Precursors for Formation of 2-Pentyl Pyridine in Processing of Soybean Protein Isolates

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ABSTRACT: Using stable isotopic ^{13}C-2,4-decadienal and ^{15}N-ammonia, both compounds were confirmed as precursors in the formation of 2-pentyl pyridine (2-pp) in processing of soybean protein isolates (SPI). The yields of 2-pp were higher when adding 2,4-decadienal and ammonia together than the yields when adding 2,4-decadienal only for soybean cultivars Stressland, LOX null, Edison, and KS4694. Arginine, lysine, asparagine, and glutamine increased 2-pp when added together with 2,4-decadienal, whereas aspartic acid, glutamic acid, glycine, and histidine had no effects. In both buffer and defatted soybean slurry, the highest level of 2-pp was found at pH 9, followed by pH 7, then pH 4.5. The results indicate that the synthesis of 2-pp may be a spontaneous reaction.

Key Words: soybean protein isolate, 2-pentyl pyridine, ^{13}C labeled 2,4-decadienal, ammonia, amino acids

Introduction

THE OCCURRENCE OF 2-PENTYL PYRIDINE (2-pp) has been reported primarily in roasted or fried foods (Ho and others 1987). Buttery and others (1977) identified it as the most abundant alkylpyridine in roast lamb fat. It has also been reported in fried chicken (Tang and others 1983), fried beef (Watanabe and Sato 1971), roasted sesame seed oil (Schieberle 1993), and soybean lecithins (Stephan and Steinhart 1999). In soybean protein isolates, 2-pp has the largest reported flavor value of any volatile compound found in soybean products (Boatright and Crum 1997). The levels of 2-pp are affected by pro-oxidants ($FeCl_3$, $CuCl_2$), UV light, and pH changes during SPI processing (Boatright and others 1998; Zhou and Boatright 1999).

It was proposed that the formation of 2-pp resulted from the interaction of ammonia with 2,4-decadienal (Buttery and others 1977). In this mechanism, ammonia condensed with 2,4-decadienal to form a Schiff base intermediate, followed by ring closure, leading to the formation of a dihydropyridine that could then be oxidized to 2-pp. Schieberle (1993) showed that the amount of 2-pp was increased by the addition of ammonium sulfate before roasting in sesame oil. However, Zhang and Ho (1989) found that free ammonia was not available in the thermal interaction of 2,4-decadienal with glutathione. They proposed an alternative mechanism in the formation of 2-pp as a result of the direct condensation of the amino group of amino acids or peptides with the aldehydic group of 2,4-decadienal. It has been known for many years that glutamine and asparagine can undergo deamidation to release free ammonia (Riha and others 1996). Therefore, amino acids may not react with 2,4-decadienal directly. But through deamidation the free ammonia can react with 2,4-decadienal to form 2-pentyl pyridine.

In this study, we used stable isotopes to study N-source and C-source in the synthesis of 2-pp when making soybean protein isolates.

Material and Methods

Preparation of defatted flour from soybeans

Stressland, Edison, and KS4694 variety soybeans were obtained from the Purdue University USDA–ARS soybean breeding and genetics program. Soybeans null for lipoxygenase 1, 2, and 3 were obtained through the University of Kentucky Agronomy Department. Defatted soybean flour was prepared by cracking the beans in a blender and removing the hulls by aspiration. The dehulled bean pieces were then ground and passed through a 20-mesh screen. One part full-fat flour was mixed with 10 parts hexane, agitated for 10 min, and centrifuged at 1000 × g for 10 min at 20 °C. The hexane micella (supernatant) was decanted and discarded. The extraction was repeated two more times on the resulting pellet. Hexane was evaporated from defatted flour in a fume hood overnight.

Preparation of soybean protein isolates

For the typical processing of soybean defatted flour to obtain protein isolate (Fig 1), defatted flour was mixed with water (1:10 w/w) from a Barnstead Nanopure 4-Module System (Fisher Scientific, Pittsburgh, Pa., U.S.A.), the pH was increased with 1 N NaOH to pH 9.0, and the mixture was held for 1 h. After centrifugation at 1500 × g for 10 min, the supernatant pH was decreased with 1 N HCl to pH 4.5 and held another 1 h. Then a second centrifugation at 1500 × g for 10 min was performed. After freezing overnight, samples were lyophilized in a Freeze-Dry System LYPH LOCR 4.5 (Labconco, Kansas City, Mo., U.S.A.) under vacuum at room temperature for 2 d.

