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Analysis of lipids in aging seed using capillary supercritical fluid chromatography

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ABSTRACT

Because of separation or detection problems associated with non-volatility and the non-chromophoric nature of lipids, capillary supercritical fluid chromatography (cSFC) was chosen over gas chromatography or high-performance liquid chromatography for the analysis of seed lipids. cSFC and flame ionization detection (FID) enabled more complete and accurate analysis of lipids extracted from onion seed. Replicated chromatography of triglyceride standards showed that retention times and quantitative chromatographic response was very reproducible. Analysis of cSFC–FID chromatograms of artificially aged and fresh onion seed lipids showed that significant changes occur in the lipid component of seed during aging. As the seed aged, there was a relative decrease of some of the larger molecular weight fractions and of the peak identified as vitamin E, tocopherol, while an increase was found in free palmitic and linoleic acids.

INTRODUCTION

Something occurs in a seed during its period of quiescence that results in premature senescence and death prior to or during the germination process. The importance of quality seed to agriculture and to the economic value of the seed industry provide reason enough to further understand the seed aging process. The quiescent seed represents life in a state of suspended animation, and thus provides a means of studying the organism in an orderly manner, unobscured by a high level of metabolism.

Previous studies have indicated that seed deterioration could be a result of genetic damage [1,2], degradation of the lipids and proteins of membranes [3,4] or degradation of other cellular organelles [5]. Free radicals, resulting from peroxidation reactions, have been implicated [6]. Although free radical reactions due to peroxidation of lipids is often mentioned in the literature, complete evaluation, quantification, and characterization of the changes in seed lipids due to this process have not been reported.

The peroxidation of seed lipids has been proposed as a fundamental mechanism

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of seed storage deterioration [3,4,7]. Lipid peroxidation is a free radical chain reaction between fatty acids and oxygen leading initially to hydroperoxides. Methylene interrupted pairs of double bonds in polyunsaturated fatty acids are especially prone to autoxidation. These same fatty acids serve as substrates for lipoxygenases which are found almost universally in plant tissues [8]. Lipoxygenase accelerates the rate of peroxidation of polyunsaturated fatty acids [9].

Also present in dicot seeds and seedlings are lyases which split hydroperoxides into aldehydes, hydrocarbons and smaller oxo-acids [9,10]. Volatile products comprise a characteristic volatile chemical signature amenable to analysis by gas chromatography (GC) [11]. Less volatile products such as large aldehydes, unsaturated aldehydes and ketones and products bearing glyceride residues have been poorly studied. Oxygenated fatty acids may accumulate during aging via autoxidation and break down to toxic aldehydes during seed hydration.

In order to study changes in the seed as it ages, accelerated aging techniques are often employed. It has been shown that the use of an elevated temperature and oxygen concentration regime sufficiently simulates natural aging [12,13]. To study seed lipids after seeds have undergone accelerated aging, many investigators have transesterified the entire lipid fraction and looked for changes in the fatty acid complement. This approach is simple and well established, but has not shed much light on causes of quality loss because whole lipid changes are obscured in the transesterification process. Less volatile compounds of complex mixtures are insufficiently volatile or stable to separate by GC at GC temperatures, and the mixtures are too complex for adequate resolution and identification using retention time in high-performance liquid chromatography (HPLC). Further, the lack of chromophoric groups limits detection after HPLC. These compounds are ideal candidates for the separation and detection by capillary supercritical fluid chromatography (cSFC) with flame ionization detection (FID). In addition, high-pressure liquefied gas extraction (HPLGE) is an attractive method for the study of seed aging because it combines low temperature and an inert atmosphere.

Since this combination of analytical methods is uniquely suited to extraction, separation and detection of lipid molecular species and their oxidation products, the objective of this study was to determine if changes occurred over time in the lipid content of onion seeds exposed to two different storage conditions. Changes were measured by monitoring seed viability (germination), seedling vigor, and making a critical comparison of lipid profiles obtained by cSFC–FID from pure, chemically unaltered extracted seed lipids.

