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Identification of Volatile Compounds in Soybean at Various Developmental Stages Using Solid Phase Microextraction

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Soybean (*Glycine max*) seed volatiles were analyzed using a solid phase microextraction (SPME) method combined with gas chromatography−mass spectrometry (GC-MS). Thirty volatile compounds already reported for soybean were recovered, and an additional 19 compounds not previously reported were identified or tentatively identified. The SPME method was utilized to compare the volatile profile of soybean seed at three distinct stages of development. Most of the newly reported compounds in soybean seed were aldehydes and ketones. During early periods of development at maturity stage R6, several volatiles were present at relatively high concentrations, including 3-hexanone, (*E*)-2-hexenal, 1-hexanol, and 3-octanone. At maturity stage R7 and R8, decreased amounts of 3-hexanone, (*E*)-2-hexenal, 1-hexanol, and 3-octanone were observed. At maturity stage R8 hexanal, (*E*)-2-heptenal, (*E*)-2-octenal, ethanol, 1-hexanol, and 1-octen-3-ol were detected at relatively high concentrations. SPME offers the ability to differentiate between the three soybean developmental stages that yield both fundamental and practical information.

KEYWORDS: Soybean; volatiles; maturity; gas chromatography; mass spectrometry; solid phase microextraction; SPME

INTRODUCTION

Soybean (*Glycine max*) is grown worldwide and is a major source of protein and oil for both human and animal consumption. Harvesting soybean seeds after proper development and maturation is a critical step in soybean production. Soybeans are considered ready to harvest when the seed moisture content is less than 14%, and this often depends on variety, planting location, planting date, and local weather conditions (*1*). During experimentation in the field, soybean plants are subjected to treatments that often depend on application at specific plant developmental stages for optimum effect. Soybean developmental stages have been previously described by Fehr et al. (*2*) whereby vegetative and reproductive growth are determined by physical characteristics typical of each stage of growth. Reproductive stages R1 and R2 are based on flowering, R3 and R4 are based on pod development, R5 and R6 are based on seed formation, and R7 and R8 are based on seed maturation. This system has long been accepted as the standard by which soybean growth is described in the literature.

Accurate determination of reproductive growth stage could be further specified by chemical analysis of soybean seeds as they develop to maturity. We initialized a study to investigate the volatile profile of soybean seeds from pod fill (R6) to physiological maturity (R8). Volatile compounds may serve as indicators of developmental maturity and as biochemical markers to determine seed quality. Several reports have already identified many volatile components in fully mature soybeans (*3*−*7*) and a commercially available fermented soybean curd (*8*). The development of objectionable off-odor detection and classification methodology for use in grain grading has stimulated research on volatile components of soybean and grains (*9*−*10*). Several compound classes identified were alcohols, aldehydes, esters and lactones, ketones, and terpenoids. Different methods were utilized to trap volatile compounds. Tenax trapping methods have been widely used for the analysis of soybean and other grains (*4*, *5*); however, solid phase microextraction (SPME) is currently replacing older techniques for volatile analysis (*11*). The SPME method is rapid, less labor intensive, uses little solvent, and is relatively inexpensive. In this paper, SPME combined with gas chromatography−mass spectrometry (GC-MS) was utilized for the identification of volatile compounds in soybean at three distinct developmental stages.

MATERIALS AND METHODS

Plant Material. Soybean (*G. max* cv. Pioneer 95B41) was grown at the Southern Regional Research Center in New Orleans, LA. Seeds were harvested at three distinct stages of maturity (*2*): R6, pod contained full size green beans at one of the four uppermost nodes with a completely unrolled leaf; R7, pods yellowing; and R8, 95% of pods brown (harvest maturity). Soybean seeds from stage R6 (25 seeds) were pooled together, milled in a Waring Pulverizer (model S110) for

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3 min, 40 mesh screen, and analyzed within 3 h of harvest. Seeds from stage R7 (20 seeds) and R8 (20 seeds) were milled in a similar manner.

Sample Preparation. Soybean samples (500 mg) were placed in 4 mL glass vials (Supelco model 27136). 3-Hexanol was used as an internal standard (IS) and was not detected in SPME GC-MS analysis of soybean to which it was not added. The 3-hexanol standard was dissolved in methanol and injected onto the milled soybean (50 ng/kg final concentration). Vials were sealed with a steel crimp cap fitted with a Teflon/silicon septum that was conditioned at 100 °C and mixed by agitation. Samples from each maturity group (R6−R8) were prepared in triplicate.