Lipid Extraction

Lipid extractions were accomplished by a modification of the well-known method using chloroform partitioning. Approximately 1 g of lyophilized sample was extracted twice, each time with 20 mL of chloroform/methanol/water (5:10:4, v/v/v). The lipids obtained were brought to near dryness with a rotary evaporator at 50 °C with vacuum followed by removal of the last traces of solvent with a stream of dry nitrogen and then suspended in 300 μL methylene chloride and stored in a freezer at −15 °C.

Measurement of 2-pentyl pyridine

The deuterium-labeled 2-pentyl pyridine (d_5/d_6) was added as internal standard. A Hewlett Packard model G1800A GCD system (Palo Alto, Calif., U.S.A.) equipped with an electron ionization detector was used to measure 2-pentyl pyridine, described by Boatright and Crum (1997). Quantification was done by the ratio of m/z 93 ion to the m/z 99 and 98 ions from the internal standard (Guth and Grosch 1990). For each treatment, 2-pp was quantified in triplicate by GC/MS.

Cool, on-column injections were accomplished by installing an on-column injection sleeve on a Hewlett Packard Model

5890 Series II GC with a 5971A mass spectrometer. Injections were made with a 12.5-cm needle directly onto an EC-5 capillary column (30 m × 0.53 mm i.d.) with 1.2 m film thickness (Alltech Associates, Inc., Deerfield, Ill., U.S.A.). The helium flow rate through the column was about 3 mL/min, with 2 mL/min vented with a post-column splitter (SGE Intl., Ringwood, Australia). The capillary column between the post-column splitter and the mass spectrometer was 0.1 mm i.d. The injection port and initial oven temperature were set at 35 °C. The column temperature was then increased by 10 °C/min to 165 °C, held for 3 min, then increased to 210 °C at 10 °C/min where it was held for 10 min The EID was set to detect in the mass range of 35 to 250 m/z.

Synthesis of stable isotopic compounds

The ^{2}H labeled 2-pentyl pyridine was prepared using the method of Tchitchibabine (1936) and substituting 2-picoline-d_7 in place of unlabeled 2-picoline.

The amino acids and ^{15}N labeled ammonia were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A.).

The ^{13}C-labeled trans,trans-2,4-decadienal (Figure 2) was synthesized and purified in the lab by the modified method of Pippen and Nonaka (1958). Through the Barbier reaction, ^{13}C-labeled trans,trans-2,4-decadienal (VI) was generated by a reaction between ^{13}C-labeled hexanal (V) and 1-methoxy-1-Z-buten-3-yne (IV). The molar ratio (2:1) of 1-methoxy-1-Z-buten-3-yne to hexanal was higher than that in previous publication. ^{13}C-labeled hexanal was prepared by the method of Olah and others (1984). 1-methoxy-1-Z-buten-3-yne was synthesized by the method of Shostakovskii and Khomenko (1960) and purified by the method of Corey and Albright (1982). In the preparation of ^{13}C-labeled hexanal, ^{13}C labeled N,N-dimethylformide (II, from Sigma-Aldrich) was reacted with pentyl magnesium bromide (I). In the preparation of 1-methoxy-1-Z-buten-3-yne, 1,4-dichloro-2-butyne (III) was mixed with methanol and KOH at 65 °C. It was then interacted with freshly prepared ethyl magnesium bromide, 1-methoxy-1-Z-buten-3-yne condensed with 1-^{13}C- hexanal, and through these sequential reactions made to form 5-^{13}C -trans,trans-2,4-decadienal.