MATERIALS AND METHODS

Seed storage treatment

Onion (*Allium cepa*) seed, breeding line MSU 2399B Lot 537879, was obtained from Dr. R. Watson, ARCO Seed Co. (El Centro, CA, USA). Until used, the seed was stored in plastic containers at 4°C, 28–30% relative humidity (%RH) at the USDA-ARS, Western Regional Plant Introduction Station (WRPIS) seed storage facility in Pullman, WA, USA.

Storage treatment conditions for the seed were: (1) the control was held in standard seed storage at 4°C, 28–30% RH in the WRPIS facility. Samples of seeds

were put into small coin envelopes and stored in drawers, the same as normal procedure for the Plant Introduction germplasm. (2) Seed exposed to heat treatment with elevated oxygen (accelerated aging) was removed from the conventional seed storage and 6–7 g seed/replicate was put into 250-ml erlenmeyer flasks. These were placed into a warm water bath (4 cm deep) maintained at constant 40–41°C. The flasks were then flooded with pure oxygen (O_2) for 2–3 min, and stoppered with rubber stoppers. A 95–99% oxygen atmosphere at 40–41°C was maintained by reflooding the flasks with oxygen every 14 days. Three replications were made for each aging time interval.

Treatment environments were monitored regularly by sampling the head space over the seed in each flask of the elevated oxygen temperature treatment for oxygen, nitrogen and carbon dioxide concentrations using GC–thermal conductivity detection (TDC). To separate the oxygen and nitrogen, injections were made into a GS-Q, 30 m × 0.53 mm I.D. capillary column (J & W Scientific, Folsom, CA, USA) which was valved in tandem to a 10 m × 0.53 mm I.D. Chrompack molecular sieve 5 A capillary column in a HP 5890A GC–TDC system. Chromatographic conditions were: 50-μl injections, using a gas-tight syringe, into the columns held at constant oven temperature of 30°C, and a helium carrier gas flow-rate of 15–20 ml/min. Air standards were run before and between sets of samples. For the first four weeks samples were taken every 48 h over the 12–14-day interval between oxygen flooding. Later, samples were taken just prior to oxygen flooding to verify the 95–99% oxygen concentration.

Germination and vigor tests

Seed was tested at regular intervals using the standard AOSA [14] methods. Seeds were germinated for viability data by plating 2 replicates of 50 seeds on blotter paper set in 15 cm diameter plastic petri plates. Plates were moistened and placed in environators (Puffer-Hubbard Environator, Grand Haven, MI, USA) set at the recommended regime of constant 20°C and constant dark. At each germination testing date, cold storage controls were also germinated. In these studies, successful germination was defined as when the seed had uniform imbibition and at least a 2 mm radical emerged. Vigor was evaluated based on comparative growth rates of the radical and hypocotyl between the treated samples and the control. Chlorosis, deformation and stunting of the radical were also considered in the vigor evaluations. Measurement of seedling vigor was based on a 1–5 scale where 5 was best vigor and 1 was very poor. Seeds that did not germinate where not rated for vigor. Germination data were analyzed with a standard analysis of variance and comparisons between treatments were made using the least significant difference (LSD) test at $P=0.05$.

Extraction

Extractions were conducted using a high-pressure Soxhlet extractor (J & W Scientific) and liquid carbon dioxide (CO_2) as the extraction solvent. A liquid CO_2 tank with an eductor tube was used as the solvent source. Seed was ground dry in a 37-ml stainless-steel container on a standard laboratory Waring blender for 20 s. Extractions were run for 48 h at 650–700 p.s.i. with a temperature differential of 38–41°C at the base of the chamber to 4°C on the cold finger. Blanks were run periodically to test for system contaminants. For each sample treatment three replicate extractions were made. Three aliquots for each extract were diluted 1:10 and 1:100 (v/v) in methylene chloride and chromatographed.

Capillary supercritical fluid chromatography

The cSFC was performed on a Lee Scientific Model 501 SFC with a flame ionization detector (Lee Scientific, Salt Lake City, UT, USA). Injection volume was 100 μl and split 12–15:1. A 20 m × 0.05 mm I.D., SB-Methyl-100 capillary column (Lee Scientific) was used. In all of the separations the mobile phase was SFC-grade carbon dioxide (Matheson Gas Products), and the following pressure program was used at 150°C isothermally: 100 atm held for 3 min then increased to 275 atm at 25 atm/min, 300 atm at 5 atm/min, 320 atm at 2 atm/min, and finally to 400 atm at 40 atm/min which was held for 5 min. The column was thermally conditioned 14 h at 280°C, and was rinsed with pentane and then with supercritical carbon dioxide by cycling between densities of 0.19 and 0.845 g/cm^3 for 12 h at 75°C. Three replicate chromatograms per dilution per extraction per treatment were made, and adjusted peak areas and retention times were statistically analyzed.

