximately 250 ppm of each antioxidant (based on an SPI lipid content of 4%). The extraction blank was prepared by repeating the extraction procedure without SPI.

Gas–liquid chromatography, olfaction, and MS. Identification of compounds that contribute undesirable odors to SPI was accomplished in a Perkin-Elmer (Norwalk, CT) Autosystem gas chromatograph with integrated autosampler, equipped with a hydrogen flame-ionization detector (FID) and a model 970 Intelligent Interface for data analysis. For olfaction, a portion of the entire lipid extract that corresponded to approximately 25 mg SPI was loaded onto the injector liner and held splitless for 30 s. The injector liner (maintained at 210°C) was packed with silanized glass wool and changed after the duplicate run for each sample. The outlet from a Hewlett-Packard (Palo Alto, CA) HP-1 capillary column (30 m × 0.25 mm i.d.) with 0.25 µm film thickness, SE-54 capillary column (Alltech Associates, Inc., Deerfield, IL) (30 × 0.25 mm i.d.) with 0.25 µm film thickness, or a DB-225 capillary column (J&W Scientific, Folsom, CA) (30 × 0.32 mm i.d.) with 0.25 µm film thickness was divided with an O.S.-2 outlet splitter system (SGE Intl., Ringwood, Australia). One-third of the splitter outlet was directed to the FID detector (maintained at 250°C), and the other two-thirds to a sniffing port (SGE Intl.). The helium flow rate through the column was 1.0 mL/min, and the makeup gas supplied to the splitter-tee was 4 mL/min. The Kovats indices of the detected odors were determined from FID response to C_{10} and C_7–C_{25} odd carbon-numbered paraffins. There was no measurable delay between detected odor and FID response.

Gas–liquid chromatography/MS was done on a Hewlett-Packard Model G1800A GCD System (Wilmington, DE), equipped with an electron ionization detector (EID) and Model G1030A Chemstation controller, and the same series of capillary columns was used for olfaction. The column temperature on both GC systems was held at 40°C for 5 min then raised at 3°C/min to 150°C, then raised to 230°C at 20°C/min. High-purity helium was the carrier gas at 1.0 mL/min. The EID was set to detect in the mass range of 35 to 175 *m/z*. The ionization voltage applied was 70 eV. Compounds were quantitated on the basis of one characteristic ion to ensure adequate resolution from any adjacent interfering compounds. The ions used were: 2-pentyl pyridine (93), 2-pentyl furan (81), hexanal (44), butyric acid (60), and 2-methyl butyric acid methyl ester (88). All determinations were performed in duplicate. Identification of lipid compounds was accomplished by comparison of mass spectra and retention times to authentic standards and olfactory response.

Size-exclusion chromatography (SEC). SEC was performed on a Sephadex LH-20 (Pharmacia, Piscataway, NJ) with 100% chloroform as the mobile phase. This was accomplished with an LKB model 2150 high-performance liquid chromatography (HPLC) pump (Pharmacia), equipped with a 100-µL injection loop attached to a 1.0 × 90 cm glass column that was covered with an ultraviolet (UV) light inhibitor. Peaks were monitored with a Beckman model 160 absorbance detector (Beckman Instruments, Inc., Fullerton, CA), set at

254 nm. The flow rate was 0.8 mL/min. Molecular weight standards used were β-carotene (MW 537), triphenyl phosphate (MW 326), and 2-pentyl pyridine (MW 149) (Fig. 1A). Each day the SEC was run, the column was recalibrated with triphenyl phosphate only.

HPLC. Samples were prepared by loading 100 mg lipid extract onto a silica solid-phase extraction cartridge in chloroform and passing 5 mL chloroform through the cartridge. The material subsequently eluted with 4 mL of 15% methanol in chloroform was collected, brought to near dryness under a stream of nitrogen at 50°C, and resuspended in 100 µL 20% isopropyl alcohol (IPA) in hexane. HPLC separations were accomplished on a Rainin Gradient HPLC System (Ridgefield, NJ) with two model HPXL pumps, model 805 pressure module, Dynamax HPLC gradient controller, Dynamax model UV-D II variable UV detector set at 260 nm, and a 50-µL injection loop. Lipids were separated on a Dynamax Microsorb 5-µm silica column (4.6 × 250 mm) with a 5-µm silica guard column (4.6 × 15 mm). The flow rate was 1.0 mL/min with a solvent gradient of 1.5% (vol/vol) IPA in hexane at the start to 6% IPA at 20 min.