Incorporation of 2,4-decadienal, ammonia and amino acids

The 2,4-decadienal-chloroform solution (2 mL) was mixed with soybean flour slurry to give a final concentration of 5.64 mM. By adding chloroform only, as a control, no increase of 2-pp was observed in the slurry. Isotopic (^{15}N-labeled) ammonia was dissolved in H_2O, then ammonium hydroxide was mixed with the slurry directly. By titration with HCl, the SPI slurry contained 8.05 mM ammonia. Amino acids were mixed with dry defatted flour (8 mM). Mixtures of 2,4-decadienal and ammonia (or amino acids) in aqueous buffer solutions were directly extracted by chloroform/methanol/water (5:10:4, v/v/v). After the solvent was removed, methylene chloride (300 μL) was added. Then the suspension was injected into a GC/MS for 2-pp analysis.

Statistical analysis

Analysis of variance (ANOVA) was performed by SAS (1995) software package. Least significant difference (LSD) values were computed at $P < 0.05$, and comparison between means was done using the Tukey-Kramer HSD test.

Results and Discussion

DEUTERATED 2,4-DECADIENAL WAS used as an internal standard in isotope dilution assays (Lin and others 1999). However, for the study of reaction precursors, deuterated compounds usually cannot provide an accurate isotopic position that contributes to the formation of products because more than one position are usually deuterated during synthesis. Since ^{13}C-labeled 2,4-decadienal was not available commercially, the crucial step of this study was the synthesis of it. To synthesize 5-^{13}C-2,4-decadienal (Figure 2), it was important to maintain the whole system anhydrous and free of oxygen. Grignard reagent and tetrahydrofuran were prepared freshly before use in the synthesis. By mass interpretation, the mass spec-

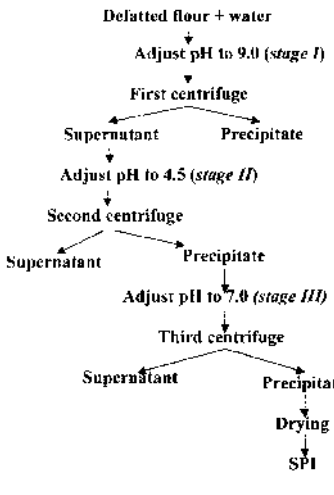

Figure 1 (above)—A typical scheme for processing defatted flour into soybean protein isolates (SPI).

