Effect of Pro-Oxidants on the Occurrence of 2-Pentyl Pyridine in Soy Protein Isolate

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ABSTRACT: Levels of 2-pentyl pyridine in hexane-defatted soybean flours prepared from Burlison, Stressland, and Probst varieties and a variety null for lipoxygenase 1, 2, and 3 were determined using an internal standard of deuterium-labeled 2-pentyl pyridine. These defatted flours contained from 0.06 to 0.51 ppm 2-pentyl pyridine. The flours from lipoxygenase-null and Stressland varieties of soybeans contained the lowest levels. Control freeze-dried soy protein isolates (SPI) prepared from defatted-flours all contained from 0.16 to 0.18 ppm 2-pentyl pyridine.

Adding pro-oxidants ($FeCl_3$, $CuCl_2$) or exposing the protein slurries to ultraviolet light during SPI processing increased the level of 2-pentyl pyridine in the protein isolates by up to 1256 percent above the control Burlison, Stressland, and Probst SPI. The $CuCl_2$ and/or UV treatment contributed the largest increase in each case. The same pro-oxidant and UV treatments contributed no significant increases in the level of 2-pentyl pyridine in the protein isolates from the lipoxygenase null SPI.
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KEY WORDS: Hexane-defatted soy flour, 2-pentyl pyridine, pro-oxidants, soybean protein isolate, stable isotopes.

Our previous investigation demonstrated that the level of 2-pentyl pyridine in commercial soy protein isolates (SPI) ranges from 23,333 to 84,167 times greater than its flavor threshold value in water and contributes a throat-catching/grassy flavor (1). Because 2-pentyl pyridine has the largest reported flavor value of any compound found in SPI it is a strong contributor to its overall flavor. While this was the first reported occurrence of this compound associated with soybean products, it had been previously found in roasted lamb fat (2) and the volatiles of fried beef (3) and fried chicken (4). No quantitative data were provided from these previous investigations. This investigation was undertaken to quantify 2-pentyl pyridine in defatted flours prepared from several different varieties of soybeans (including a variety null for lipoxygenase 1, 2, and 3) and corresponding SPI. This was done to confirm the occurrence of 2 pentyl pyridine in soy products from a noncommercial source and to investigate the effect of soybean variety differences on the occurrence of 2-pentyl pyridine. Also, because a proposed mechanism for the synthesis of 2-pentyl pyridine involves both lipid and protein degradation products (2), the effect of added pro-oxidants and ultraviolet (UV) light treatment on its occurrence in SPI was examined.

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MATERIALS AND METHODS

Soybeans and protein products. Burlison, Stressland, and Probst variety soybeans were obtained from the Purdue University USDA-ARS soybean breeding and genetics program (Purdue, IN). Soybeans null for lipoxygenase 1, 2, and 3 (5) were obtained through the University of Kentucky Agronomy Department. Defatted soybean flour with a particle size of ≤0.825 mm 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.

The laboratory SPI were prepared by dispersing the laboratory-prepared hexane-defatted soybean flour in water (1:10, flour/water) followed by additions of 1 N sodium hydroxide, as needed, until a pH of 9 is achieved and maintained for 15 min. After centrifugation at 1500 × *g* for 10 min, the supernatant was adjusted to a pH of 4.5 with 1 N HCl to precipitate proteins. Following centrifugation at 1500 × *g* for 10 min, the precipitate was washed with water and the protein isolate was adjusted to pH 7 with 1 N sodium hydroxide and freeze-dried.

$FeCl_3$ and $CuCl_2$ were added during SPI processing by substituting 25 μM solutions, individually or in combination, for the water used at each step of SPI processing. Treatment of SPI with UV light was processed by exposing the protein slurry during the entire process up to the point where the slurries were frozen to direct UV radiation from a dual wavelength UV light (365 and 254 nm).

Chemicals. Sodium amide, 1-chlorobutane, and 2-picoline-d_7 were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI, and St. Louis, MO). Lancaster Synthesis Inc. (Windam NH) provided 2-pentyl pyridine.