SPME Analysis. Two commercially available SPME fibers suitable for volatile analysis available from Supelco (Bellefonte, PA) were examined for this study. These were poly(dimethylsiloxane) (PDMS; 100 μm) and divinylbenzene/carboxen/PDMS (DCP, 50/30 μm). Vials containing samples were placed in a VWR aluminum heating block (model 35756) at 40, 50, and 60 °C with the SPME fiber inserted into the headspace above the soybean sample. Adsorption was timed for 1 h.

GC-MS Parameters and Analyses. SPME fibers were desorbed at 230 °C for 2 min in the injection port of an HP5890/5989A GC-MS (Hewlett-Packard, Palo Alto, CA) with a HP-5 (cross-linked 5% phenyl methyl silicone, Hewlett-Packard) column (50 m, 0.2 mm i.d., 0.5 μm film thickness). GC-MS runs were 40 min, and the fiber remained in the injection port for 20 min after each run. The injection port was operated in splitless mode with a He inlet flow pressure of 42 psi. The initial oven temperature was 40 °C, held for 3 min, ramped at 10 °C min^{-1} to 60 °C, then ramped at 3 °C min^{-1} to 150 °C, and ramped at 20 °C min^{-1} to 250 °C and held for 5 min. The HP5989A quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV, a source temperature of 200 °C, quadrupole temperature of 100 °C, and an interface temperature of 200 °C, with a continuous scan from *m*/*z* 40 to 500.

Positive identification of a component was performed by comparison of its retention time or retention index (RI) and mass spectrum with that of an authentic compound (when available). The RI for each identified compound was calculated using a series of straight chain alkanes (C_5−C_{20}). Tentatively identified compounds were uniquely identified on the basis of the mass spectra from the Wiley (v.7 NIST98) library of mass spectral database (Palisade Corp., Newfield, NY). Samples were run in triplicate, and integrated areas based on selected ions were normalized on 3-hexanol and averaged.

RESULTS AND DISCUSSION

Volatile analysis of grains, including soybean, has been described using a Tenax trap combined with GC-MS (*3*, *5*). The use of tenax adsorbents for analyses is labor intensive and expensive and requires special hardware when interfacing the trap to the GC injection port. A newer analytical technique, SPME, has been described for the analysis of oats (*11*) that eliminates the many problems encountered with tenax trapping methods. In this study, a DCP fiber showed higher adsorption of all solutes with a 1 h exposure time when compared to two other SPME fibers. We therefore performed a preliminary analysis of SPME volatile extraction from the headspace above mature soybean seed by varying SPME fiber (DCP and PDS), extraction temperature, and exposure time. Our preliminary analyses indicated higher adsorption of volatiles using a DCP fiber with a 1 h exposure time and a temperature of 60 °C (data not shown).

Using SPME to analyze volatiles from a commercial soybean variety, a total of 49 volatile compounds (**Table 1**) were recovered. These compounds belonged to six classes of compounds, including aldehydes (2), esters and lactones (4), alcohols (8), ketones (7), terpenoids (3), furans (2), and miscellaneous compounds (2). Of these compounds, 30 were previously reported in soybeans (*3*−*8*). Nineteen compounds not previously reported in raw soybean were also recovered. Of these compounds, nine were confirmed using authentic standards, whereas the remainder are listed as tentative in **Table 1**. The compound dihydro-5-pentyl-2(3*H*)-furanone identified in raw soybean was previously identified in fermented soybean curd (*8*).