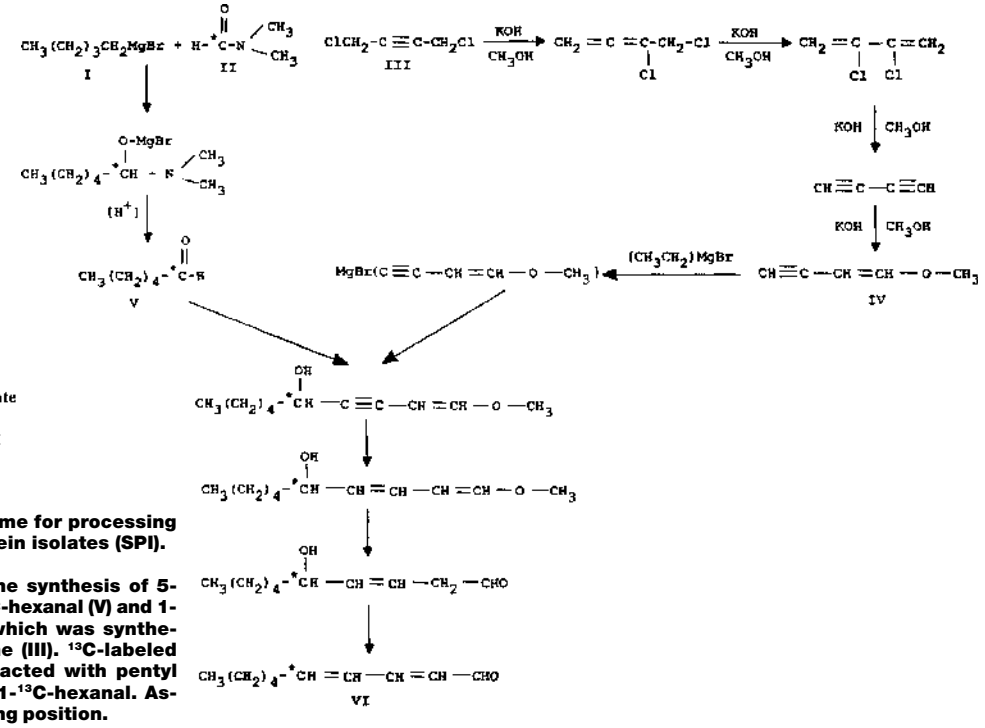

Figure 2 (right)—Schemes for the synthesis of 5-^{13}C-2,4-decadienal (VI) from 1-^{13}C-hexanal (V) and 1-methoxy-1-Z-buten-3-yne (IV), which was synthesized from 1,4-dichloro-2-butyne (III). ^{13}C-labeled N,N-dimethylformide (II) was reacted with pentyl magnesium bromide (I) to form 1-^{13}C-hexanal. Asterisk (*) indicates the ^{13}C-labeling position.

tra in Figure 3 were identified as 1-methoxy-1-Z-buten-3-yne (a), 1-^{13}C-hexanal (b) and 5-^{13}C-2, 4-decadienal (c).

Figure 4 shows the spectra of 2-^{13}C-2-pentyl pyridine (b) when adding 5-^{13}C-2,4-decadienal and of 1-^{15}N-2-pentyl pyridine (c) when adding ^{15}N-ammonia. The molecular ion was 149 instead of 148 for standard 2-pp, and after the first loss of methylene group, the ion in the labeled spectra was 135 instead of 134. When adding unlabeled 2,4-decadienal and ammonia, the base peak was m/z 93 $[M]^+$ (a) rather than m/z 94 $[M+1]^+$ when adding labeled precursors. The spectra of labeled 2-pp provided direct evidence for the first time, using ^{13}C- and ^{15}N-labeled isotopes, to confirm that 2-pp was synthesized from the interaction of ammonia with 2,4-decadienal in soybean protein isolates.

Table 1 shows the changes in 2-pp by adding 2,4-decadienal only, but sampling at stage I, II and III (Figure 1) during the SPI process for soybean varieties Stressland, LOX null, Edison, and KS4694. No significant differences were observed among various stages for LOX null and KS4694. For Stressland cultivar, a slight difference occurred between stages I and II. And for Edison, there was a slight difference between stages II and III. All the statistical differences were negligible compared to the increase in values by adding 2,4-decadienal. The results suggested that 2-pp was formed shortly after adding 2,4-decadienal, and remained during SPI processing.

When adding ammonia only, the levels of 2-pp increased slightly for all varieties, much less than the increase when adding 2,4-decadienal only (Table 2). However, when adding ammonia together with 2,4-decadienal, 2-pp increased greatly to the level that was much larger than the sum of adding 2,4-decadienal only and adding ammonia only. It was possible that the amount of 2,4-decadienal was very limited, but ammonia existed in soybean defatted flours. When adding ammonia only, the amount of original 2,4-decadienal was not adequate to synthesize 2-pp. On the other hand, the relatively high level of original ammonia could form 2-pp when 2,4-decadienal was added. When both precursors were added together, the added 2,4-decadienal reacted with both original and additional ammonia, and the concentration of 2-pp therefore increased to a high level.

Table 3 shows the results of 2-pp formation when adding 8 different amino acids. Like adding ammonia only, 2-pp did not increase significantly when adding any amino acid alone. When adding each amino acid together with 2,4-decadienal, arginine, lysine, asparagine, or glutamine induced the increase of 2-pp levels, whereas no significant increases were observed for aspartic acid, glutamic acid, histidine, or glycine. Compared to aspartic acid and glutamic acid, asparagine and glutamine induced higher levels of 2-pp, respectively. However, in neutral buffer solutions, arginine, lysine, asparagine,

Table 1—Formation of 2-pentyl pyridine (ppm) when adding 2,4-decadienal

	Stage I[c]	Stage II	Stage III
Stressland	2.330 ± 0.042[a] (4.426 ± 0.061a')[d]	2.546 ± 0.038b (4.483 ± 0.066b')	2.600 ± 0.032[b] (4.550 ± 0.049[b'])
LOX null[e]	2.122 ± 0.038[a]	2.378 ± 0.029[a]	2.077 ± 0.026[a]
Edison	2.427 ± 0.051[a]	2.432 ± 0.036[a]	2.798 ± 0.032[b]
KS4694	2.309 ± 0.035[a]	2.466 ± 0.029[a]	2.368 ± 0.040[a]

