Carotene and Tocopherol Content of Dehydrated and Sun-Cured Alfalfa Meals

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An analytical procedure for the measurement of total carotenes and total tocopherols in dehydrated alfalfa has been used to survey the carotene and tocopherol content of commercial alfalfa meals from various areas throughout the United States. Wide variability was found in these components in different samples of dehydrated alfalfa and sun-dried alfalfa. Much of this variability probably was due to deteriorative changes which occurred after production of dehydrated meal. Sun-cured alfalfa meal may be a better source of tocopherol than its carotene content would indicate.

DEHYDRATED ALFALFA remains an important source of certain nutrients in animal feeds. Analytical methods for one of these, vitamin E, in forage crops appear to be in need of improvement (10). Although the several tocopherol isomers vary in biological potency, total tocopherol values for alfalfa are sufficient for nutritional evaluations, since 95% of the tocopherol in alfalfa is α-tocopherol (5, 14).

COMBINED ASSAY OF CAROTENE AND TOCOPHEROL IN ALFALFA MEALS

A procedure is described which is an improvement in terms of utility over previously available methods for dehydrated alfalfa samples. This procedure provides an opportunity for simultaneous measurement of total carotene by a widely used chromatographic procedure (2), and is applicable to samples containing antioxidants. Specifically, it eliminates interference in carotene assay (3) from DPPD (N, N'-diphenyl-p-phenylenediamine), and in tocopherol assay from either DPPD or Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline). At the same time, it takes advantage of their protective (antioxidant) action in early stages.

Experimental

For the purposes of the project in which this method was developed, there was available a comprehensive series of dehydrated alfalfa meals. This included samples from several widespread points in the United States, samples from various cuttings, and several sun-dried field samples for comparison. All samples were analyzed for both carotene and tocopherol on an "as received" basis; and stored samples were re-analyzed after 12 weeks and again after 24 weeks of storage at 25° C. in sealed containers. Aliquots treated on receipt with DPPD, and others with Santoquin, were analyzed initially and after the

same intervals of storage under the same conditions.

Extraction. A number of variations in extraction technique were compared before the present one was selected. Using a 50–50 (v./v.) mixture of Skellysolve and absolute ethyl alcohol as a base, replacement of small amounts of ethyl alcohol by water was studied, in both Soxhlet and mechanical types of extraction. The extracted residues contained more dark material and appeared less thoroughly leached out when water was absent, yet these water-free extracts generally gave slightly higher analytical values. At this stage, because of many unanswered questions at other steps in the procedure, it was impossible to be sure whether a higher analytical value meant more efficient extraction of tocopherol and carotene or also extraction of interfering substances. Under the circumstances, it was determined that no extraction procedure would be acceptable if it did not leave a completely exhausted looking residue. Since mechanical extraction was more convenient and gave higher values than Soxhlet extraction, the latter was not considered further.

Watering of the extractant was undesirable, but reconstitution of the dry samples just prior to extraction with a limited amount of water led to more thorough maceration and leaching of the samples, as judged by appearance of residues.

Extraction devices with blades mounted through the bottom of the vessel tended to develop leaks when used with the solvents employed in this work. Heat developed by friction was transferred directly to the volatile, flammable solvents. Accordingly, an ordinary malted milk machine was adapted by mounting a special cutting blade at the end of the shaft from the overhead motor. Wide-mouthed Erlenmeyer flasks containing reconstituted samples and extractant were raised from below to place the contents in the desired relation to the cutting edge. The latter was occasionally sharpened with a fine file.

During such extraction, materials are violently agitated in contact with air and there was no convenient way to evaluate possible losses of carotene and tocopherol. Several factors, however, oppose more than negligible losses: a short extraction time of 8 minutes, low temperature, and presence of two antioxidants in all samples during extraction. These were DPPD and Santoquin, each at a level of 150 p.p.m. of alfalfa.

Antioxidants were added in Wesson oil as a vehicle. Blanks were run to check on possible additions of tocopherol from the oil. It was necessary to add two and a half times as much oil as recommended in the adopted procedure to deflect the Evelyn galvanometer measurably (98 to 99 with an oil-free tocopherol blank at 100). Hence, introduction of tocopherol or reactive compounds from the oil, at the recommended level, was taken as insignificant.

The extraction procedure quantitatively removed both carotene and tocopherol. This was shown by re-extraction of extracted residues on the same day by the same technique, and pooling of extracts until a measurable amount of

[1] Deceased.

the vitamins was accumulated. This indicated removal at first extraction of 99.2% of carotene, and 98.5% of tocopherol.

Preparation of Extract for Chromatography. After extraction it was advisable to eliminate antioxidants from the extracts, since either or both might interfere, particularly in the chromatographic and colorimetric steps. The extracts were washed vigorously in separatory funnels with a large volume of $0.25N$ HCl solution, containing 5% of Na_2SO_4 to eliminate emulsion formation. Soluble solutes—water, alcohol, and dilute acid—were removed, leaving the vitamins in Skellysolve solution which was dried by passage through a column of anhydrous sodium sulfate.

Chromatography. The carotene analysis described is similar to the official AOAC method (2) using Waring Blendor extraction, but with one important modification—use of Micron brand activated magnesia No. 2641 instead of No. 2642, and the chromatography of an extract containing no acetone. This possibly represents a different way of balancing column retentiveness against solvent eluting power. The No. 2641 adsorbent was not sufficiently retentive with solutions containing 9% of acetone.

The washing procedure applied to the extract prior to chromatography eliminated interference by DPPD (3) in the determination of carotene. The test of Smith et al. (15) has been applied repeatedly to eluates obtained by this procedure, with consistently negative results. If this should not always prove to be the case, the procedure of Kaler (9) for removal of the interfering DPPD derivative could be applied, although it was not necessary in the present work.

