

Rapid Determination of Carotene and Xanthophyll in Dried Plant Materials

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A method is presented for the rapid determination of carotene and xanthophyll in dried plant materials by stirring or reflux extraction followed by chromatography on a magnesia column. Procedures are

specified which adequately exclude chlorophyll from the column eluates. Results obtained are in good agreement with analytical values obtained by overnight extraction at room temperature.

Until recent years, carotenoid analysis of dehydrated alfalfa for production quality control was limited to determination of carotene. A variety of methods have been used in different laboratories for this analysis, even though two official methods—employing hot extraction or overnight soaking—were recommended by the Association of Official Agricultural Chemists in 1950 (Quackenbush, 1950) (for carotene analysis only).

The increasing importance of forages as poultry pigmentsers has made analysis for xanthophylls as important as that for carotene. An improved total xanthophyll method (WU-1971) providing increased extraction has recently been published (Livingston *et al.*, 1971). At the present time collaborative evaluation of a tentative AOAC method by Quackenbush *et al.* (1970) for carotene and pigmentsing xanthophylls is also underway. Recent studies at this laboratory have indicated that the tentative AOAC procedure and the WU-1971 procedure give similar carotene and total xanthophyll analyses for dehydrated alfalfa meal samples (Livingston *et al.*, 1972). However, both of these procedures as described require overnight extraction. Quackenbush and Miller (1972) have recently proposed hot extraction of corn gluten meal, dehydrated alfalfa meal, and commercial mixed feeds in the presence of methanolic KOH, using the solvent of the tentative AOAC procedure. However, the data presented therein seem insufficient for the conclusion drawn that hot saponification may be substituted in that method for overnight treatment.

In order to expedite commercial operations, a rapid procedure giving an analysis within approximately 2 hr would be of real value to the dehydration industry. The following method has been successfully used for analysis of dehydrated forages including alfalfa, turf grass clippings (custom Kentucky Bluegrass), cauliflower leaf, and PRO-XAN [an alfalfa protein-xanthophyll concentrate (Knuckles *et al.*, 1971)].

APPARATUS

Chromatographic columns are constructed of borosilicate glass tubing, 12.5 mm i.d. \times 30 cm length, with a tapered capillary tube 2 mm i.d. \times ca. 8 cm length sealed to the lower end and fitted to a vacuum filtration bell jar with a rubber stopper. The condensers are cold-finger type, to fit into 100-ml volumetric flasks loosely (*e.g.* Pyrex Cat. #91300, size 1).

REAGENTS

The extractants are hexane-acetone mixtures, 7:3 or 1:1 (v/v). The column adsorbent consists of magnesium oxide (Sea Sorb 43, Fisher Scientific Co.)-diatomaceous earth (Hyflo Super-Cel, Johns Manville Co.), 1:1 (w/w), used as received, mixed by tumbling 200 times. The eluant used for carotene is hexane-acetone, 9:1 (v/v), and that used for

xanthophylls is hexane-acetone-methanol, 8:1:1 (v/v/v). The solvent mixtures are prepared with "High Purity" grade (or equivalent) hexane (Phillips Petroleum Co.), reagent grade acetone, and absolute anhydrous methanol, all used without further purification.

The chromatographic column is packed dry under reduced pressure, firmly tamped with a flat-end rod to a final height of 7 cm, and a 2-cm layer of anhydrous sodium sulfate powder is added to the top of the column.

PROCEDURE

All operations are performed under reduced light. Two grams of sample, ground through a 40-mesh screen, are placed in a 100-ml volumetric flask. Thirty milliliters of hexane-acetone, 7:3, are added plus 0.5 ml of H₂O per 2 g of sample, with gentle swirling. The sample is then extracted either by refluxing or stirring. (Exceptions: Use 1 g of sample if xanthophyll content is greater than approximately 200 mg/lb; a 60-mesh screen for grinding dehydrated grass; 1:1 hexane-acetone for extraction of PRO-XAN; and no added water during extraction of freeze-dried materials.)