[a-b(a'-b')]Means (± standard deviation) within a row series with no common superscripts differ (P < 0.05; n = 3)
[c]See Figure 1
[d]Values in parenthesis from the samples when adding 2,4-decadienal and ammonia together
[e]Cultivar null for lipoxygenase 1, 2, and 3

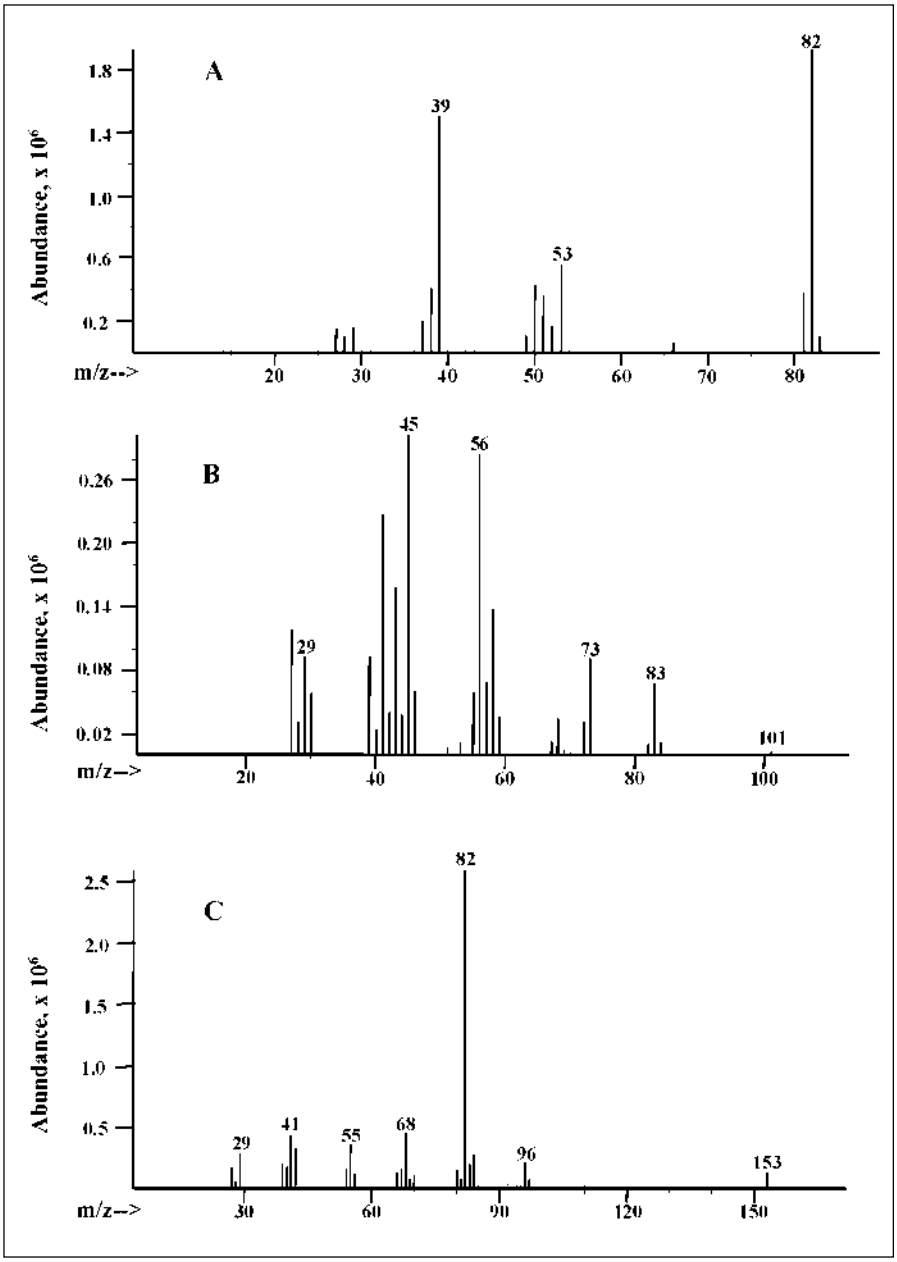

Figure 3—Mass spectra of 5-^{13}C-2,4-decadienal (c) synthesized from 1-methoxy-1-Z-buten-3-yne (a) and 1-^{13}C-hexanal (b).