2-Pentyl pyridine-d_6/d_5 was prepared using the method of Tchitchibabine (6), substituting 2-picoline-d_7 in place of unlabeled 2-picoline. The deuterated standard was purified [99.9%

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pure by gas chromatography/mass spectrometry(GC/MS)] by preparative thin-layer chromatography using ethyl ether/hexane (4:6, vol/vol) on silica gel G (1.0 mm thickness).

Concentration of deuterium-labeled 2-pentyl pyridine was determined by GC/MS with unlabeled 2-pentyl pyridine as an internal standard. The standard curves were prepared with mixtures containing known amounts of the labeled and unlabeled compound in the appropriate range. The ratio of the relative abundances of the ions selected for quantifying (*m/z* 93 for 2-pentyl pyridine and *m/z* 98 and 99 for the labeled 2-pentyl pyridine with five and six deuteriums, respectively) were plotted against the weight ratio of the labeled compounds over the sum of the labeled and unlabeled compounds as described by Guth and Grosch (7).

Lipid extraction. Lipid extractions from SPI and defatted flours were accomplished by a modification of the method of Bligh and Dyer (8) as previously described (9,10). Approximately 1 g of SPI was extracted twice, each time with 20 mL of chloroform/methanol/water.

The lipids obtained from each extraction were combined and brought to near dryness with a rotary evaporator at 50°C with 0.7 kg/cm^2 vacuum, followed by removal of the last few milliliters of solvent with a stream of dry nitrogen, and then suspended in 300 μL methylene chloride and stored in a freezer at –15°C. Throughout the extraction procedure these materials came into contact only with glass and Teflon rinsed with methanol/chloroform (2:1, vol/vol) and chloroform. Labeled 2-pentyl pyridine was added during the first solvent/soy product mixture at a level corresponding to 0.202 ppm of the soy product.

GC/MS. GC/MS was done on a Hewlett-Packard Model G1800A GCD System (Wilmington, DE) equipped with an electron ionization detector (EID) maintained at 250°C and a Model G1030A Chemstation controller. Separations were performed on a DB-225 capillary column (30 × 0.25 mm i.d.) with 0.25 μm film thickness (J&W Scientific, Folsom, CA), with a 1 min splitless injection. The injector liner (maintained at 210°C) was packed with silanized glass wool and changed after the duplicate run for each sample. The column temperature was held at 40°C for 5 min, raised 3°C/min to 165°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*. 2-Pentyl pyridine was quantified by the ratio of *m/z* 93 ion (at the appropriate retention time) in the lipid extracts to the *m/z* 99 and 98 ions for the internal standard 2-pentyl pyridine-d_6/d_5. *Trans,trans*-2,4-decadienal was quantified using a standard curve of the ratio of its primary ion (*m/z* 81) and the ions *m/z* 99 and 98 for the internal standard 2-pentyl pyridine-d_6/d_5. All determinations were performed in duplicate.

Statistical analyses. Statistical evaluation was done using the Statistical Analysis System (15) software package. Analysis of variance was performed. Least significant difference (LSD) values were computed at $P \leq 0.05$, and comparison between means was done using the Tukey-Kramer HSD test.

RESULTS AND DISCUSSION

Compositional data for the defatted flour and SPI are presented in Table 1. As with previous samples of SPI (11,12), the lipid content in the prepared SPI was greater than the concentration found in the defatted flour. The protein contents of all SPI ranged from about 78 to 84% protein, which is similar to other commercial and laboratory-prepared SPI (11–13). This variation may relate to the variety of soybeans with the lipoxygenase-null variety producing the highest protein content SPI.