This work was aimed at determination of both carotene and tocopherol. Numerous attempts were made to devise a chromatographic procedure which would yield each component quantitatively in a fraction devoid of the other. Modifications of the benzene-Floridin chromatographic system (17, 19) were tried in an attempt to get a tocopherol fraction free of carotene. All such attempts failed.

It was easy, however, to obtain eluate fractions from the magnesia columns, one containing all of the carotene and no other colored substance (fraction 1), and a second one containing all of the remaining tocopherol (fraction 2). By making each to known volume, mixing for homogeneity, and combining half of one fraction with half of the other, a combined eluate (fractions 1 and 2) was made available containing precisely half of the eluated tocopherol. Carotene was determined by direct colorimetry in another aliquot of eluate fraction 1. Although nearly all of the tocopherol was present here, it did not interfere, since tocopherol is colorless.
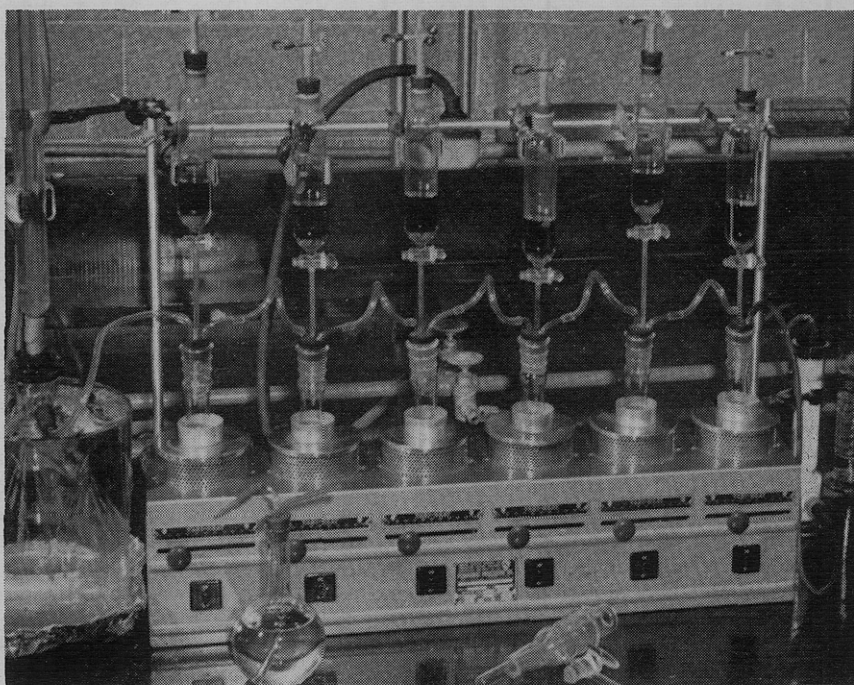

Figure 1. Special apparatus for concentration of eluates

Table I. Tocopherol Obtained in Various Eluate Fractions

Fraction	No Added Tocopherol		500 γ Tocopherol/G. Alfalfa			1000 γ Tocopherol/G. Alfalfa		
	γ/g. alfalfa	% of total	γ/g. alfalfa	% of total	% rec. of added amount	γ/g. alfalfa	% of total	% rec. of added amount
1	216	90.2	776	96.0		1312	97.65	
2a	18.8	7.85	24.7	3.06		26.1	1.94	
2b	4.7	1.95	7.7	0.94		5.5	0.41	
Total	239.5	100.00	808.4	100.00	113.8	1343.6	100.00	110.4

That all tocopherol was contained in these eluate fractions was established by further exhaustive elution of columns from which these fractions had been taken. These further eluates were collected on many occasions as an addendum to regular analyses and were carried through the remainder of the procedure for tocopherol. They did not reduce the Devlin-Mattill reagent (7), which concurrently was reduced by the combined fraction 1 and 2 eluates.

Further, the recovery of added tocopherol in eluate fractions was checked, as follows: Several 2-gram samples of a market sample of dehydrated alfalfa were extracted and prepared for chromatography in the manner described, then were pooled and mixed. Six 25-ml. aliquots (each ca. 1 gram of alfalfa) were taken; two were fortified with 500 γ each of α-tocopherol and two with 1000 γ each of α-tocopherol. The additions were made with a graduated 1-ml. pipet. The six solutions were chromatographed as usual, except fraction 2 which was collected as two subfractions: fraction 2a, containing all bands subsequent to fraction 1, except the prominent canary yellow one referred

to in the procedure; and fraction 2b, consisting of this canary yellow band. (All three fractions—1, 2a, and 2b—collectively constitute the combined eluate used routinely for tocopherol assay.) The separate fractions were carried through the remainder of the procedure for tocopherol, with results given in Table I.

Recovery of added tocopherol was essentially the same at both added levels. It thus appears that the column is unaffected, within reasonable limits, by load size of tocopherol. Nearly all of the tocopherol comes off in fraction 1, and there is quantitative delivery in combined fractions 1 and 2. The fact that the recovery figures for added tocopherol exceed 100% is puzzling. The most likely explanation is evaporative loss of solvent from the standard solution employed.

Since a great many substances affect the Devlin-Mattill reagent, it was necessary to purify the combined eluate further before analysis for tocopherol. For this purpose two further treatments were applied: short-path ("molecular") distillation followed by sulfuric acid washing.

Concentration of Eluates. For short-path distillation the small volumes of eluates needed had to be "dry"—that is, essentially free of solvents. Concentration to small volume by ordinary means in the presence of air could lead to large losses of tocopherol by oxidation. Hence a special apparatus was assembled in which six combined eluates could be concentrated simultaneously under a nitrogen atmosphere in the "flasks" to be used for short-path distillation. The operation of the apparatus shown in Figure 1 requires the practice and development of a skilled technical routine.