and glutamine did not increase 2-pp levels at room temperature. Zhang and others (1993a, 1993b) demonstrated the nonenzymatic deamidation of glutamine and asparagine residues from soy protein at 100, 115, and 130 °C. The activation energies of soy proteins deamidation decreased sharply as the pH went up from pH 5 to pH 11. Kim and others (1996) and Kim and Ho (1998) found that, in aqueous or oil model systems (180 °C), respectively, ammonia was generated by the deamidation of amide side chains (^{15}N-labeled) of asparagine and glutamine. Then free ammonia reacted with 2,4-decadienal to synthesize 2-pp. However, this does not explain the increases induced by arginine and lysine in our results by similar deamidation processes. The contribution of amino nitrogen to the formation of pyrazines was reported previously (Izzo and Ho 1992). Because glycine produced no significant increase in 2-pp, it was also difficult to explain the increases induced by arginine and lysine through the release of ammonia from α-amino groups.

Free ammonia was found in the basic fraction from raw soybean (Arai and others 1966), but not in the acidic fraction. Figure 5 shows the effects of pH on the synthesis of 2-pp in the model buffer system and the Stressland defatted flour slurry when adding 2,4-decadienal and ammonia together. Under basic conditions, yields of 2-pp were higher than yields under either neutral or acidic conditions. At pH 9, ammonia was the main form with some ammonium hydroxide, whereas at pH 4.5 the ammonium ion was predominant. Our previous investigation (Zhou and Boatright 1999) found that the level of 2-pp did not increase when the defatted flour solution was not subjected to alkaline extraction. It suggested that alkaline condition assisted the removal of insoluble components, but on the other hand induced the formation of 2-pp. These results imply that ammonia is the active form in the synthesis of 2-pp with 2,4-decadienal, and the formation of 2-pp from 2,4-decadienal and ammonia was a spontaneous reaction at room temperature. Previous investigations by Kim and others (1996) and Kim and Ho (1998) showed the synthesis of 2-pp at elevated temperature (180 °C), but they did not deal with the reaction at room temperature in an alkaline medium.

It is important to ensure that 2-pp is not being formed as a result of exposing 2,4-decadienal and residual ammonia to the 210 °C GC injector. Since a stream of dry nitrogen was used to remove the solvents after extraction of 2-pp, ammonia was completely flushed out before GC injection. By sniffing, the odor of 2-pp was confirmed before GC/MS analysis. Also performing cool, on-column injections of the reaction products further confirmed that 2-pp was not an artifact caused by the high temperature of the injection port.

It has been thought that lipid oxidation is the major process contributing to the flavor of SPI. The results in this study show that 2,4-decadienal, a lipid oxidation product, is the carbon source for the formation

Table 2—Formation of 2-pentyl pyridine (ppm) when adding ammonia[e]

	No addition	2,4-decadienal only	Ammonia only	2,4-decadienal + ammonia
Stressland	0.214 ± 0.004[a]	2.375 ± 0.038[b]	0.295 ± 0.004[c]	4.269 ± 0.056[d]
LOX null[f]	0.138 ± 0.002[a]	2.015 ± 0.042[b]	0.189 ± 0.003[c]	4.189 ± 0.048[d]
Edison	0.172 ± 0.003[a]	2.591 ± 0.041[b]	0.194 ± 0.003[c]	4.593 ± 0.065[d]
KS4694	0.198 ± 0.003[a]	2.229 ± 0.035[b]	0.236 ± 0.003[c]	4.568 ± 0.055[d]

[a-d]Means (± standard deviation) within a row series with no common superscripts differ (P < 0.05; n = 3)
[e]Sampling at stage I, see Fig 1
[f]Cultivar null for lipoxygenase 1, 2 and 3