To accurately quantify 2-pentyl pyridine in these samples, an internal standard of 2-pentyl pyridine labeled with deuterium was synthesized. The mass spectra of the labeled and unlabeled 2-pentyl pyridines are shown in Figure 1. The *m/z* ion used for quantifying the unlabeled compound was 93 and for the labeled compounds was 98 for 2-pentyl pyridine-d_5 and 99 for 2-pentyl pyridine-d_6. The presence of major peaks at *m/z* 98 and 99 from the labeled compounds demonstrates that one of the six possible deuterium labels was lost during the synthesis reactions. This occurred to about a third of the material. Thus, 2-pentyl pyridine in the lipid extracts was quantified on the basis of the ratio of ions *m/z* 99 and 98 (from the internal standards 2-pentyl pyridine-d_5 and 2-pentyl pyridine-d_6, respectively) to *m/z* 93 (from 2-pentyl pyridine) using the standard curve shown in Figure 2. The labeled 2-pentyl pyridines were added during the extraction process so they would be subjected, as nearly as possible, to the same conditions as the 2-pentyl pyridine extracted from the soy products.

The level of 2-pentyl pyridine in the defatted flour ranged from 0.06 ppm in the Stressland to 0.51 ppm in the Probst variety (Table 2). At 0.06 ppm, 2-pentyl pyridine's flavor value is 5000 owing to its low flavor threshold value (0.000012 ppm). The SPI prepared from these flours contained about the same level, ranging from 0.16 to 0.18 ppm. Because 2-pentyl pyridine is a water-soluble compound and no references to its protein binding characteristics were available, it is difficult to predict if the 2-pentyl pyridine in the SPI has its origin in the defatted flour or whether it was synthesized during the SPI preparation. All lipid extractions and subsequent analyses to quantify 2-pentyl pyridine were accomplished within 3 wk of the SPI preparation.

TABLE 1
Composition of Defatted Flours and SPI from Selected Soybeans Varieties

Variety/product	Moisture (%)	Protein (%) (N × 5.71)[a]	Lipid[a] (%)
Burlison/flour	8.20 (0.31)[b]	45.57 (0.27)	3.12 (0.01)
Burlison SPI	1.98 (0.17)	81.81 (0.04)	3.54 (0.04)
Stressland/flour	7.72 (0.42)	45.22 (0.16)	3.05 (0.03)
Stressland/SPI	6.45 (0.22)	77.95 (0.04)	3.87 (0.07)
Probst/flour	6.86 (0.28)	42.53 (0.16)	2.93 (0.01)
Probst/SPI	1.74 (0.05)	81.42 (0.11)	4.07 (0.09)
LOX-null flour	9.02 (0.32)	44.13 (0.08)	3.52 (0.02)
LOX-null/SPI	6.89 (0.02)	84.47 (0.09)	3.98 (0.02)

[a]Percentage by weight dry basis.
[b]Figures in parentheses are standard error. LOX, lipoxygenase; SPI, soy protein isolates.