Flavor threshold values. A 12-member panel, composed of 10 women and 2 men, ages 24 to 54, was familiarized with the typical odor and flavor of each compound by performing an open group discussion prior to individual evaluation. This was done to establish a common description for each compound and to determine if panelists were capable of detecting the compound being evaluated. The initial evaluation of each compound was accomplished at a level in water corresponding to that found in SPI, except that 2-pentyl pyridine, because of its repulsive flavor, was evaluated initially at only 0.05 ppm. Panelists who could not detect a specific compound at the initial level examined were excused from its evaluation if their numbers did not exceed 15% of the total number of panelists participating. If more than 85% of panelists could not detect the compound at the initial concentration, no further testing was done on that compound. Only compounds with "generally recognized as safe" (GRAS) status were used for sensory evaluations. The room where individual sensory evaluations were performed was equipped with individual booths that were separated from the sample preparation room by individual passage doors for sample presentation.

To determine compound flavor thresholds, samples of water from a Barnstead Nanopure 4-Module System (Fisher Scientific, Pittsburgh, PA) were presented to the panelists, along with the samples in water (at 27 ± 1°C) by using the three-alternative forced choice model (25). Samples were presented in 1.5-ounce glasses. Each panelist performed six replications at each concentration of each compound spaced by a factor of 10 in increasing order of concentration. PROC NLIN (26) was used to analyze the threshold data for each panelist and compound individually by fitting the nonlinear regression models by means of the least squares method. To calculate detectable flavor thresholds for each panelist, the percentage correct responses (above chance) were calculated at each concentration for each compound (27). The concentration at which a panelist gave the correct response 50% of the time (above chance) was considered to be the detectable flavor threshold for that particular panelist for that specific compound. Group flavor thresholds were calculated from the geometric means of the individual detectable flavor thresholds.

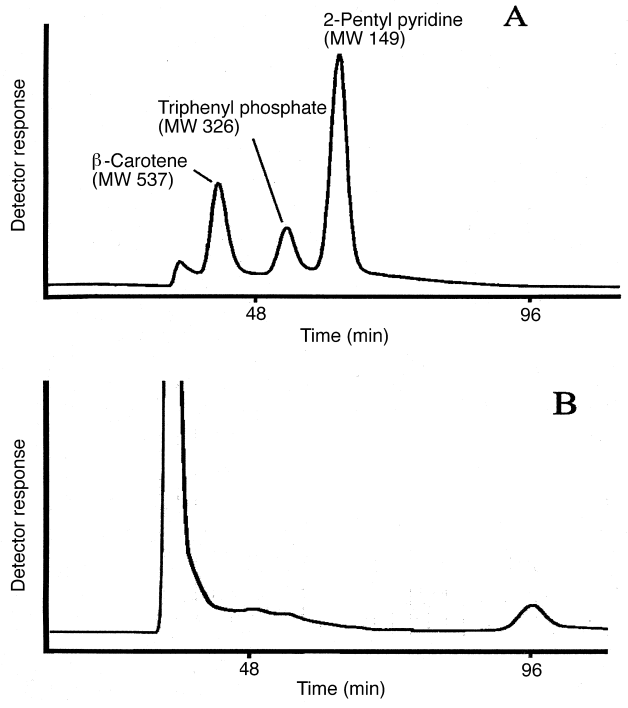


FIG. 1. Gel permeation chromatography on Sephadex LH-20 (Pharmacia, Piscataway, NJ) of (A) standards and (B) total lipid extract from soy protein isolates.

RESULTS AND DISCUSSION

This investigation incorporated an evaluation of the entire volatile-lipid contents extracted from SPI by combined GC, MS, and olfaction. This technique, which has been used in the analysis of components of other foods, has not been included in any published data concerning the components associated with soy protein products (28). The scope of this procedure is limited because it uses detection by smell. Compounds whose contribution to the flavor of soy protein products is perceived primarily through oral receptors may be underestimated. Also, samples with a boiling point below that of the solvent (methylene chloride, 40°C) and those that are not strongly retained by the stationary phase may not be detected. However, after careful consideration of available methods that provide for the analysis of a broad range of compounds, combined GC/MS/olfaction provided the most comprehensive results.