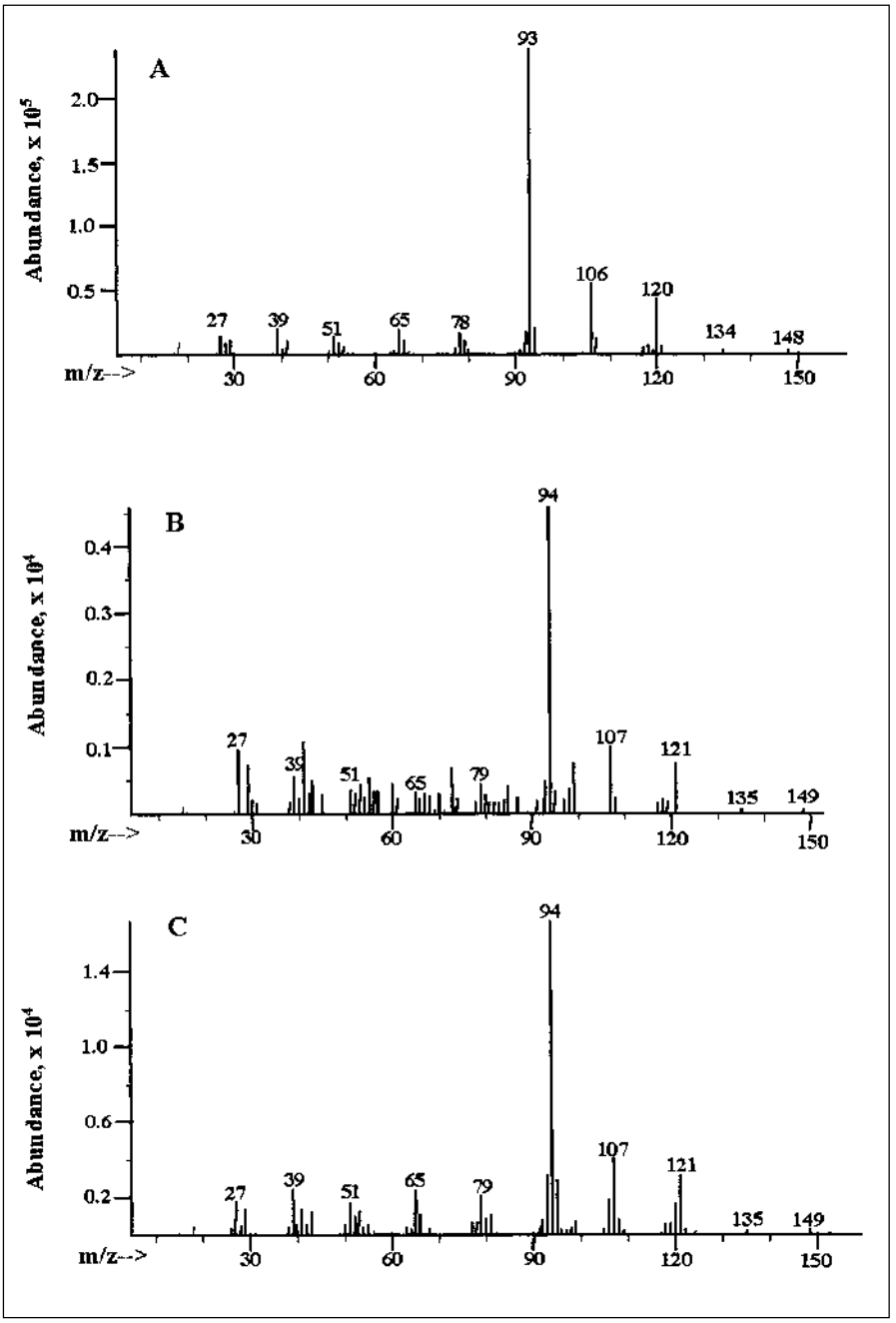

Figure 4—Comparison of mass spectra from unlabeled 2-pentyl pyridine (a), 2-^{13}C -2-pentyl pyridine (b) and 1-^{15}N-2-pentyl pyridine (c).