Component identification by mass spectrometry

The 70-eV electron impact mass spectra were obtained on a VG 7070E spectrometer with a source temperature of 200°C and 6000-V ion acceleration. An aliquot of 2 μl of oil was put into a melting point capillary and heated by the solids probe in 25°C increments to 400°C. Scans were made between 30–750 mass/charge (m/e) at 1.5 s/decade. Spectra were obtained and selected scans were compared to the National Bureau of Standards Library of standard spectra.

RESULTS AND DISCUSSION

Germination and vigor tests

Initial germination upon receipt of the onion seed was 99.5%. At the beginning of the aging experiments the germination was 96%. There was no significant change in germination in the cold treatment over the duration of the experiment (Table I). There was a significant loss of viability in response to the heat (41°C) and elevated oxygen treatment as compared to the control, 14% and 99% respectively over 11

TABLE I

GERMINATION AND VIGOR OF ONION SEEDS STORED AT CONSTANT 4°C, OR AT 41°C IN ELEVATED OXYGEN ATMOSPHERE

Crop	Treatment			Mean percent[a] germination					Mean vigor[a]				
	Temp. (°C)	%O_2	%RH	T_0	T_3	T_6	T_9	T_{11}	T_0	T_3	T_6	T_9	T_{11}
Onion	4	21	28	96	97	98	98	99a[b]	5.0	4.7	5.0	4.8	4.8a[b]
	41	97	22	95	65	28	19	14b	4.7	2.5	1.0	1.0	0.8b

[a] Values represent mean percent germination or mean vigor from 2 replications of 50 seeds. T subscripts represent the number of months from the beginning of the aging treatment to the time sampled for testing.

[b] Comparisons among treatments followed by the same letter do not differ significantly from each other according to Duncan's new multiple range test, $P = 0.05$.

months. Vigor data indicated that 41°C and under the elevated oxygen environment, tested seed quickly lost seedling vigor when compared to the control.

Extraction and chromatography

The onion seed had an extractable lipid content within the normal range for its species. The average lipid component on a dry weight basis for the onion seed was 10–12%.

In replicated studies of the chromatography of lipids from onion seed stored under the two aging environments, significant differences were found in the quantitative comparisons of peaks from the chromatograms. Degradation of the triglyceride peaks in the aged seed lipids and an increase in the smaller components free fatty acids were found. In addition, there was a quantitative increase in the last few peaks. Statistical analysis showed no significant differences for the retention times of corresponding peaks in chromatograms of replicate sub-samples from either within or between sample treatments.

At the 1:100 dilution of seed lipids from onion seed which underwent accelerated aging there was a significant decrease in two of the triglyceride peaks (peaks 5 and 7) of 3.8 and 19.5%, respectively compared to the 4°C control, and increases in peaks 1, 2 and 3. As shown in Fig. 1, new peaks, numbered 1 and 2, appeared after 11 months of accelerated aging. There was a three fold increase of peaks 8 and 9 in the aged seed over the control. As shown in Table II, all but one of the peaks from the aging treatments were significantly different from those of the cold storage control.

As seen in Table III these differences were further amplified in the chromatography of the 1:10 dilutions of the same onion seed lipid samples. Of 20 integrated peaks from the oxygen treated aged sample, all but 2 (peaks 3 and 10) were significantly different from the room temperature treatment. There were large relative decreases in the two major peaks 11 and 12 of 4.7 and 22.1% respectively. Degradation in these