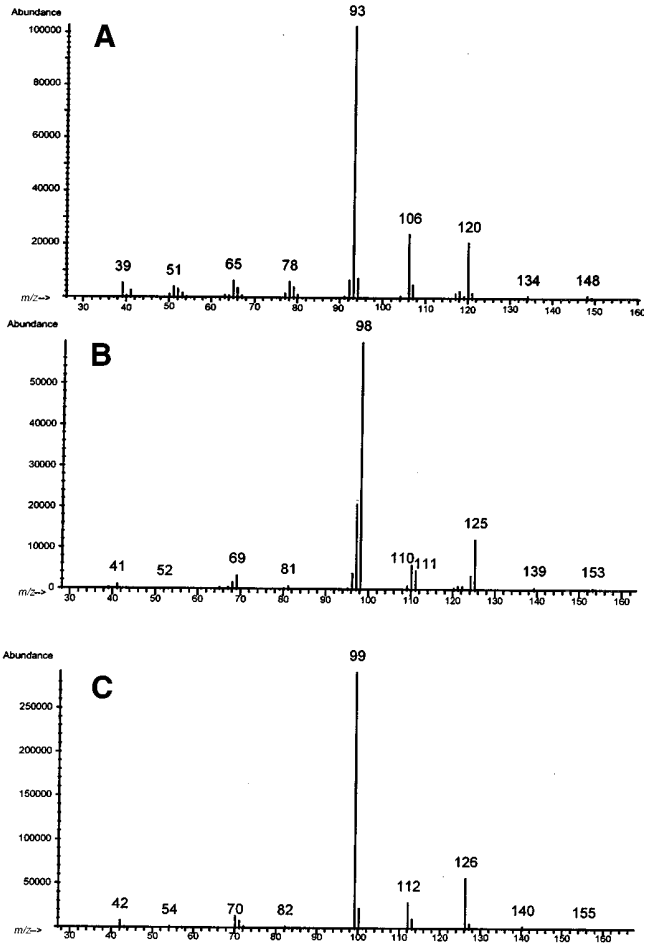

FIG. 1. Mass spectra for (A) 2-pentyl pyridine, (B) 2-pentyl pyridine-d_5, (C) 2-pentyl pyridine-d_6.

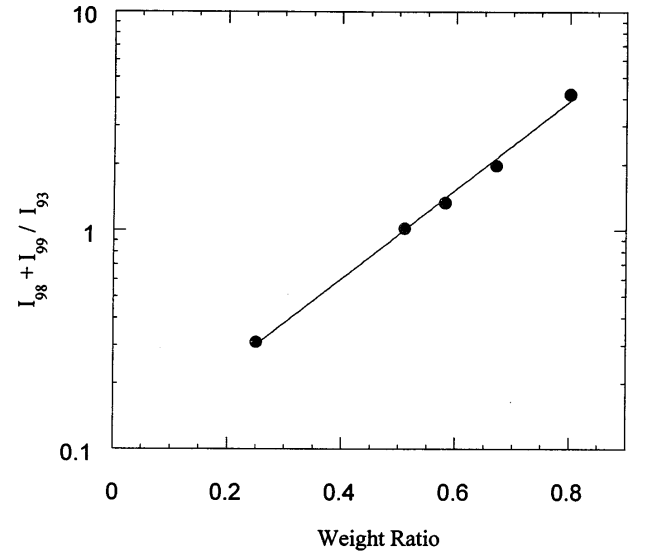

FIG. 2. Standard curve for the determination of the response factor of 2-pentyl pyridine by gas chromatography/mass spectrometry (GC/MS). The ratio of the relative abundance of *m/z* (I) 98 + 99 (2-pentyl pyridine-d_5 + 2-pentyl pyridine-d_6)/*m/z* 93 (2-pentyl pyridine) was plotted in a logarithmic scale vs. the weight ratio of ng (2-pentyl pyridine-d_5 + 2-pentyl pyridine-d_6)/(2-pentyl-pyridine-d_5 + 2-pentyl pyridine-d_6 + 2-pentyl pyridine). (R = 0.99591).

TABLE 2
Content (ppm) of 2-Pentyl Pyridine in Hexane-Defatted Flours and Corresponding Freeze-Dried SPI

Variety	Defatted flour[a]	SPI[a]
Burlison	0.30[a]	0.16[a]
Stressland	0.06[a]	0.17[a]
Lipoxygenase-null	0.07[b]	0.18[a]
Probst	0.51[c]	0.16[a]

[a]Means with the same roman superscript letter in columns indicate no statistically significant difference at $P \geq 0.05$. For abbreviation see Table 1.