To implement the evaluation of the total lipid extracts from SPI by GC/MS/olfaction, quantitative extractions were performed on two different code dates of commercial SPI. The composition of these isolates was similar to the commercial isolates in a previous investigation (24). These isolates contained 3.9 and 4.1% total lipids by weight on a dry basis. Total

TABLE 1
Distinct Odors Identified from Soy Protein Isolates (SPI) by Gas Chromatography/Olfaction and Their Mean Kovats' Indices on Various Stationary Phases

Odor description	Relative Intensity[a]	Kovats' indices on HP-1	Kovats' indices on SE-54	Kovats' indices on DB-225
Fruity	++	756	753	945
Oxidized/nutty	+	759	771	n.d.
Burnt sulfur	++	786	796	998
Varnishy	+	n.d.	n.d.	1013
Beany	+	n.d.	n.d.	1029
Burnt	+	n.d.	n.d.	1142
Sweaty feet[a]	+++	796–830	805–828	1237–1259
Old sweaty socks	++	894	922	1349
Mushrooms	+	959	976	n.d.
Burnt	++	1053	1096	1343
Moldy fruit	+++	1144	1156	1396
Penetrating/ grassy	++	1187	1192	1468

[a]Odor was detected over the entire range shown. Abbreviations: n.d., not detected; + = mild; ++ = medium; +++ = strong.

lipid extracts from each soy product were analyzed by combining GC/olfaction with GC/MS. Compounds detected by olfaction were correlated with their mass spectra on a given stationary phase by using Kovats' indices. Compounds with Kovats' indices greater than 1500 were not examined. This analysis was repeated twice for each sample on each of three different stationary phases of different polarity (HP-1, SE-54, and DB-225). Table 1 lists the distinct odors detected, their relative intensities, and their Kovats' indices. The clearly distinguishable odors and their relative intensities, described in Table 1, were similar in both samples of SPI examined. There were some inconsistencies between results obtained on one stationary phase compared to another. For example, the mushroom odor was detected on the HP-1 and SE-54 columns (973 and 959 Kovats' indices, respectively) but not on the DB-225. This mushroom odor was not due to 1-octen-3-ol, the compound usually associated with this type of odor in soybean products. The varnishy, beany, and burnt odors were detected on the DB-225 column (Kovats' indices of 1013, 1029 and 1142, respectively) but not on the other two stationary phases. Each of these inconsistencies involves an odor that was relatively mild and may be due to radically different retention times between the nonpolar columns (HP-1 and SE-54) and the mid/high polarity DB-225 column (e.g., the varnishy, beany, and burnt odors appear to elute too early on the nonpolar stationary phases to be observed). All compounds that were detected as medium to strong odors were consistently detected regardless of the column used.

The compounds that were identified as corresponding with the observed odors through matching Kovats' indices on all three stationary phases are presented in Table 2. Correlating the Kovats' indices of the odor with the corresponding mass spectra was not achieved for some compounds, owing to coelution, significant overlapping of the various mass spectra, lack of documented matching mass spectra, or perhaps insufficient material to be detected with the mass spectrometer. After compounds were tentatively identified based on their mass spectra, authentic standards were obtained and their retention times, Kovats' indices, odor intensities, and odor descriptions (at the level found in the protein isolates) were confirmed by both GC/olfaction and GC/MS. The concentrations of these identified compounds in the final lipid extracts were similar in extracts performed with added antioxidants. This indicates that no significant oxidative degradation of the examined lipids occurred during the extraction process.