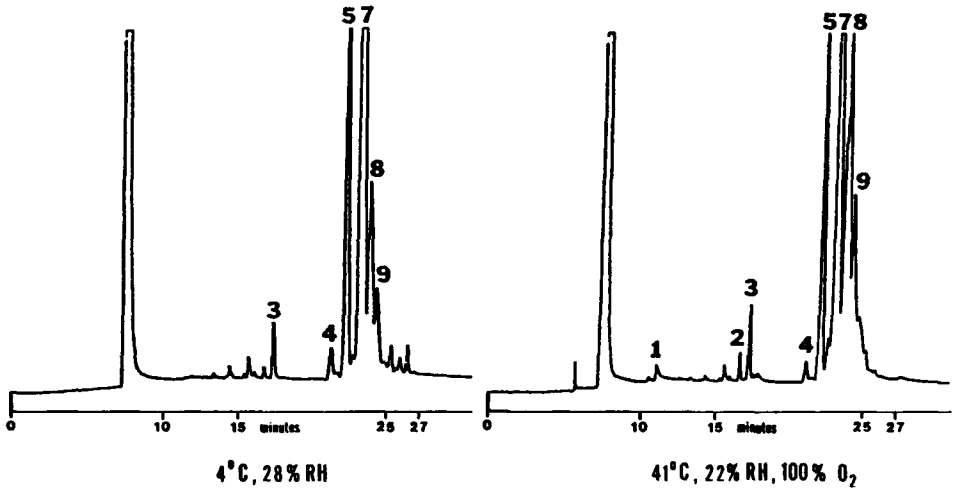

Fig. 1. cSFC–FID chromatograms of lipids from onion seeds which had been stored in different environments at 1:100 dilution in methylene chloride.

TABLE II

COMPARISON OF LIPIDS CHROMATOGRAPHED WITH cSFC–FID in 1:100 DILUTION OF SEED LIPID IN METHYLENE CHLORIDE

Allium cepa seeds were stored at 4°C in ambient atmosphere or 41°C in elevated oxygen atmosphere.

Peak No.	Relative areas[a]	
	Treatment temperature (°C)	
	4°C	41°C and O_2
1	NP	0.919 ± 0.083*
2	NI	0.636 ± 0.020*
3	1.068 ± 0.016	1.805 ± 0.089*
4	0.766 ± 0.130	0.621 ± 0.025*
5	16.181 ± 0.311	12.336 ± 0.202**
6	NI	1.297 ± 0.096*
7	72.554 ± 0.132	52.988 ± 1.31*
8	7.084 ± 0.274	21.015 ± 0.360*
9	2.771 ± 0.073	10.100 ± 0.916*

[a] Relative areas = (peak area) (total area of lipid components) (100%). Data are followed by the standard deviation. Data calculated from 3 runs per 3 replicate extractions. Treatment comparison where * = significantly different at $P = 0.05$, and ** = significantly different at the $P = 0.01$ level using Student's paired *t*-test. NP = no peak in chromatogram. NI = Peak is in chromatogram but not large enough to be integrated.

TABLE III

COMPARISON OF LIPIDS CHROMATOGRAPHED WITH cSFC–FID IN 1:10 DILUTIONS OF SEED LIPID IN METHYLENE CHLORIDE

Allium cepa seeds stored at 4°C in ambient atmosphere or 41°C in oxygen atmosphere.

Peak No.	Retention time	Normalized peak areas[a]		
		Treatment temperature (°C)		Ratio 41°C/4°C
		4°C	41°C & O_2	
1	10.89	–	0.590 ± 0.054	–
2	11.25	0.366 ± 0.024	3.043 ± 0.338	8.319
3	14.49	0.509 ± 0.025	0.285 ± 0.036	0.561
4	15.71	1.000	1.000	1.000
5	16.71	0.614 ± 0.030	1.685 ± 0.132	2.743
6	17.32	3.054 ± 0.169	5.000 ± 0.421	1.637
7	17.71	–	0.437 ± 0.085	–
8	18.12	–	0.953 ± 0.206	–
10	21.65	2.751 ± 0.235	2.133 ± 0.255	0.755
11	22.74	44.356 ± 2.41	30.963 ± 0.418	0.698
12	24.11	196.959 ± 8.89	136.810 ± 9.11	0.694
13	24.37	19.832 ± 2.10	18.786 ± 1.16	0.947
14	24.88	9.951 ± 0.505	38.037 ± 3.03	3.822
15	25.22	2.188 ± 0.188	28.320 ± 2.05	12.943
15′	25.41	–	10.245 ± 2.12	–
16	25.62	1.781 ± 0.189	5.211 ± 0.427	2.895
17	25.94	0.273 ± 0.023	0.597 ± 0.121	2.197
18	26.22	0.663 ± 0.238	0.895 ± 0.022	1.350
19	26.67	1.171 ± 0.039	–	–
20	27.84	–	2.544 ± 0.613	–

[a] All peaks were normalized to peak 4 since relative peak areas were equivalent for this peak. Data are followed by the standard deviation. Data calculated from 3 runs per 3 replicate extractions.