Reflux Extraction (All Meals). A cold-finger condenser is fitted to the flask, which is then suspended in a 60° water bath just to the depth of the solvent surface. The sample is refluxed for the time indicated in Table II. The sample is then cooled briefly, 10 ml of hexane is added, and the sample is swirled for 1 min. Two milliliters of 40% methanolic KOH (w/v) is added and the sample is thoroughly mixed for 1 min by careful vigorous swirling. Following this it is held for 30 min, protected from light; then 1.5 ml of H₂O is added (1.75 ml for 1-g sample, 2 ml for freeze-dried) and the sample is again thoroughly mixed by swirling for 1 min. It is then diluted with hexane to a volume of 100 ml, stoppered, and shaken vigorously five times, by hand. The sample is set aside in the dark for 15 min prior to being chromatographed.

Stirring Extraction (Freeze-Dried Alfalfa, Dehydrated Whole or Leaf Alfalfa, PRO-XAN). A 1.5-in. Teflon stirring bar is gently inserted into the flask. The flask is stoppered, suspended over a magnetic stirrer, and smoothly stirred at room temperature (see Table II for time required). Two milliliters of 40% methanolic KOH is added and the sample is again stirred for 1 min. The mixture is set aside in the dark for 30 min; then 1.5 ml (or see above) of H₂O is added with additional stirring for 1 min. A stirring bar retriever is employed to remove the stirring bar which is rinsed within the flask with a few milliliters of hexane. The sample is diluted to 100 ml with hexane, stoppered, and vigorously mixed five times, then set aside in the dark for 15 min prior to being chromatographed.

Chromatographic Separation. With a 25-ml volumetric flask in place beneath the column on a vacuum filtration apparatus, 10 ml of sample extract is transferred without remixing to the chromatographic column and vacuum is ap-

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Table I. Effect of Fineness of Grind on Carotene and Xanthophyll Analysis

	Screen mesh	Carotene, mg/lb		Xanthophyll, mg/lb	
		Reflux ^a	O.N. ^b	Reflux ^a	O.N. ^b
Dehy leaf cauliflower	20	151.5	162.8	250.2	267.1
	40 ^c	163.9*	168.5*	278.6	277.5
	60	160.4	167.1	272.0	274.4
Dehy whole alfalfa	20	99.0	102.2	155.1	157.2
	40 ^c	101.7	98.0	158.6	155.6
	60	102.0	100.5	160.6	156.0
Dehy turf grass	20	166.7	180.7	381.2	451.5
	40	176.3	192.6	431.0	477.5
	60 ^c	191.3	188.7	474.6	491.8

^aRefluxed 1 hr with (7:3) hexane-acetone + 0.5 ml of H₂O. ^bWU-1971 O.N. procedure. ^cReflux vs. O.N. analytical results for this mesh are not significantly different ($p = 0.05$, by Duncan's multiple range test) excepting those marked (*), which agree within 4%.

Table II. Comparisons of Procedures and Extraction Time Effects on Xanthophyll^a Analysis^b

	Reflux, hr			Stir room temperature, hr			O.N. Soak, hr
	0.25	0.5	1	0.25	0.5	1	16
Dehy whole alfalfa	149.6 ^c	153.3	155.8	148.5	152.0	153.2	156.0
Dehy leaf alfalfa	136.3	138.6	142.7	127.8	131.8	136.5	135.6
Dehy leaf cauliflower	251.2	267.2	278.6	237.5	260.3	262.7	277.5
Dehy turf grass ^d	411.1	435.6	474.6			434.3	491.8
Freeze-dried alfalfa	318.4	319.1	310.5	317.7	324.6	303.4	312.0
PRO-XAN	281.5	282.1		274.1	282.0		278.5

^aXanthophyll values are given in mg/lb of sample; each value is the average of duplicates. ^bThirty milliliters of hexane-acetone (7:3) plus 0.5 ml of H₂O was employed for dehy meals, the same solvent except no H₂O for freeze-dried alfalfa, and 20 ml of hexane-acetone (1:1) plus 0.5 ml of H₂O for the PRO-XAN. ^cValues underlined are not significantly different from results by WU-1971 O.N. Soak extraction ($p = 0.05$, Student's *t* test). ^d60-mesh grind.