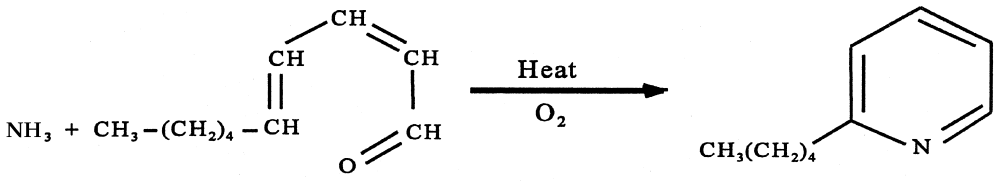
Because compounds that are present at relatively small quantities can have significant impact on odor and flavor, it is important to ensure that the compounds identified as significant contributors to the odor of soy products are not being formed as a result of exposing a complex mixture of lipids, primarily phospholipids (29), to the 210°C GC injector. To minimize the possibility of artifact formation, the low-MW compounds (≤350 Daltons and eluting beyond 48 min) were separated from the higher-MW compounds (e.g., phospholipids; MW ~760) by gel permeation chromatography on lipophilic Sephadex LH-20 (Fig. 1). Compounds in the MW range of ≤350 were collected and subjected to separation by GC/olfaction and GC/MS. Each identified odor contributor was also found by this method. The peak at 96 min was predominantly isomaltol (MW 126). This longer-than-expected retention time for a compound of this size indicates its interaction with the column packing by means other than strict permeation.

Additional precautions were taken to ensure that 2-pentyl pyridine was not being formed in the GC injector, because one proposed mechanism for the formation of 2-pentyl pyridine involves the heating of ammonia and 2,4-decadienal in

TABLE 2
Compounds Identified in SPI, Corresponding Odor Description, and Kovats' Indices of Standards

Odor description	Compound	Kovats' indices on HP-1[a]	Kovats' indices on SE-54[a]	Kovats' indices on DB-225[a]
Fruity	2-Methyl butyric acid methyl ester	756	760	945
Oxidized/nutty	Hexanal	769	773	1041
Sweaty feet[b]	Butyric acid	796–840	805–828	1237–1259
Penetrating/grassy	2-Pentyl pyridine	1187	1201	1465

[a]Chromatographic columns are identified in the Materials and Methods section.
[b]Odor was detected over the entire range shown. See Table 1 for abbreviations.



SCHEME 1

the presence of oxygen (30) (Scheme 1). The total lipid extracts from SPI were fractionated by silica solid-phase extraction with 100% chloroform to elute the nonpolar lipids (including 2,4-decadienal), then 15% methanol in chloroform to elute compounds of intermediate polarity. The 2-pentyl pyridine standard was found to elute with 15% methanol by following this procedure. This lipid fraction was then applied to normal-phase HPLC, and the eluting compounds that corresponded to the retention time of standard 2-pentyl pyridine were collected and analyzed by GC/MS. Fractions from the SPI extracts contained 2-pentyl pyridine at approximately the same level as the original whole-lipid extract. This confirms that 2-pentyl pyridine was not an artifact of GC analysis. In addition to running appropriate blanks in solvent-washed glass and Teflon containers, the presence of 2-pentyl pyridine in the residue of ethyl ether extracts (at approximately 0.005 ppm of SPI) confirms that it is not an artifact of the chloroform/methanol/water extraction process. This considerably lower amount is most likely due to the inefficiency of the ethyl ether extraction.

After the odor-contributing compounds from SPI were identified, they were evaluated for their flavor characteristics in water. 2-Pentyl furan, commonly noted as a contributor to the beany flavor of soy products (but not identified by GC/olfaction), was also evaluated. The flavor description, threshold, and mean content of each compound in SPI are given in Table 3. Because its flavor threshold was above its mean content in SPI (0.113 and 0.089 ppm), the sensory properties of 2-methylbutyric acid methyl ester were not investigated further. The primary interest of this portion of the investigation was to identify compounds that contribute an undesirable flavor at a level corresponding to one-tenth their concentration found in SPI (10% solutions of the commercial SPI examined by this laboratory have a strong undesirable flavor).

The mean contents of 2-pentyl pyridine in the two samples of SPI were above its flavor threshold by factors of 23,333 and 84,167. Furthermore, its flavor characteristic is extremely repulsive, being described by our sensory panelists as throat-catching and grassy with a fishy odor at 0.05 ppm in water. In contrast, the concentrations of 2-pentyl furan in SPI were 433 and 592 times greater than its flavor threshold value, and the concentrations of hexanal were 112 and 201 times greater than its flavor threshold value. Thus, neither 2-pentyl furan nor hexanal would make a significant flavor contribution in a 10% SPI solution.