Table 3—Formation of 2-pentyl pyridine (ppm) when adding amino acids[d,e]

	2,4-decadienal only	amino acid only	2,4-decadienal + amino acid (in buffer)[f]
Arginine	2.469 ± 0.029[a]	0.179 ± 0.003[b]	4.668 ± 0.056[c] (2.398 ± 0.033[a])
Lysine	2.544 ± 0.031[a]	0.184 ± 0.003[b]	3.819 ± 0.046c (2.466 ± 0.028[a])
Aspartic acid	2.444 ± 0.022[a]	0.191 ± 0.005[b]	2.491 ± 0.029[a] (2.461 ± 0.022[a])
Asparagine	2.512 ± 0.030[a]	0.216 ± 0.004[b]	4.025 ± 0.044[c] (2.529 ± 0.029[a])
Glutamic acid	2.169 ± 0.019[a]	0.188 ± 0.003[b]	2.292 ± 0.022[a] (2.206 ± 0.021[a])
Glutamine	2.306 ± 0.022[a]	0.200 ± 0.002[b]	3.835 ± 0.039[c] (2.332 ± 0.031[a])
Glycine	2.288 ± 0.028[a]	0.178 ± 0.003[b]	2.334 ± 0.025[a] (2.296 ± 0.028[a])
Histidine	2.299 ± 0.031[a]	0.169 ± 0.003[b]	2.318 ± 0.030[a] (2.278 ± 0.026[a])

[a-c]Means (± standard deviation) within a row series with no common superscripts differ (P < 0.05; n = 3)
[d]Sampling at Stage I, see Fig 1
[e]Soybean cultivar was Stressland.
[f]Values in parentheses from the samples when adding 2,4-decadienal and amino acids in pH 7 buffer solution

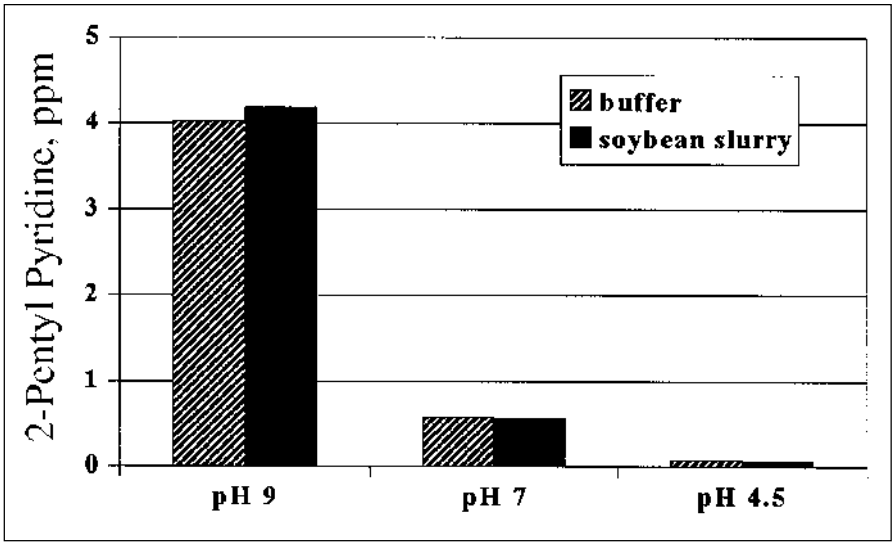

Figure 5—pH effects on formation of 2-pentyl pyridine in buffer systems and Stressland defatted flour slurries when adding 2,4-decadienal and ammonia.

of 2-pp. The nitrogen source was ammonia, which may be derived from amino acids through deamidation reactions. Ammonia can react with 2,4-decadienal to form 2-pp in a buffer system, especially at pH 9.0. However, amino acids do not contribute to the synthesis in buffer solution at room temperature (Table 3). Soybean protein may provide the matrix for deamidation of amino acids, possibly by enzymatic means. Both lipids and proteins are attributed to the formation of 2-pp and the flavor of soybean protein isolates.

In conclusion, the synthesis of 2-pp is a spontaneous reaction from 2,4-decadienal and ammonium hydroxide. Some amino acids may possibly provide ammonia in soybean protein isolates. To further understand the formation of 2-pp during processing of SPI, the binding properties of 2-pp to soy proteins may provide more conclusive evidence.

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