Several significant differences were observed in the volatile profiles recovered at different stages of maturity. **Figure 1** shows the total ion chromatograms of soybean seeds at maturity stages

Table 1. Volatile and Semivolatile Compounds in Cv. Pioneer Soybean Recovered by SPME[a]

peak ID[b]	RI[c]	ref[d]	compound	maturity R6	R7	R8
1	<500		acetaldehyde	C	C	C
2	<500	*3*, *4*	ethanol	C	C	C
3	515	*3*, *5*	methyl acetate	L	L	L
4	621	*3*, *4*	ethyl acetate		L	L
5	640		2-butenal	T	T	T
6	687	*3*, *4*	pentanal	C	C	C
7	691		2-ethylfuran	T	T	
8	772	*3*–*5*	1-pentanol	C	C	C
9	791		3-hexanone	C	C	C
IS	802		3-hexanol			
10	805	*3*–*5*	hexanal	C	C	C
11	861	*3*–*5*	(*E*)-2-hexenal	C	C	C
12	876	*3*–*5*	1-hexanol	C	C	C
13	898	*3*, *4*	2-heptanone	C	C	C
14	903	*3*, *4*	heptanal	C	C	C
15	916		(*E*,*E*)-2,4-hexadienal	C	C	C
16	925	*5*	methyl hexanoate	L	L	L
17	928	*4*	γ-butyrolactone			L
18	954		(*E*)-2-heptenal	C	C	C
19	960	*3*, *4*	benzaldehyde	C	C	C
20	967		sabinene			C
21	969	*3*–*5*	1-octen-3-ol	C	C	C
22	975	*3*, *4*	3-octanone	C	C	C
23	978	*4*	2-pentyl furan	C	C	C
24	982	*3*, *4*	3-octanol	C	C	C
25	985		(*E*,*Z*)-2,4-heptadienal	T	T	T
26	986	*4*	octanal	C	C	C
27	994		(*E*,*E*)-2,4-heptadienal	C	C	C
28	994	*4*	α-terpinene			C
29	1023	*4*	*p*-cymene			C
30	1028	*5*	2-ethyl-1-hexanol			C
31	1043		3-octen-2-one	C	C	C
32	1052	*3*	phenylacetaldehyde		L	L
33	1064	*5*	(*E*)-2-octenal	C	C	C
34	1069		2-methyl-3-octanone	T	T	T
35	1083	*4*	(*E*,*E*)-3,5-octadien-2-one	L	L	L
36	1109		1-nonen-4-ol	T	T	T
37	1113	*4*, *5*	nonanal	C	C	C
38	1124		(*E*,*E*)-2,4-octadienal	T		
39	1141		3-hydroxy-2-methyl pyrone			T
40	1167		(*E*,*Z*)-2,6-nonadienal	C	C	
41	1172	*5*	(*E*)-2-nonenal	C	C	C
42	1180		2,6-nonadien-1-ol	T		
43	1206		(*E*,*Z*)-2,4-nonadienal	T	T	T
44	1224		(*E*,*E*)- 2,4-nonadienal	C	C	C
45	1260	*5*	(*E*)-2-decenal	L	L	L
46	1285	*3*	(*E*,*Z*)-2,4-decadienal	L	L	L
47	1298	*3*	(*E*,*E*)-2,4-decadienal	C	C	C
48	1312		3,4-dihydropyran	T	T	T
49	1318	*6*	dihydro-5-pentyl-(3*H*)-furanone	T	T	T

[a] C, identification based on correspondence of retention time and mass spectrum with genuine standard. L, compound previously observed in soybean and identified using a mass spectral library with a quality match exceeding 84%. T, compound identified using a mass spectral library with a quality match exceeding 84% and not previously reported in soybean. [b] ID, identification; used for labels in **Figure 1**. [c] RI based on identified compound retention times calculated from a linear equation between each pair of straight chain alcanes (C_5–C_{20}). [d] Articles in which the compounds were reported.