The supposed sensitivity of tocopherol to alkali was tested, in order to have results which are not mistaken for oxidative losses. The study was conducted at the step when the apparatus (Figure 1) was available for provision of an oxygen-free atmosphere—namely, after chromatography and before short-path distillation. Six regular eluates were combined and mixed. Twenty-milliliter aliquots (each ca. 0.4 gram of alfalfa) were concentrated—three as usual, and three with saponification (2 ml. of $0.1M$ KOH in absolute ethyl alcohol) and subsequent neutralization with the calculated amount of hydrochloric acid in alcohol; all were included as part of the concentration step under nitrogen. All six eluates were carried through the remainder of the procedure as usual. The average values obtained were 225 γ of tocopherol per gram of alfalfa for the "unsaponified" samples and 208 γ per gram for those treated with potassium hydroxide. This represents destruction of 7.55% of the tocopherol by mild treatment with potassium hydroxide in an oxygen-free atmosphere.

Short-Path Distillation and Sulfuric Acid Treatment. Short-path distillation is an established means for purification of tocopherols (6, 12, 13). Molecular stills are not all identical in design. The one used in this work had, between the header tank, where pressure measurements were made, and the actual distillation units, a dry ice-cooled trap and connecting tubing (approximately $^3/_{16}$ inch in inside diameter) aggregating 110 $\pm$ 10 cm. for any one of the four distillation units connected and used simultaneously on four samples. It is assumed that the actual distillation pressure was higher than that read on the gage for the header tank. This difference was small as indicated by high responsiveness of the gage to the slightest lack of seal at the connections of the distillation units which were sealed with Dow-Corning silicone high-vacuum grease. The distillates obtained on cold fingers were yellow to orange in color, containing carotenes and other carotenoids, which were capable of reducing the reagent in

place of tocopherol. The distillates after being dissolved in acid-washed Skellysolve as described later were shaken vigorously with 85% sulfuric acid, which by chemical addition at carotenoid double bonds converted the latter to derivatives soluble in the acid phase (11).

The complete lack of color after one wash with sulfuric acid indicated that a single acid treatment was completely removing carotenoids. Nevertheless, a uniform batch of composited short-path distillates was distributed equally among 10 centrifuge tubes. Two were treated once with 85% H_2SO_4 in the usual manner. Two more were treated a second time with a second portion of sulfuric acid after removal of the first. Another pair was given three washes; a fourth pair, four washes; and a fifth pair, five washes. All were treated simultaneously with Devlin-Mattill reagent, and gave galvanometer readings against the same blank as shown below:

	Galvanometer Readings				
	1	2	3	4	5
Tube	wash	washes	washes	washes	washes
1	86	86	86	86	85.5
2	86	86	86	85	86

It was concluded that a single treatment with 85% H_2SO_4 under the prescribed conditions was sufficient. As a further check on loss of tocopherol in this treatment, a solution of pure α-

tocopherol in Skellysolve was distributed and handled as described above. Again the galvanometer readings remained constant through five sulfuric acid washes.

Recovery of Added Tocopherol. It was essential to test the recommended procedure for recovery of tocopherol added to sample material and carried through the entire procedure. This was done on several occasions during the course of the regular series of analyses. Data from several recovery trials are summarized in Table II.

Comparison with Other Methods. Tocopherol values (micrograms per gram of dehydrated alfalfa) for these three collaborative samples of dehydrated alfalfa by the proposed new procedure were compared with those obtained by three other methods in different laboratories:

WARF, Sample No.	D.P.I. Method (16)	WARF, Modified (16)	Connecticut Method of Bunnell	Colorado Proposed Method
5020 533	171	165	240	232
5020 573	148	155	195	210
5030 254	125	133	155	149

The lower values by the Stern and Baxter (16) procedure result from inadequate protection of tocopherol from oxidation and photolysis in the preliminary steps. Their procedure considers only the colorimetric aspect of the analysis and is described in application only to soybean oils or to concentrates obtained by short-path distillation as starting materials. More is involved in analysis of cruder materials such as alfalfa. Experience in this laboratory indicates that it is fallacious to assume either complete extraction or lack of destruction of tocopherol during extraction by classical procedures. Soxhlet extraction gave large losses of tocopherol, and contact with alkali, even under nitrogen, produced losses.

Table II. Recovery of Added Tocopherol

Date of Analysis	No. Description of Sample	γ/Gram			
		Added	Found[a]	Recovered	Recovery, %
10-3-55	301 Atlantic-Ohio, untreated, not stored	None	273		
		500	805	532	106
10-19-55	507 Colo. common-Colo., sun-dried, untreated not stored	None	90		
		200	314	214	107
		400	474	384	96
10-20-55	202-S12 SW common-Tex. Santoquin Tr, stored 12 weeks	None	215		
		100	309	94	94
		200	395	180	90
10-27-55	402-S12 Ranger-Neb. DPPD-Tr., stored, 12 weeks	None	216		
		50	266	50	100
		100	319	103	103
12-2-55	Mixed sun-dried, untreated, not stored	None	13		
		200	199	186	93
		200	204	191	96
11-29-57	5020533 Collab. sample, 2 years old	None	135		
		100	230	95	95

[a] Each figure is an average of two replicate measurements.

Reproducibility of Results. Typical analytical results for several samples analyzed in replicate on different days are shown in Table III.

Thus an idea can be gained of the reproducibility and general validity of the proposed procedure. The variability encountered might be due to lack of homogeneity of sample materials, leading to sampling error; particularly since low-potency samples—often observed to be somewhat coarse and stemmy—gave the most difficulty. Several such samples were hammer-milled to pass a fine screen and were re-analyzed in replicate. The data in the lower section of Table III illustrate that in some cases (sample 207) the powdering of samples did have a marked effect on analytical results. These data may even indicate that sampling variation, particularly from day to day, probably constituted a major source of error under conditions employed in this study. Consideration of all aspects indicated the desirability of either running the assays on replicate subsamples or finely grinding samples. The latter was undesirable for the present study; on the other hand, data from replicate samples were considered desirable in this study, aimed as it was at establishment of a valid assay method.