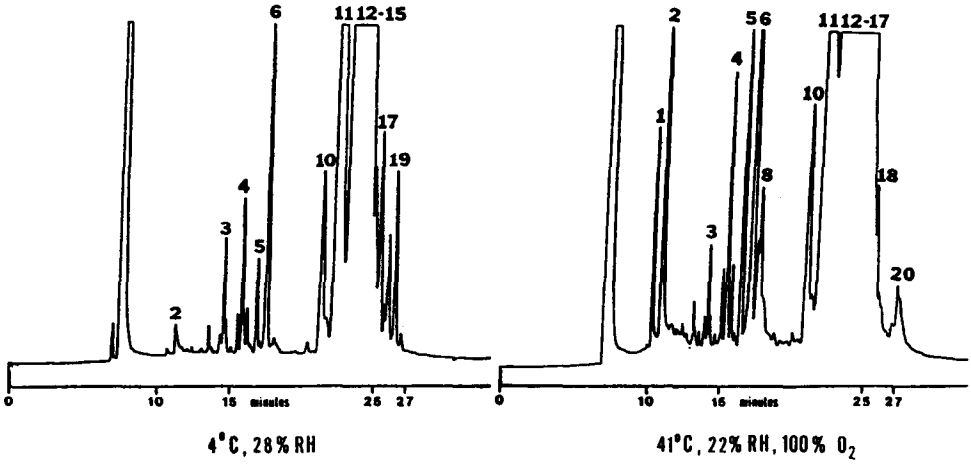

Fig. 2. cSFC–FID chromatograms of lipids from onion seeds which had been stored in different environments at 1:10 dilution in methylene chloride. Peaks: 1 = palmitic acid, 2 = linoleic acid, 3 = tocopherol, and 4 = sitosterol.

peaks was reflected in significant increases in the lower-molecular-weight fractions at peaks 1, 2, 5 and 7–9, and the larger-molecular-weight peaks at 14, 15, 16 and 17. In the control and room temperature treatments there was one small, non-integrated coalesced peak at a retention time which would span the retention times for peaks 7–9 of the oxygen treatment. Although there was not good separation for peaks 7–9 in the oxygen treatment, they can be seen in the chromatogram (Fig. 2).

Results from electron impact (EI) mass spectrometry gave a very clear spectra at scan 400 between 250 and 700 mass units. Using a cut of this scan in the mass range of 370–460 the spectra matched the NBS library spectra for α-tocopherol (vitamin E) and γ-sitosterol. In a comparison of standards for these two compounds and the 4°C control onion lipid sample run on cSFC–FID there was a match for retention times for both compounds (Fig. 3). These peaks are represented by peaks 3 and 4 in the onion lipid chromatogram (Fig. 2).

CONCLUSIONS

These studies represent the first time that the seed lipid components have been compared in fresh and artificially aged seed using capillary supercritical fluid chromatography and flame ionization detection to generate chromatographic pictures and data for intact lipid molecular species reflecting the aging process. Results from these studies show that under less than optimal storage conditions there are significant changes in the lipid component of normal, healthy onion seed. During the aging process, compounds are either fragmented or chemically redistributed. Therefore, when the lipid components are chemically hydrolysed and prepared for fatty acid analysis, changes in the larger triglyceride species are not readily evident. As has been found previously by many others, there was a corresponding loss in seed viability and vigor during the aging process [12]. Under high-temperature and oxidative conditions the change of seed lipid components was accelerated, and the patterns of these chang-

