Changes in the Lipids of Alfalfa Prior to and after Dehydration

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Alfalfa was freeze-dried immediately after harvest and after being held for 24 and 48 hours in the fresh state at room temperature. Samples of the lyophilized products were stored with and without added ethoxyquin for 46 days at 37° C. All were analyzed for total extractable lipid, β-carotene, fatty acids, and lipid components. During storage, ethoxyquin protected β-carotene but did not modify the rate of change of other lipid components. For maximum retention of β-carotene, dehydration should be achieved as soon as possible after harvest.

Thompson (1950) and Bickoff *et al.* (1952) showed that the addition of ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, EQ) effectively retarded the rate of loss of β-carotene during storage of dehydrated alfalfa. However, little work has been done on the other alfalfa lipid constituents. The present study was undertaken to compare the effects of various handling and storage treatments on carotene and on several other alfalfa lipids.

Experimental

Samples of fresh alfalfa were harvested at the University of California Experimental Station, Albany, Calif. Control samples were immediately frozen in the field by packing them in dry ice. Additional samples (approximately 100 grams) were held at room temperature for 24- and 48-hour periods. All samples were lyophilized for 20 hours in a Virtis freeze dryer. The resulting meal was stored in brown screw-capped bottles at −18° C. until used.

Each dehydrated meal was divided into three 6-gram parts. One was held at −18° C. as a control. A second was treated with EQ (0.0125%) as previously described (Van der Veen and Olcott, 1964), and a third was used for a storage control. Triplicate samples of treated and untreated samples were then stored at 37° C. for 46 days.

Lipid analyses were performed as follows: Two grams of material were analyzed for β-carotene by the method of Thompson and Bickoff (1951) except that a 6- instead of a 4-cm. column was needed to give reproducible results with the batch of MgO (Westvaco Mineral Products Division, Food Machinery and Chemical Corp., Newark, Calif.) used by the authors. The remaining 4 grams of material from each sample were extracted in a Soxhlet extractor with chloroform for 24 hours. Chloroform extracted only small amounts of nonlipid materials.

The amount of extractable lipid was determined by weighing the residue after evaporating the solvent in a rotating evaporator under reduced pressure. Fatty acid composition of the total lipids was determined by gas chromatography following methylation by transesterification with methanol containing 1% sulfuric acid. The methyl esters were applied to an F & M Model 810 gas chromatograph equipped with dual columns with hydrogen flame detectors. Two 6-foot columns, 4-mm. i.d., packed with 10% DEGS (diethylene glycol succinate, Varian Aerograph) on Chromsorb Q 80- to 100-mesh (Applied Science), were employed. Column temperature was 186° C., and N_2 flow was 75 ml. per minute. The columns were equilibrated at 200° C. for 2 days and standardized with NIH standards or equivalent (Applied Science Lab) as described by Horning *et al.* (1964). The amount of each ester was estimated by the method of Carroll (1961) and also by triangulation or planimetry of peak areas. The methods agreed. Standards were run repeatedly to detect column changes, if any. All methyl ester preparations were stored under N_2 with a few crystals of hydroquinone added as antioxidant. Occasional samples of alfalfa lipid fatty esters were hydrogenated (Farquhar *et al.*, 1959) to help identify the carbon chain length of fatty acids.

The composition of a second part of the extracted lipid sample was determined by the thin-layer chromatographic (TLC) method of Nichols (1963) with chloroform-methanol-water (85:25:3) as the eluting solvent. After the plates were developed and dried, they were sprayed with saturated $K_2Cr_2O_7$ in H_2SO_4 and charred at 180° C. (oven) for 25 minutes, after which a second glass plate was taped onto the developed plate to protect the silica gel surface. The developed plates were scanned with a densitometer (Photovolt, New York). Each spot gave a peak on the recorder, and the integrated area under each was mechanically recorded. Areas of unknowns were calculated by comparing them to those of known standards (Applied Science Lab.). A detailed explanation of the method is given by Blank *et al.* (1964). The solvents were reagent grade and redistilled prior to use.

Results

β-Carotene was lost very rapidly during the holding period prior to dehydration (Table I); 75% disappeared during the first 24 hours after harvest. Ethoxyquin protected the β-carotene that remained and seemed to be slightly more effective in the 24- and 48-hour samples, respectively, than in the control.

The amount of extractable lipid also decreased with increased holding time after harvest (Table II). The extractable lipid from stored samples was larger when the meal was protected with EQ; however, EQ appeared to be more protective in the zero hour control than in the 24- or 48-hour samples.

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Table I. Retention of β-Carotene in Fresh, Lyophilized, and Stored Alfalfa Samples

Storage of Dried Alfalfa[a]	Storage of Fresh Alfalfa[b]					
	No Storage		24 Hours		48 Hours	
	P.p.m.	%	P.p.m.	%	P.p.m.	%
None	240, 267		67, 58, 56		48, 38, 38	
Average	(253)	100	(60)	24	(41)	16
With EQ	156, 199, 190		42, 49, 46		33, 29, 42	
Average	(182)	72	(46)	18	(34)	14
Without EQ	129, 142, 144		29, 28, 28		19, 19, 21	
Average	(138)	55	(28)	11	(20)	8

[a] The lyophilized samples were stored for 46 days at 37° C. with and without added EQ, 0.7 μmole/gram (0.015%).
[b] Stored at room temperature.