Paper Chromatographic Studies. As a check on the possibility that some of the colorimetric response was due to artifacts, several composite batches of final sulfuric acid–treated short-path distillate were collected and spotted on coated paper with and without, and alongside, standard α-tocopherol, and chromatographed by the techniques of Eggitt and Ward (8).

Results obtained by spraying developed chromatograms with Devlin-Mattill reagent showed clearly that nearly all color developed by the reagent was at a single spot at $R_f = 0.31$, standard α-tocopherol had this same R_f value, and α-tocopherol spotted together with the short-path distillate did not separate from the latter during chromatography. In a few instances a very faint secondary spot was observed at $R_f = 0.82$. This probably was δ-tocopherol, since a calculated R_f for δ-tocopherol, based on the authors' value of R_f for α-tocopherol and the R_f ratios of Eggitt and Ward, gives $R_f = 0.84$. Thus, it appears that the method proposed is not invalidated by responses to nontocopherol artifacts.

Procedure

It is recommended in view of the known properties of tocopherols that all operations be carried out in dim, diffuse light; that alkali be avoided throughout; and that all steps be expedited where sample materials are in contact with air.

Table III. Typical Replicate Analyses
(γ tocopherol/g. of alfalfa)

Date	104	207	304	508	703	705	708
			Sample Number				
11-30-55		85, 83ᵃ					
12-6-55			237				
12-7-55		103, 97				255, 276	
12-8-55		62, 63	240, 248				
12-14-55	185, 191						137, 132
12-16-55	185, 191						135, 139
12-19-55							137, 144
2-16-56				128, 144			
2-22-56					199, 194		
2-23-56						259, 259	
2-24-56				130, 127			
2-27-56					187, 206		

Date	207	208	307	508	607
	Hammer-Milled, Sample Number				
7-11-56	122, 120 114	122, 125 107, 105			
7-13-56	113, 113	115	112, 114	120, 118	
7-16-56			104, 105	113, 113	93, 98
7-17-56					91

ᵃ Each figure represents a separately weighed sample, subjected to the entire analytical procedure.

1. Weigh duplicate 2.0-gram samples into 250-ml. wide-mouthed Erlenmeyer flasks.

2. Add, directly to each sample in its flask, 10 ml. of distilled water. Let stand for 4 minutes, in order for the sample to become uniformly wetted. Then add 300 γ of crude DPPD and 20 mg. of Wesson oil (dissolved in a small volume, x, of low boiling Skellysolve) to each sample not previously treated with DPPD; or 300 γ of Santoquin and 20 mg. of Wesson oil (dissolved in a small volume, y, of Skellysolve) to each sample not previously treated with Santoquin. Add both solutions to each sample not previously treated with either. Place in the extraction flask 50 ml. of absolute ethyl alcohol, and 50 ml. less x ml. or y ml. or (x plus y) ml. of Skellysolve as the case requires.

Raise and support the flask in such a position under a Hamilton-Beach malted milk mixer that the end of the shaft and cutting blade mounted thereon are immersed in the solvent, and about $^{1}/_{4}$ to $^{1}/_{2}$ inch above the bottom of the flask. Start the mixer and leave on for 4 minutes. Stop the mixer and rinse down the sides of the flask with Skellysolve from a wash bottle. Run the mixer for another 4 minutes. Lower the flask, rinsing shaft, and cutter into it with Skellysolve.

Using moderate vacuum (Fisher Filtrator, Whatman No. 1 paper) filter the contents; wash the extracted residue on the filter thoroughly with Skellysolve. Transfer the extract and washings quantitatively into a 500-ml. separatory funnel, keeping the volume of Skellysolve at the minimum necessary for quantitative transfer.

3. Remove alcohol, water, and dilute acid-soluble solutes as follows: Add 150 ml. of a 5% solution of anhydrous Na_2SO_4 in 0.25N HCl. Shake the funnel vigorously. Allow the phases to separate, and drain off the aqueous phase into a beaker (save). Wash the Skellysolve phase with 50 ml. of 5% solution of anhydrous Na_2SO_4 in distilled water. Drain off the aqueous phase and combine with the first aqueous phase. Drain off the Skellysolve phase into a flask (save). Return the aqueous phase to the separatory funnel. Rinse the beaker into the funnel, and extract with 25 ml. of Skellysolve. Discard the aqueous phase. Combine the Skellysolve extracts. Dry by drawing through a 1 × 5 cm. column of anhydrous sodium sulfate into a 100-ml. viscosity flask. At this point, the volume can be reduced if necessary by applying a beaker of hot water to the flask. Rinse the column with a small volume of Skellysolve. Make up to 50 ml. in a volumetric flask with Skellysolve, stopper tightly, mix for uniform distribution of solutes, and keep in a dark place until needed.

4. Chromatograph a 15-ml. aliquot on a 1:1 (by weight) magnesia-Supercel 15 × 100 mm. column topped with 15 mm. of anhydrous sodium sulfate with suction applied through the receiving flask—a 50-ml. viscosity flask. Elute with a solution of two volumes of absolute ethyl alcohol in 98 volumes of acid-extracted Skellysolve. This is prepared by shaking, twice, approximately 3.5 liters in a large separatory funnel with 75 ml. (each time) of concentrated sulfuric acid, then once with 100 ml. of a 2% sodium hydroxide solution, finally twice with 200 ml. (each time) of distilled water. Remove only the first continuous color band, carotene. This usually appears as a dual colored band, and actually is a complex of carotenes; but they do not separate completely under the conditions described. As soon as the carotene is completely removed, replace the receiver by a similar one. Using a 2% (v./v.) solution of ethyl alcohol in Skellysolve, elute the next five bands to appear, the last of which is a prominent canary yellow one. This is eluate fraction 2. The first eluate

(eluate fraction 1) contains all the carotene. The two together contain all the tocopherol. Make up each to 50 ml.

5. Determine the carotene in an Evelyn or other suitable colorimeter, by direct measurement of its color in 5 ml. of eluate fraction 1 diluted to 15 ml. (or as necessary) with Skellysolve. Calculate the carotene content of the sample by reference to a standard curve prepared with dilutions of β-carotene in Skellysolve.