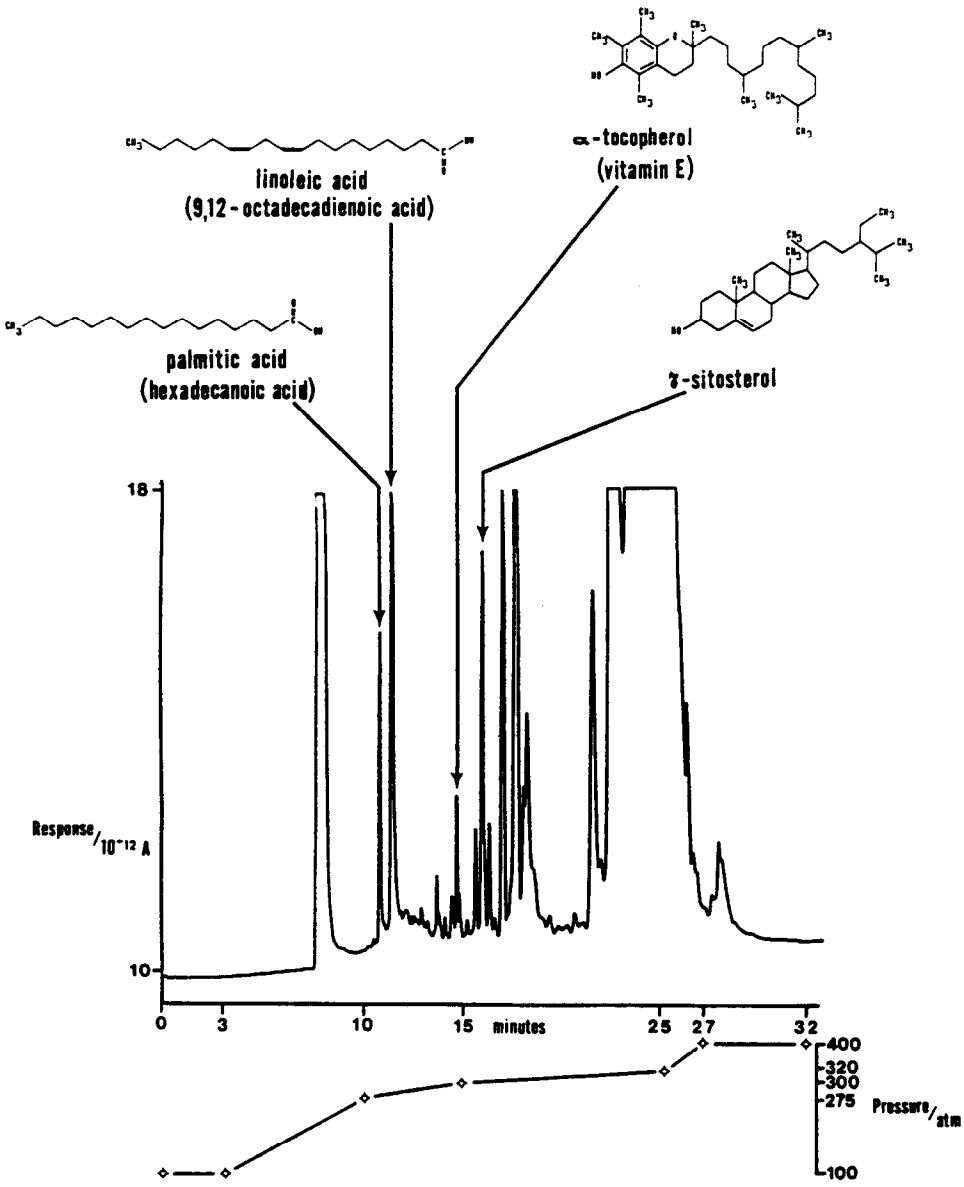

Fig. 3. Combination of mass spectrometry and cSFC retention time matching of standards indicated that two products of accelerated aging were palmitic and linoleic acid. Also, tocopherol and sitosterol were identified in both the control and aged seed lipid samples.

es was similar to what has been found in naturally aged seed [15]. The gradual loss in germination and appearance of seedlings with poor vigor was similar to what is found when testing a broad selection of genotypes from the same species from naturally aged germplasm. There was a general chlorosis, slow growth of the radical, and often

deformation of the primary leaves found in the seedlings from accelerated aged seeds. This was a similar response to what is often found in seeds of the same species which have been stored under optimal maintenance storage conditions for a long period of time.

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