Table II. Losses of Extractable Lipid in Treated Alfalfa

Storage of Dried Alfalfa	Storage of Fresh Alfalfa[a]					
	No Storage		24 Hours		48 Hours	
	Lipid, %	Retention, %	Lipid, %	Retention, %	Lipid, %	Retention, %
None	5.2, 5.3, 5.3		4.9, 4.4, 4.5		4.0, 4.0	
Average	(5.3)	100	(4.6)	88	(4.0)	75
Stored with EQ	5.1, 5.4, 5.0		4.8, 4.0, 4.1		3.5, 3.7	
Average	(5.2)	98	(4.3)	81	(3.6)	69
Stored without EQ	4.7, 4.7, 4.6		4.2, 3.7, 3.8		3.5, 3.4	
Average	(4.7)	88	(3.9)	74	(3.5)	66

[a] See footnotes to Table I.

The fatty acid composition of the extractable lipids changed considerably during the holding periods after harvest (Table III). The linolenic acid content dropped sharply and the linoleic and palmitic acids showed relative increases. However, after drying, the stored EQ-treated samples had the same fatty acid composition as did the untreated samples, suggesting that the fatty acids were probably oxidized uniformly and irrespective of their chemical moiety.

There were noticeable changes in the relative amounts of the various lipid constituents prior to lyophilization and during storage after dehydration (Table IV). The major changes that occurred prior to lyophilization were decreases in the phospholipids (PL), digalactodiglyceride (DGDG), and monogalactodiglyceride (MGDG) and an increase in the amount of free fatty acids (FA). There were little changes in the neutral lipids (NL). During storage, EQ did not affect the relative amounts of PL, DGDG, or MGDG in the extractable lipids. However, there was a further decrease in the NL and increase in the FA fractions. EQ did not affect the changes.

Discussion

The critical time to protect the lipids of alfalfa is during the first few hours after harvesting. Booth (1960) has already shown that there is an enzymatic loss of carotene amounting to 10 to 20% during the first 1/2

Table III. Effect of Various Treatments of Alfalfa on the Fatty Acids in the Lipid Extracts[a]

Fatty Acid[c]	Storage of Fresh Alfalfa[b]								
	None			24 Hours			48 Hours		
	Control	Stored with EQ	Stored without EQ	Control	Stored with EQ	Stored without EQ	Control	Stored with EQ	Stored without EQ
14:0	1	1	1	2	2	1	1	1	tr
16:0	10	9	10	15	17	18	13	15	16
16:1	1	1	1	2	2	2	1	2	2
18:0	2	1	1	2	2	2	3	3	3
18:1	1	2	2	3	4	4	4	5	6
18:2	9	11	10	16	17	17	13	18	17
18:3	64	65	63	45	45	44	39	40	41
Others	12	12	12	15	11	12	26	16	16

[a] Fatty acids expressed as % of total peak area in the GLC curve.
[b] See footnotes to Table I for treatment.
[c] Chain length: number of unsaturated bonds.

Table IV. **Effect of Various Treatments on the Composition of the Lipids in Alfalfa Extracts**

	Storage of Fresh Alfalfa[a]								
	None			24 Hours			48 Hours		
	Control	Stored with EQ	Stored without EQ	Control	Stored with EQ	Stored without EQ	Control	Stored with EQ	Stored without EQ
PL[b]	8	8	8	5	6	6	4	4	5
DGDG	10	9	8	4	5	6	4	3	4
MGDG	16	17	16	4	5	7	4	6	5
FA	11	13	20	26	30	38	24	31	30
NL	44	40	36	42	32	30	47	35	37
Others	12	10	11	18	22	15	15	21	20

[a] Analysis by quantitative TLC; the values represent averages of duplicate densitometric analyses of each sample run in triplicate and are expressed as per cent of the total number of counts by the densitometer. See footnotes to Table I for treatments.
[b] PL = phospholipids.
DGDG = digalactolipid.
MGDG = monogalactolipids.
FA = fatty acids.
NL = neutral lipids.

hour. When the alfalfa was boiled or dehydrated immediately, no loss occurred.

The losses of some of the other constituents measured in this study during the holding period prior to lyophilization are probably also due to enzyme action. The rapid decreases in amounts of PL, DGDG, MGDG, and linolenic acid shortly after harvesting are indications of this enzymatic action. The apparent stability of these same constituents after lyophilization suggests that they are protected against oxidation by naturally occurring antioxidants. However, to what extent these lipids were oxidized is difficult to ascertain since only the extractable lipids were measured. Any oxidized lipid would be less likely to be extracted. The galactolipids contain large amounts of linolenic acid, and their oxidation should show a change in this fatty acid. None was observed. The galactolipids apparently were not oxidized any faster than the other lipid constituents measured.

Recently, Thafvelin and Oksanen (1966) studied the vitamin E and linolenic acid content of hay under different drying conditions. Hay that was sun-dried lost 50% of its vitamin E during a 1-month storage period, but the linolenic acid composition remained largely unchanged. However, in hay dried under more moist conditions, the vitamin E loss was greater (60%) and the linolenic acid loss increased (50%). The apparent stability of the linolenic acid in dried hay agrees with results of the present study with alfalfa. The large decrease of linolenic acid in slow-drying, moist hay was similar to that found in alfalfa kept moist after harvest. Sastry and Kates (1964) have shown an enzymatic hydrolysis of MGDG and DGDG in the chloroplasts of damaged runnerbean leaves. Although these processes probably occur also in the intact leaf, the breakdown products are reconverted by the biosynthetic systems in the chloroplasts (Neufeld and Hall, 1964).

The combined evidence suggests that the most important period to protect the lipid constituents is immediately after harvesting. During subsequent storage there is a continued loss of β-carotene and extractable lipids. These losses can be minimized with EQ but the longer the period prior to drying, the less effective is the EQ in protecting the lipid constituents.

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