6. Combine 25 ml. of eluate fraction 1 with 25 ml. of eluate fraction 2 (4, above) and evaporate to dryness in a short-path distillation flask. For the evaporation of the mixed eluate, incorporate the flask into a special apparatus over a small sand bath on a hot plate, from which apparatus air and evaporated solvent can be removed by a stream of nitrogen previously scrubbed by passing through alkaline pyrogallol, water, and a calcium chloride drying tube, in that order. After air has been displaced, and with a stream of scrubbed nitrogen flowing through the apparatus, admit the Skellysolve solution slowly into the flask through the stem of a dropping funnel. Maintain a layer in the flask about 5 to 15 mm. deep throughout the concentration process, rinsing the dropping funnel into the flask twice with small additional amounts of acid-extracted Skellysolve. When the last of the Skellysolve has been admitted into the flask, allow it to evaporate to dryness. Raise the apparatus from the hot plate and allow it to cool with nitrogen still flowing.

Proceed at once as follows:

7. Connect the flask containing the sample to the rest of the specially constructed still (sublimation apparatus fitted for evacuation through a side arm with stopcock, and having a built-in cold finger, 17 mm. in outside diameter, as a condenser). Quickly evacuate the still by means of an ordinary aspirator, attached through a trap to the still. This saves time in the following step, as well as needless wear and tear on the high vacuum apparatus. Before applying heat to the still or placing coolant in the cold finger, attach to the diffusion pump and evacuate to 1 micron of mercury or less for 5 minutes. Charge the cold finger with dry ice and acetone (or alcohol). Then lower the still into an oil bath at 205° C., immersing to such a level that the oil surface is at the same level as, or slightly higher than, the end of the cold finger. Leave it in this position for 30 minutes, during all of which time the cold finger is kept well charged with coolant. During the 30-minute distilling period, low pressure must be maintained (0.25 to 0.45 micron of mercury).

8. Close the side-arm stopcock and remove the still from the hot bath. Remove coolant from the cold finger and allow it to warm, while still evacuated. Open the stopcock to relieve the vacuum. Open the still and dissolve the condensate from the cold finger in 10 ml. of acid-washed Skellysolve from a fine-tipped wash bottle, collecting washings and rinsings through a funnel into a 15-ml. graduated centrifuge tube. Add 4 ml. of 85% H_2SO_4. (An automatic buret is convenient.) Tightly insert a rubber stopper and invert two or three times immediately. Shake vigorously and repeatedly over a period of 45 minutes at a temperature of 30° to 35° C. The supernatant liquid, although slightly turbid, must be colorless. Centrifuge at 1800 r.p.m. for 10 minutes at a mean radius of 15 cm.

9. Pipet 5 ml. of the supernatant liquid, which must be absolutely clear and colorless, into an Evelyn colorimeter tube. Add 13 ml. of freshly prepared color reagent, shake the tube, and let stand for 20 minutes or more (up to 50 minutes). Prepare the reagent by dissolving in glacial acetic acid, 50 mg. of α,α-dipyridyl, and 25 mg. of $FeCl_3$. $6H_2O$ per 100 ml. of acid (7). Measure the color developed against a Skellysolve blank by direct galvanometer deflection. Calculate the total tocopherol content of the sample by reference to a standard curve prepared on the same basis with dilutions of α-tocopherol in Skellysolve.

CAROTENE AND TOCOPHEROL CONTENT OF DEHYDRATED AND SUN-CURED ALFALFA MEALS IN THE UNITED STATES

The importance of vitamin E in animal nutrition has long been recognized. Interest in vitamin E (especially α-tocopherol) is intensified because of the variety of properties it possesses. Chemically it may serve as a typical antioxidant. Physiologically its lack or deficiency may produce a series of symptoms, many of which are irreversible.

Alfalfa meal has been recognized as a rich source of α-tocopherol (1, 4). Yet little has been done to evaluate the vitamin E quality of dehydrated alfalfa meal from various sources. It is recognized that tocopherols are readily susceptible to oxidation. Kohler, Beier, and Bolze (10) reported that losses of carotene in dehydrated forage crops were high at ordinary storage temperatures. Vitamin E losses were not so great, but are of concern. Thompson (18) has shown that certain antioxidants greatly decrease the rate of destruction of carotene in alfalfa and other crops. Kohler et al. (10) reported that storage of dehydrated forage crops under nitrogen reduced the rates of loss of vitamin E and carotene.

The project plan included obtaining first and third cutting samples of commercially dehydrated alfalfa meal from six widely distributed areas in the United States. Sun-cured alfalfa meals from the same areas were also included. These samples were obtained directly from the processor. Histories of these samples were to be obtained in so far as available. Samples, as received in Fort Collins, were to be assayed for carotene and vitamins E and K. Aliquots from these samples were removed for storage experiments to be described later.

In addition to the first and third cutting samples from six areas, 10 open market samples were procured at different locations within the United States. They underwent the same initial analyses.