plied. When all of the extract has entered the sodium sulfate layer, 10 ml of 9:1 hexane-acetone is added to the column. Elution is continued until this solvent has all entered the sodium sulfate; then 8:1:1 hexane-acetone-methanol is added to fill the column. The receiving flask is changed when the carotene band is completely eluted and the xanthophyll band is approximately halfway down the column. The xanthophylls are then eluted into a 25-ml volumetric flask with approximately 20 ml of the same solvent. At least 2 ml of acetone is then added to the xanthophyll eluate to ensure that only one liquid phase is present. [This elution procedure may be used also in previously published overnight-soak carotene and xanthophyll methods (Kohler *et al.*, 1967; Livingston *et al.*, 1971) if preferred.]

Determination. The eluates are adjusted to 25 ml with 9:1 hexane-acetone for carotenes, and with acetone for xanthophylls. The carotenes absorbance is measured in a 1-cm cell at 436 $m\mu$; the xanthophylls absorbance is measured at 475 $m\mu$. The absence of chlorophyll can be checked by measuring the absorbance of the xanthophyll eluates at 665 $m\mu$. Non-epoxide xanthophyll (NEX) may then be readily determined (Livingston *et al.*, 1969) on the same xanthophyll solutions (employing the absorptivity of 210 for the acidified xanthophyll solutions instead of 196 as described in that publication).

CALCULATIONS OF RESULTS. Carotene (mg/lb) = $(A_{436} \times 454 \times F)/(196 \times L \times W)$, where F = extract volume correction (0.95 for 2 g of meal, 0.958 for 1 g of meal used), L = cell length in cm, and W = (g of meal extracted/100 ml) \times (volume extract chromatographed/final volume of solution read in spectrophotometer). Total xanthophyll (mg/lb) = $(A_{475} \times 454 \times F)/(236 \times L \times W)$. NEX (mg/lb) = $(A_{475} \times 454 \times F)/(210 \times L \times W)$, where $W = 10/25 \times W$ for total xanthophyll.

RESULTS AND DISCUSSION

Sample Preparation. Dehydrated alfalfa, cauliflower, and turf grass meals were ground through 20-, 40-, and 60-mesh screens, and the efficiency of extraction by reflux was com-

pared with the WU-1971 overnight-soak extraction. The data presented in Table I indicate that 40 mesh is sufficient fineness for extraction of cauliflower and alfalfa; however, turf grass meal requires a 60-mesh grind in order to assure complete carotenoid extraction.

Extraction. Hexane-acetone, 7:3, with H₂O added was satisfactory for rapid extraction of alfalfa, cauliflower, and turf grass meals. For PRO-XAN, Knuckles *et al.* (1971) have found that hexane-acetone, 1:1, is a more effective extractant and it was therefore used for this product. Analyses of various meals initially yielded lower values by reflux extraction than by overnight soaking. However, the addition of a 10-ml increment of hexane to the refluxed sample just prior to KOH treatment resulted in significantly improved carotene and xanthophyll recoveries. The addition of more than 10 ml of hexane made the saponification of chlorophyll much more difficult, resulting in frequent chlorophyll contamination of the xanthophyll fraction during chromatography, and is therefore not recommended. Higher values were obtained by this addition of hexane in the reflux extraction trials only.

Table II indicates that dehydrated whole or leaf alfalfa, freeze-dried alfalfa, and PRO-XAN can be extracted by refluxing for 0.25 hr, and dehydrated cauliflower leaf can be extracted by refluxing for 1 hr. Dehydrated turf grass is more difficult to extract, but 1 hr refluxing gives results less than 4% below the overnight soaking extraction.

It was also found that freeze-dried alfalfa and PRO-XAN can be satisfactorily extracted by simply stirring at room temperature in the recommended solvent system for 0.25 hr, and that dehydrated whole or leaf alfalfa can be thus extracted in 0.5 hr. Since the extraction by stirring requires less equipment and manipulation than heating in a water bath, this may be the preferred procedure for these materials in many laboratories. Surprisingly, no increase in the rate or degree of extraction was obtained by stirring during refluxing. It appears that agitation due to boiling is sufficient, with no enhancement achieved by mechanical stirring.