To determine if the presence of pro-oxidants would contribute to the occurrence of 2-pentyl pyridine, $FeCl_3$ and $CuCl_2$ were added, and exposure of the protein slurries to UV light during SPI processing was performed. These treatments were considered in response to the proposed mechanism for the synthesis of 2-pentyl pyridine from a lipid oxidation product (2,4-decadienal) and ammonia from protein degradation. The SPI obtained from these treatments of Burlison and Stressland flours resulted in a pronounced increase in the level of 2-pentyl pyridine over the corresponding controls (Table 3). Owing to a shortage of materials, certain treatments for these two varieties were omitted. The increases in the Probst SPI were less pronounced and occurred only with the $CuCl_2$ and UV treatments. The treatments of the SPI from the lipoxygenase-null soybeans resulted in no increases in 2-pentyl pyridine. This indicates that formation of 2-pentyl pyridine is linked to the activity of lipoxygenase enzymes and is accelerated in the presence of pro-oxidants. The lipoxygenase-null SPI with four different pro-oxidant treatments ranged in protein content from 84.7 to 86.8%, dry basis. This provides further indication that the lipoxygenase-null soybeans may produce a higher protein content SPI.

Because the proposed mechanism for the formation of 2-pentyl pyridine involves 2,4-decadienal and is related to the presence of pro-oxidants, the level of 2,4-decadienal was estimated in the lipid extracts from the SPI using the ratio of the major ion from 2,4-decadienal (*m/z* 81) with the internal standard of labeled 2-pentyl pyridine (Fig. 3). This can only be considered as an estimate, since the internal standards were added during the lipid extraction process and have different solubility characteristics from those of 2,4-decadienal. If a portion of the internal standard equilibrates in the methanol/water phase during the phase separation of the lipid

TABLE 3
Content (ppm) of 2-Pentyl Pyridine in Freeze-Dried SPI Treated with Pro-Oxidants[a]

Variety	Control	$FeCl_3$ (25 μM)	$CuCl_2$ (25 μM)	$FeCl_3$ (25 μM) + $CuCl_2$ (25 μM)	Ultraviolet
Burlison	0.16[a]	—	—	0.62[b]	2.17[c]
Stressland	0.17[a]	0.44[b]	1.51[c]	1.00[d]	—
Lipoxygenase-null	0.18[a]	0.12[a,b]	0.16[a,b]	0.08[b,c]	0.15[a,b]
Probst	0.16[a]	0.17[a]	0.25[b]	0.17[a]	0.28[b]

[a]Means with the same roman superscript letter in rows indicate no statistically significant difference at $P \geq 0.05$. For abbreviation see Table 1.

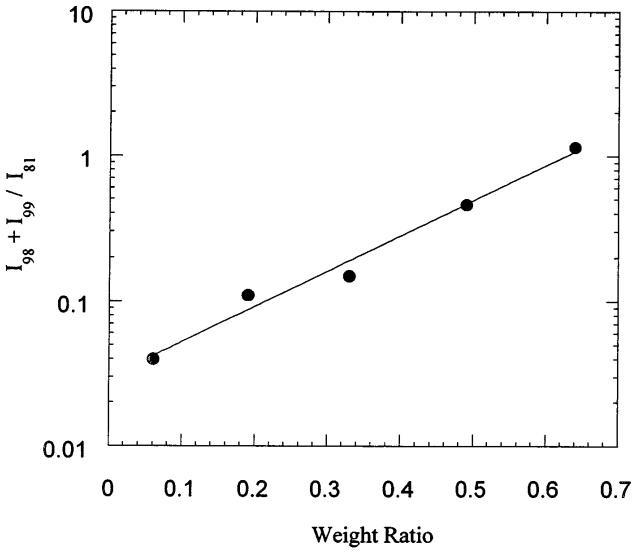

FIG. 3. Standard curve for the determination of the response factor of 2,4-decadienal by GC/MS. The ratio of the relative abundance of *m/z* (I) 98 + 99 (2-pentyl pyridine-d_5 + 2-pentyl pyridine-d_6)/*m/z* 81 (2,4-decadienal) was plotted in a logarithmic scale vs. the weight ratio of ng (2-pentyl pyridine-d_5 + 2-pentyl pyridine-d_6)/(2-pentyl pyridine-d_5 + 2-pentyl pyridine-d_6 + 2,4-decadienal) (R = 0.99803). For abbreviation see Figure 2.