Because of the large number of to-

Table IV. Source of Storage Series Samples

Supplier	Location	Series Numbers
Dixon Dryer Co.	Davis, Calif.	100's
Grayson Alfalfa Dehydrating Mills	Grayson, Tex.	200's
Rohloff Bros., Inc.	Graytown, Ohio	300's
Nebraska Alfalfa Farms, Inc.	Lexington, Neb.	400's
National Alfalfa Dehydrating and Mfg. Co.	Johnstown, Colo.	500's
Hayward, Inc.	El Centro, Calif.	600's

Table V. Source of Open Market Samples

Supplier	Market Location	Sample Source	Number
Chas. M. Cox Co.	Boston, Mass.	Calvert Milling Co., Hoytville, Ohio	701
Quaker Oats Co.	Cedar Rapids, Iowa	Dawson Co. Feed Products, Lexington, Neb.	702
Ralston Purina Co.	Ft. Worth, Tex.	Bert and Wetta, Gothenburg, Neb.	703
Uncle Johnny Mills	Houston, Tex.	H. E. Co. (Hackeny, Kan.), Winfield, Kan.	704
Allied Mills, Inc.	Chicago, Ill.	Allied Mills, Inc., Cozad, Neb.	705
McMillen Feed Mills	Ft. Wayne, Ind.	Reimer Alfalfa, Wakenda, Mo.	706
Poultry Producers of Central Calif.	Petaluma, Calif.	Jerry Fielder's Dehydrator, Dixon, Calif.	707
H. K. Webster Co.	Lawrence, Mass.	W. J. Small Co., Kansas City, Mo.	708
H. K. Webster Co.	Richford, Vt.	Saunders Mill, Toledo, Ohio	709
Poultry Producers of Central Calif.	Petaluma, Calif.	Poultry Producers Dehydrator, Ryar Island, Calif.	710

copherol analyses that were essential for the experiments planned, it was considered essential to evaluate current analytical procedures extensively. The procedure developed as a result is presented in the first part of this paper.

The source and origin of the samples outlined are indicated in Tables IV and V.

Sample Handling. The samples representing areas and markets approximated 100 pounds. The dehydrated alfalfa meal samples had been sacked and shipped by the processor from regular commercial mill channels. The sun-cured alfalfa hay samples had been hammer-milled to meal when received.

Each master sample was thoroughly mixed and blended prior to subdivision. The commercial mixer used accomplished this operation by a combination of screw conveying and tumbling. Fractionally large portions of the sample were repeatedly removed from and returned to the mixer during the process to ensure complete blending.

After thorough mixing the master sample was taken to the laboratory, where it was subdivided and placed in slip-covered 10-pound metal cans. The covers were sealed with freezer tape. All material not immediately used was placed in low temperature rooms at −20° F.

Samples for initial determination of moisture, carotene, and tocopherol were largely removed after subdivision, and analyzed. When master samples arrived during week-end or holiday intervals, the subdivided samples sealed as indicated were held at −20° F. until the following week for initial analysis. Such containers were brought to room temperature before opening.

Historical Information Regarding Processors' Samples. This information is presented in Table VI. The truck strike during the summer of 1955 had an influence on the length of time between date of cutting and receipt of sample, since most of the samples were forwarded by truck.

Information regarding histories of the market samples was far from complete, except as to the sources indicated. Results obtained suggested that quality compared favorably with the area samples just presented.

Results

The means of analytical values for carotene, tocopherol, and percentage of dry matter are presented in Tables VII, VIII, and IX. To show the agreement and dispersion of the data, Figure 2 compares carotene and tocopherol values of paired analyses calculated on the vacuum-oven dry basis. The coefficient of correlation for linear regression is 0.865. When carotene is plotted along the abscissa and tocopherol along the ordinate, the least squares

Table VI. Histories Assembled on Processors' Samples

Series No.	100	200	300	400	500	600
A. Location and Transportation Variables						
General source location	Davis, Calif.	Grayson, Tex.	Graytown, Ohio	Lexington, Neb.	Johnstown, Colo.	El Centro, Calif.
1st cutting date	4-1-55	5-2-55	5-3-55	4-26-55	6-20-55	11-14-55
Shipped	4-15-55	5-7-55	5-8-55	6-2-55	7-22-55	11-17-55
Received	4-29-55	5-14-55	5-16-55	6-21-55	7-22-55	11-23-55
1st cutting						
Days in transit	14	7	8	19	1	6
Days after cutting	28	12	13	56	32	9
3rd cutting date	6-14-55	7-7-55	7-18-55	8-19-55	9-8-55	4-2-56
Shipped	6-22-55	8-30-55	7-19-55	8-27-55	10-21-55	4-9-56
Received	7-12-55	9-12-55	7-27-55	9-2-55	10-21-55	4-21-56
3rd cutting						
Days in transit	8	13	8	6	1	12
Days after cutting	28	67	9	14	40	19
Sun-cured cutting and 1955 date	1st (5-14)	1st (4-5)	1st (6-5)	1st (6-1)	3rd (9-8)	3rd (4-5)
B. Other Available Historical Information (Furnished by Processors)						
Variety						
1st cut	Buffalo	SW Common	Atlantic	Ranger, Cert.	Colo. Common	African Common
3rd cut	Buffalo	SW Common	Atlantic	Ranger, Cert.	Colo. Common	Calif. Common
Yields, tons/acre						
1st cutting	1.25	0.75	0.95	1.75	. . .	0.5
3rd cutting	1.0	. . .	1.0	1.5	2.0	1.25
Sun-cured	. . .	1.0	1.0	1.75	2.0	1.0
Irrigation, 1st and 3rd cuttings	Yes	No	No	Yes	Yes	Yes
Age of stands, years						
1st and 3rd cuttings	3	5	3	3	3	3
Sun-cured	4	4	1	2	3	2
Fertilizer, lb./acre						
1st and 3rd cuttings	None	0-20-0 200	0-12--12 400	Manure 70	None	None 100
Sun-cured	None	0-20-0	None	Avail. P	None	Liquid P.
Protein, %						
1st cutting	20.3	24.7	22.0	19.3	20.0	. . .
3rd cutting	23.7	18.3	. . .	17.1	19.6	20.9
Carotene, IU/lb.						
1st cutting	204	235.2	204	177.5	175	. . .
3rd cutting	167.7	133.3	. . .	131	153	185
Condition						
1st cutting	LB[a]	LB	VLB	B	L $\frac{1}{2}$ B1	VLPB
3rd cutting	LPB	. . .	S $\frac{1}{2}$ B1	LSFB1	. . .	LB

[a] LB = leafy bud; VLB = very leafy bud; B = bud; L $\frac{1}{2}$B1 = leafy $\frac{1}{2}$ bloom; VLPB = very leafy prebud; LPB = leafy prebud; S $\frac{1}{2}$B1 = stemming $\frac{1}{2}$ bloom; LSFB1 = leafy stemmy full bloom.