Other pyridines tentatively identified in SPI were 2-methyl pyridine, 2-methyl-6-propyl pyridine, and 2-methyl-5-butyl pyridine. These compounds were detected at considerably lower levels than 2-pentyl pyridine but may have a synergistic effect. None of these alkylpyridines has previously been reported in soy products.

The flavor thresholds of both hexanal and 2-pentyl furan were close to their published odor threshold values in water. The flavor threshold of 2-pentyl pyridine (0.000012 ppm) is approximately 50 times lower than its published odor threshold value (0.0006 ppm). Also, its odor profile, described as fatty or tallowy by Buttery *et al.* (30), is greatly different from its throat-catching flavor profile observed by our panelists. These observations indicate that, when 2-pentyl pyridine is swallowed, it provokes a sensory response in the region of the mouth as well as in the olfactory epithelium. The flavor of 2-pentyl furan at levels well above its threshold was relatively mild. This is likely why it was not among the clearly distinguishable odors detected by olfaction.

TABLE 3
Flavor Description, Threshold in Water, and Content of Selected Compounds in SPI

Compound	Flavor description	Flavor threshold (ppm)	Published odor threshold (ppm)	Mean Content in SPI (ppm)[a]	
				A[a]	B[a]
Butyric acid	Sweaty feet	3.19	0.2[b]	5.64	23.96
Hexanal	Oxidized oil	0.0075	0.0045[c]	1.51	0.84
2-Pentyl furan	Grassy	0.0048	0.006[c]	2.84	2.08
2-Pentyl pyridine	Throat-catching grassy	0.000012	0.0006[d]	0.28	1.01

[a]Samples described in the Materials and Methods section.
[b]Reference 35.
[c]Reference 36.
[d]Reference 30. See Table 1 for abbreviation.

The reported occurrence of 2-pentyl pyridine in foods is primarily in roasted or fried meats. Buttery *et al.* (30) identified 14 alkylpyridines from roast lamb fat. The most abundant was 2-pentyl pyridine. They suggested that these alkylpyridines contribute to the undesirable taste that is often associated with lamb and mutton. Also, 2-pentyl pyridine has been reported in the volatiles of fried beef (31) and fried chicken (32). No quantitative data were provided in these investigations.

One proposed mechanism for the formation of 2-pentyl pyridine is the interaction of 2,4-decadienal, which was quantitated in SPI at 0.022 to 0.036 ppm (24), with ammonia (Scheme 1). Bains and Mlotkiewicz (33) suggested that the availability of ammonia may be the determining factor in the formation of the alkylpyridines, and noted that lamb contains a higher concentration of amino acids and has a lower sugar content than other species. They suggested that, with a high protein content and a relatively low sugar content, amino acids are more likely to be degraded to ammonia than to be involved in the Maillard reaction. In the Maillard reaction, the nitrogen from amino acid degradation is more likely to be incorporated into compounds such as pyrazines, which contribute more desirable flavors. A subsequent investigation of beef fat heated with glycine demonstrated that both 2-butyl pyridine and 2-pentyl pyridine were formed. This reaction mixture possessed an undesirable odor, in contrast to a pleasant odor produced when the system contained sugar (34). Reaction mixtures that contained an amino acid (either glycine, lysine, or cysteine) and ribose with egg lecithin also produced 2-pentyl pyridine. The mixture with cysteine produced from 20- to 100-fold greater amounts of 2-pentyl pyridine, compared to the glycine and lysine mixtures, respectively. Ammonia is believed to be produced from the Strecker degradation of cysteine. Also, mixtures of glycine and lysine with ribose produced greater amounts of alkylpyrazines than those with cysteine, indicating that ammonia is not a major intermediate through which nitrogen enters the pyrazine ring in these systems. These model systems involve the application of heat (e.g., 200°C for 4 h or 140°C for 2 h).

Of the odors identified in Table 1, several corresponding compounds have not yet been identified. These observed odorous compounds will be investigated further. However, because of the strong undesirable sensory characteristics of 2-pentyl pyridine, reducing its occurrence in SPI should result in significantly improved SPI flavor.

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