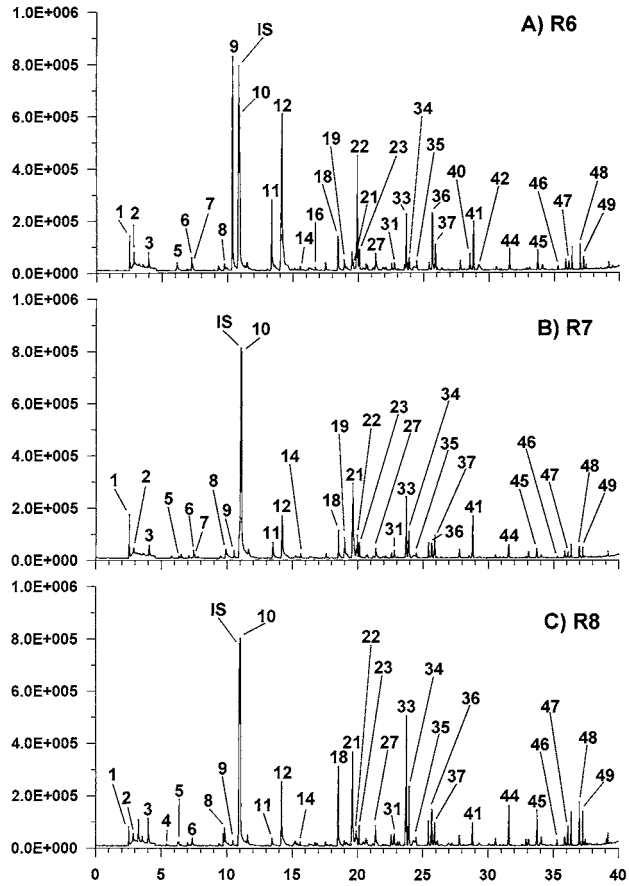

Figure 1. Total ion chromatograms of soybean (*G. max* cv. Pioneer) at three different maturity stages: (A) maturity stage R6, bean just forming in pod; (B) maturity stage R7, pod turns yellow with full size bean formed; (C) maturity stage R8, bean fully mature.

R6, R7, and R8. In general, all three maturity stages had only a few predominant peaks; however, the concentration of several of these compounds changed as the seed matured. At maturity stage R6 (**Figure 1A**), the soybean seed consisted mainly of the aldehydes (*E*)-2-hexenal, nonanal, (*E*)-2-nonenal, and (*E*)-2-decenal; the ketones 3-hexanone and 3-octanone; and the alcohol 1-hexanol. At R7 (**Figure 1B**), decreasing amounts of these three classes of compounds were observed. At R8 (**Figure 1C**), relatively higher levels of the aldehydes hexanal, (*E*)-2-heptenal, and (*E*)-2-octenal and the alcohol 1-octen-3-ol were observed. In addition, several terpenoids were detected at trace levels, including sabinene, α-terpinene, and *p*-cymene at maturity stage R8.

Careful examination of the ion traces for specific compounds listed in **Table 1** led to identification of several compounds that significantly changed in amount during seed development. To quantify the differences observed between maturity stages, the change in peak area relative to the IS (IS peak area = 100) for a number of volatile compounds identified is presented in **Table 2**. The significant difference of a specific compound between each maturity stage (R6 to R7 and R7 to R8) was calibrated by Student's *t*-test. The two ketones selected, 3-hexanone and 3-octanone, decreased as the soybean seed matured. A significant decrease in peak area was observed for 3-hexanone between R6 and R7, and a further significant decrease was observed between R7 and R8. A similar decrease in peak area was observed for 3-octanone between R6 and R7 and between R7 and R8. These two ketones are excellent markers of maturity stage R6. As shown, the aldehyde (*E*)-2-hexenal decreased in concentration with increasing stage of maturity. However, the aldehydes hexanal and (*E*)-2-heptenal increased as the soybean seed reached maturity, particularly between maturity stages R7 and R8. The aldehyde (*E*)-2-octenal decreased slightly in concentration between R6 and R7; however, it increased significantly between R7 and R8. Of the alcohols selected, ethanol increased significantly from R7 to R8. Also, a significant increase in concentration of 1-octen-3-ol was observed from R6 to R7 and from R7 to R8. Concentrations of 1-hexanol decreased significantly from R6 to R7 and increased between R7 to R8. These data show that several compounds, including several aldehydes, alcohols, and ketones, significantly changed in concentration as the seed developed to maturity. Of the compounds selected for quantification, 3 hexanone, 3-octanone, (*E*)-2-hexenal, 1-octen-3-ol, and 1-hexanol are all good indicators (significant change in peak area) of the change between maturity stages R6 and R7. Ethanol, 1-octen-3-ol, 3-hexanone, 3-octanone, hexanal, (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*)-2-octenal are useful indicators of the maturity stage changes between R7 and R8. Also, the appearance of several terpenoids is also indicative of maturity stage R8.