Table VII. Dehydrated Alfalfa Meal from Six Areas

(Carotene and tocopherol values in mg./100 g. dry basis. Analyses of unstored samples, Histories Table V)

Area Series No.	Mean Carotene,[a] Mg./100 G.	Mean Tocopherol,[a] Mg./100 G.	% Dry Matter[a] in Samples
Cutting 1			
101	20.4 ± 0.09	19.1 ± 0.23	91.70 ± 0.01
201	25.1 ± 0.43	23.7 ± 0.13	94.76 ± 0.01
301	21.5 ± 0.33	20.2 ± 0.59	93.18 ± 0.02
401	22.8 ± 0.35	23.0 ± 0.10	93.01 ± 0.01
501	10.1 ± 0.05	18.5 ± 0.08	95.61 ± 0.01
601	19.7 ± 0.48	19.8 ± 0.62	97.06 ± 0.01
Cutting 3			
103	19.8 ± 0.08	17.6 ± 0.06	94.24 ± 0.01
203	12.0 ± 0.27	23.1 ± 0.30	93.27 ± 0.01
303	24.9 ± 0.23	25.1 ± 0.67	95.12 ± 0.01
403	17.3 ± 0.31	19.3 ± 0.18	93.68 ± 0.04
503	23.2 ± 0.63	19.2 ± 0.48	94.96 ± 0.01
603	27.9 ± 0.45	28.0 ± 0.30	97.27 ± 0.02
Mean 1st cut	19.9 ± 0.99	20.7 ± 0.43	94.22 ± 0.54
Mean 3rd cut	20.7 ± 1.06	21.9 ± 0.80	94.76 ± 0.39
Mean (cuttings combined)	20.3 ± 1.09	21.3 ± 1.05	94.49 ± 0.33
Std. dev.			
Mean, 1st cut	0.99	0.43	0.54
Mean, 3rd cut	1.06	0.80	0.39
Mean, both cuts	1.09	1.05	0.33
1st cutting array	4.89	2.10	1.87
3rd cutting array	5.20	3.92	1.36
Total arrays	5.01	3.31	1.62

[a] ± standard deviation.

Table VIII. Sun-Cured Alfalfa Hay Meal

(Carotene and tocopherol values, in mg./100 g. dry basis)

Area Series No.	Mean Carotene,[a] Mg./100 G.	Mean Tocopherol,[a] Mg./100 G.	Mean %[a] Dry Matter in Samples
107	4.8 ± 0.15	13.6 ± 0.21	93.85 ± 0.02
207	2.3 ± 0.09	12.3 ± 0.09	92.19 ± 0.01
307	2.7 ± 0.09	12.2 ± 0.25	90.67 ± 0.02
407	2.7 ± 0.09	9.7 ± 0.19	91.84 ± 0.03
507	4.6 ± 0.16	11.3 ± 0.17	94.49 ± 0.02
607	3.0 ± 0.04	9.9 ± 0.16	95.41 ± 0.02
Mean	3.3 ± 0.07	11.5 ± 0.10	93.06 ± 0.05
Std. dev.			
Means	0.07	0.10	0.05
Arrays	1.00	1.46	1.72

[a] ± standard deviation.

Table IX. Carotene, Tocopherol, and Solids Values for Dehydrated Alfalfa Meal from 10 Widely Separated Markets

| Market Sample No.	Mean Carotene,[a] Mg./100 G.	Mean Tocopherol,[a] Mg./100 G.	Mean %[a] Dry Matter
701	18.4 ± 0.40	19.6 ± 0.38	93.92 ± 0.01
702	13.5 ± 0.51	18.0 ± 0.92	95.69 ± 0.01
703	14.2 ± 0.48	19.3 ± 0.14	95.06 ± 0.02
704	17.2 ± 0.02	21.6 ± 0.16	95.15 ± 0.01
705	30.6 ± 0.47	22.8 ± 0.59	95.13 ± 0.01
706	13.9 ± 0.09	13.4 ± 0.97	92.84 ± 0.02
707	26.9 ± 0.68	21.5 ± 0.13	93.00 ± 0.02
708	14.9 ± 0.19	15.9 ± 0.76	93.59 ± 0.02
709	23.3 ± 0.63	25.3 ± 0.46	95.61 ± 0.01
710	25.5 ± 0.55	23.7 ± 0.14	87.68 ± 0.03
Grand mean	19.8 ± 0.96	20.1 ± 0.58	93.77 ± 0.52
Std. dev.			
Means	0.96	0.58	0.52
Arrays	5.42	3.65	2.32

[a] ± standard deviation.
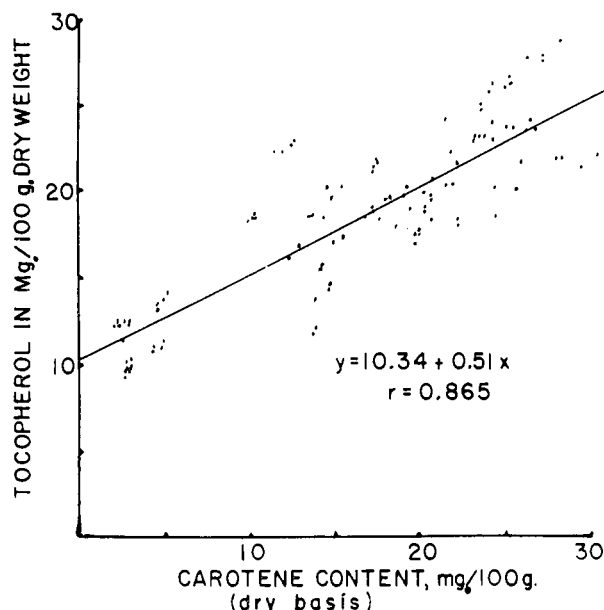
$$y = 10.34 + 0.51 x$$
$$r = 0.865$$

Figure 2. Comparison of carotene and tocopherol content of alfalfa meal

line relating them is described by the equation $y = 10.34 + 0.51 x$.