TABLE 5
Content (ppm) of 2,4-Decadienal in Freeze-Dried SPI Treated with Pro-Oxidants[a]

Variety	Control	$FeCl_3$ (25 μM)	$CuCl_2$ (25 μM)	$FeCl_3$ (25 μM) + $CuCl_2$ (25 μM)	Ultraviolet
Burlison	0.12[a]	—	—	0.18[a,b]	0.29[b]
Stressland	0.19[a]	0.28[a]	>4.84	>4.84	—
Lipoxygenase-null	0.19[a]	0.17[a,b]	0.12[b,c]	0.13[a,b]	0.13[a,b]
Probst	0.24[a]	0.18[a]	0.17[a]	0.15[a]	0.20[a]

[a]Means with the same roman superscript letter in rows indicate no statistically significant difference at $P \geq 0.05$.

TABLE 6
Iron and Copper Contents (ppm) of Commercial and Laboratory SPI[a]

Variety	Iron	Copper
Commercial A	64.46 (3.54)	14.66 (5.11)
Commercial B	87.40 (15.25)	13.15 (6.67)
Burlison	119.91 (0.16)	13.08 (0.60)
Stressland	74.92 (0.11)	24.61 (1.66)
Lipoxygenase-null	114.41 (0.71)	20.19 (0.71)
Probst	112.76 (0.35)	57.60 (3.79)

[a]Figures in parentheses are standard errors.

extraction, the level of 2,4-decadienal will be overestimated. However, the comparison between the relative level of 2-pentyl pyridine to 2,4-decadienal should be consistent among these samples. The level of 2,4-decadienal was lowest in the Probst and lipoxygenase-null defatted flour, similar to the occurrence of 2-pentyl pyridine (Table 4). However, in the SPI there appear to be erratic correlations between the level of 2,4-decadienal and 2-pentyl pyridine. For example, the highest level of 2-pentyl pyridine occurred in the Burlison SPI treated with UV light. This SPI had a relatively low level of 2,4-decadienal (Table 5). The next-highest levels of 2-pentyl pyridine, which occurred in the Stressland SPI treated with $CuCl_2$ or a mixture of $CuCl_2$ and $FeCl_3$, correspond to a level of 2,4-decadienal greater than the upper level of the standard curve. The levels of 2,4-decadienal in the SPI were either ≤0.29 ppm or >4.84 ppm, the upper limit for the standard curve.

The levels of 2-pentyl pyridine in the control laboratory SPI of this investigation (0.16–0.18 ppm) were lower than in the commercial SPI of our previous investigation (0.28 to 1.01 ppm) (1). Because pro-oxidant treatments can cause elevated levels of 2-pentyl pyridine, the copper and iron contents of the two commercial SPI used in our previous investigation [two different code dates of Archer Daniels Midland Co. (Decatur, IL) Pro Fam 970] and the control laboratory SPI of this investigation were determined (Table 6). These values for copper and iron content are similar to the values for SPI reported by O'Dell (14), 160 ppm for iron and 12 ppm for copper. The level of copper in the Probst SPI was much higher than the other control SPI but appeared to have little effect on the level of 2-pentyl pyridine. This variety also showed little reaction to the addition of added pro-oxidants. Because the levels of 2-pentyl pyridine in laboratory SPI were assayed within 3 wk of their preparation, it is conceivable that the amount in the commercial SPI was also initially low and increased over time during storage or due to other commercial processing factors.

TABLE 4
Content (ppm) of 2,4-Decadienal in Defatted Flours and Corresponding Freeze-Dried SPI[a]

Variety	Defatted flour	SPI
Burlison	>4.84	0.12[a]
Stressland	0.08[a]	0.19[a,b]
Lipoxygenase-null	0.06[b]	0.19[a,b]
Probst	0.32[b]	0.24[b]

[a]Means with the same roman superscript letter in columns indicate no statistically significant difference at $P \geq 0.05$. For abbreviation see Table 1.

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