Several volatile compounds that cause the green beanlike flavor in raw soybeans have been identified, including the aldehyde hexanal (*1*, *6*). Hexanal and other aldehydes detected by the SPME method may have been formed as a result of oxidation during sample preparation. Lipoxygenase catalyzes the peroxidation of linoleic acid and other polyunsaturated lipids that contain the *cis*,*cis*-1,4-pentadiene moiety (*1*, *6*). The initial products of lipoxygenase activity may be degraded into a variety

Table 2. Relative Peak Areas for Selected Compounds Recovered in Cv. Pioneer Soybean Harvested at Three Distinct Maturities ($n = 3$)[a]

compound	fragment used[b] (*m*/*z*)	harvest maturity relative peak area[c] R6	R7	R8
ethanol	45	15.78 ± 4.72	32.08 ± 11.17	414.46 ± 36.46*
3-hexanone	57	1122.54 ± 179.03	68.46 ± 4.21*	41.03 ± 3.02*
hexanal	82	116.14 ± 6.97	123.90 ± 8.19	159.63 ± 9.62**
(*E*)-2-hexenal	55	120.60 ± 25.24	47.20 ± 1.87**	26.12 ± 4.30**
1-hexanol	56	1000 ± 169.84	447.42 ± 132.04***	708.36 ± 113.81
(*E*)-2-heptenal	83	57.84 ± 8.86	59.07 ± 4.26	213.27 ± 36.67**
1-octen-3-ol	57	210.04 ± 30.78	783.80 ± 119.39 **	1368.28 ± 232.03***
3-octanone	43	526.36 ± 40.65	151.16 ± 37.41*	32.88 ± 7.53**
(*E*)-2-octenal	55	191.07 ± 22.31	163.70 ± 11.72	542.55 ± 210.37***

[a] Recovery of each compound based on specific MS target ions (*m*/*z*) used for quantification. [b] Specific fragment used for calculation of relative peak area of a specific compound. [c] Significant differences between maturity stages (as compared with earlier maturity stage) for each compound at $P = <0.001$ (*), <0.01 (**), and <0.05 (***), respectively (Student's *t*-test).

of C-6 and C-9 products through the action of hydroperoxide lyases and include aldehydes (hexanal), ketones, and alcohols (*1*, *6*). During grinding of raw soybeans, the lipoxygenase enzyme as well as the lipid substrate are liberated; however, excess water is necessary to catalyze the instant oxidation of the substrate (*1*). Our results show that hexanal concentrations are present throughout R6−R8 but are highest at R8. Another volatile compound that has been reported to impart a musty odor characteristic of mushrooms is 1-octen-3-ol. 1-Octen-3-ol was reported to be enzymatically formed during soaking of soybeans (*12*). In work by Ram et al. (*5*), the amount of 1-octen-3-ol obtained from soybeans increased as the amount of water added to a sample increased. High concentrations of 1-octen-3-ol can be an indication that the seed has matured to R8. The observation of 1-octen-3-ol and hexanal in soybean at maturity stages R6−R8 may be due to the high moisture content of the seeds at early stages of development. Moisture content can be as high as 83% at maturity stage R6 but drops to lower levels (15%) as the seed matures to R8 (*13*). Enzymatic reactions during milling also contribute to the formation of hexanal and 1-octen-3-ol, particularly the high concentrations observed at maturity stage R8.

In summary, the SPME method can be used to determine volatile compounds in soybean at different stages of development. Of the 49 compounds observed, several specific volatile compounds, particularly aldehydes, ketones, and alcohols, can be used as key indicators of soybean maturity as the seed progresses from maturity stage R6 to fully mature stage R8. Also, volatile profiles may be useful in the detection and classification of seed quality and objectionable off-odors (*10*). High concentrations of several select aldehydes (3-hexanone and (*E*)-2-hexenal), ketones (3-hexanone and 3-octanone), and one alcohol (1-hexanol) were observed at maturity stage R6. Significant changes in the type and amount of several volatile compounds were observed as the soybean seed matured from growth stage R6. The ketones 3-hexanone and 3-octanone decreased significantly during early stages of growth, and both of these compounds are useful indicators of maturity stages R6 and R7. Further changes were observed as the soybean seed fully developed from R7 to R8. High concentrations of several select aldehydes (hexanal, (*E*)-2-heptenal, and (*E*)-2-octenal) and alcohols (ethanol and 1-hexanol) were observed at maturity stage R8 and are useful indicators of fully mature seeds.

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