Discussion

It is believed that no adequate survey has been made previously to evaluate the carotene and tocopherol content of commercial alfalfa meal as it is produced in the United States. Hence variations that might arise from difference in variety, climate, culture, production, handling, and transportation were evaluated. During the year these samples were collected, a transport strike occurred. This variable may be inferred to have had a somewhat larger than usual effect. The widely dispersed points near the center of Figure 2 arose from samples that lay on transportation docks for a considerable time in summer heat. The data suggest that, if carotene were being rapidly lost, tocopherol was being spared relatively.

The means of the two cuttings from the six areas lie close together. The bulk of the variation is found between the various areas. This same situation exists among the open market samples. These observations coupled with the background information leads one to infer that a large portion of the variability was probably introduced after production.

A large gap lies between the carotene and tocopherol content of sun-cured alfalfa meal and these values in dehydrated alfalfa meal. Nevertheless, the values from the sun-cured meal had a marked influence in establishing the regression between the carotene and tocopherol values of these samples. Figure 2 shows the gap in data is considerable. The equation derived to estimate tocopherol content from carotene evaluation should be used with caution. Results suggest that it is possible to develop a relationship between these two components, so that knowledge of one will lead to a reasonably accurate estimate of the other.

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PLANT ANALYSIS

Determination of Saponins in Alfalfa

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A reasonably rapid procedure is presented for determining total saponins in alfalfa. Selective influences of several solvent systems upon the adsorptive behavior of activated carbon are used to separate saponins from nonsaponins. Applied to plant samples the method yields reproducible results, although the analytical values are somewhat higher than the true values. The method has been used in studying the relation between chick growth and saponin content of alfalfa in feeds.

UNDER SOME CIRCUMSTANCES triterpenoid saponins of alfalfa (5, 7) can inhibit growth in chicks and reduce egg production by hens (1, 3). There is also evidence that alfalfa saponins and those of some other legume forages may contribute to the occurrence of bloat in ruminants (2, 6).

Investigations into the importance of saponins in dehydrated alfalfa fed to chicks created a need for a quantitative method of determining alfalfa saponins.

Because alfalfa contains a number of different saponins in unknown and presumably varying proportions (6) as well as other substances of similar properties, the early methods of assay which depend upon hemolytic properties or surface activities of saponins (4) are not applicable. The tendency of cholesterol to form addition compounds with saponins has been used to recover saponins from alfalfa (6, 7), but attempts to base an analytical method on the same principle were abandoned when preferential recovery of some and incomplete recovery of all alfalfa saponins could not be avoided. For a time a method was used whereby sapogenins rather than intact saponins were estimated (6). However, the procedure required much precise manipulation and far too much time for routine application.

The method presented here uses selective influences of several solvent systems upon the adsorptive behavior of activated carbon toward saponins and other alfalfa extractives. It allows estimation of saponins rather than sapogenins

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with fair rapidity and with sufficient accuracy for use in studies of their occurrence in alfalfa and their significance in animal diets.

Procedure

Analytical Method. In a 1-liter Erlenmeyer flask, mix 15 grams of dried, coarsely ground or chopped sample with 75 ml. of water. Let the mixture stand 5 hours. Add 210 ml. of 95% ethyl alcohol, mix by swirling, stopper, and let the mixture stand 20 hours; then add and mix 53 ml. of 95% ethyl alcohol and 162 ml. of water. The volume of liquid added is now 500 ml. and the concentration of ethyl alcohol is 50%. Allow 1 hour for equilibration, then suction-filter the liquid through medium speed paper. If the dry plant material is finely ground, as little as 3 grams of sample and proportionally smaller volumes of liquids may be used to prepare this extract solution.

To a 50-ml. portion of the alcoholic extract solution (equivalent to 1.5 grams of sample) add 1.0 gram of activated carbon and warm over steam with occasional stirring for 15 minutes. Suction-filter through a 5.5-cm. medium speed filter paper. Complete the transfer and wash with 100 ml. of 50% ethyl alcohol. Evaporate the filtrate and washings to near dryness over steam, warm the residue with 20 ml. of water until solution is complete, and then add 1.5 grams of activated carbon. Stir the mixture occasionally and continue warming over steam for 5 minutes. Suction-filter through a 5.5-cm. smooth, hardened paper. If the filtrate contains

carbon, return it to the funnel, repeating the filtration if necessary until a carbon-free filtrate is obtained. Wash the carbon in the funnel with four 20-ml. portions of water followed by two 20-ml. portions each of 10 and 20% ethyl alcohol. Discard the filtrate and washings. To elute adsorbed saponins from the carbon wash the filter with 200 ml. of a mixture of pyridine (purified grade) and absolute ethyl alcohol. 3 to 7 (v./v.). Throughout filtration and elution do not permit the liquid level to reach the filter cake between additions. Evaporate the pyridine-alcohol eluate in a tared dish, vacuum-dry the residue 16 hours at 65° C., and weigh as saponins.

Commercial activated carbons differ in their suitability for this procedure not only by brands and grades, but also within the same brand and grade. Each lot that is to be used has to be selected by test. Mixed alfalfa saponins for testing activated carbons can be prepared according to the procedure that follows.

Preparative Recovery of Saponins

Heat a continuously stirred mixture of 500 grams of alfalfa meal and 4 liters of water to 95° C. and suction-filter the hot liquid. Heat the filtrate to boiling and add 80 grams of powdered cholesterol. Stir and boil the mixture gently for 5 minutes, then cool it to room temperature and add 40 grams of analytical grade diatomaceous earth. In a 15-cm. Büchner funnel, suction-filter the liquid through medium speed paper that has been precoated with 20 grams of analytical grade diatomaceous earth in the form of a filter cake. To avoid