Table III. Comparison of Methods of Analysis for Carotene^a and Xanthophyll^a

Sample	WU-Rapid Reflux		WU-1971 (O.N.)		Tentative AOAC (O.N.)	
	Carotene ^b	Xanthophyll ^c	Carotene ^b	Xanthophyll ^c	Carotene ^b	Xanthophyll ^d
Dehy whole alfalfa	82.1	124.4	84.0	126.8	80.6	131.9
	95.3	148.6	101.8	146.9	97.9	148.1
	38.6	175.9	40.7	177.9	37.8	174.9
	108.8	165.9	115.5	161.8	108.8	170.2
Dehy leaf alfalfa-1	140.8	227.2	148.1	234.8	147.1	233.2
Dehy leaf alfalfa-2	110.8	169.1	112.8	168.2	106.4	175.4
Dehy stem alfalfa	70.0	124.0	69.4	126.6	69.7	131.5
Freeze-dried alfalfa	131.6	253.0	129.4	253.3	130.9	263.6
Dehy cauliflower leaf	163.9	278.6	168.5	277.5	161.8	282.0
Dehy turf grass ^e -1	161.1	373.4	159.5	379.1	153.3	396.6
Dehy turf grass ^e -2	189.5	474.6	187.2	491.8	190.6	506.5
PRO-XAN	135.9	239.7	127.3	229.7	131.2	232.4

^a Carotene and xanthophyll are given in mg/lb mfb; each value is the average of duplicate analyses. ^b Measured at 436 m μ . ^c Measured at 475 m μ . ^d Measured at 474 m μ . ^e 60-mesh grind.

Discussion of Results. Carotene and total xanthophyll values obtained by the above described WU-Rapid method compare favorably with WU-1971 analyses, as well as with the tentative AOAC overnight soak analyses (Table III). In all but two cases, [dehy leaf alfalfa-1 (carotene), dehy turf grass-2 (xanthophyll)] WU-Rapid analyses were equivalent ($p = 0.05$, by student's t test) to results by one or both of the overnight extraction methods; in the two exceptions, the reflux procedure results were within 5% of one or both of the overnight analyses. Accordingly, most control laboratories may use either rapid or overnight extraction and expect close agreement. Measurement of xanthophyll absorbance at 475 m μ is specified since this is the long wavelength peak position for all-trans lutein; the absorptivity 236, used in the calculation, is that of all-trans lutein.

Pigmenting xanthophyll values determined by the non-epoxide xanthophyll analysis on WU-Rapid or WU-1971 extracts and by the dihydroxy pigment equivalent (DHPE) analysis of the tentative AOAC procedure are presented in Table IV. In all trials, the WU-Rapid NEX analyses were equal to results by one or both of the other methods ($p = 0.05$, Duncan's multiple range test); in the two cases (freeze-dried alfalfa, dehydrated turf grass) where there was a significant difference from one of the overnight methods, the difference was less than 5%. In the NEX procedure, the absorptivity 210 is employed for the calculations, instead of using 196 as formerly described (Livingston *et al.*, 1969). The 196 absorptivity had been selected in order to yield an NEX value, for low-epoxide dehydrated alfalfa meals, equivalent to the WU-1967 total xanthophyll analysis of Kohler *et al.* [The latter procedure had provided a total xanthophyll determination which, applied to dehydrated alfalfa, correlated well with poultry broiler pigmentation in feeding trials (Kuzmicky *et al.*, 1968).] With the improved extraction obtained by the WU-1971 analysis, use of the 210 absorptivity (correct for 0.02 N acid-treated lutein) in NEX calculations gives results which, for high-epoxide xanthophyll meals, provide better pigmentation potency estimates than the total xanthophyll analyses. For commercially dehydrated alfalfa meals in general, however, the WU-1971 total xanthophyll analysis is suitable. In limited feeding trials at this laboratory, analyses of the same test rations by these two methods provided similar broiler skin pigmentation prediction (Livingston *et al.*, 1972).

The data presented in Tables II, III, and IV demonstrate that analysts may now use either the overnight WU-1971 or the WU-Rapid method for carotene and xanthophyll analysis, followed, if desired, by pigmenting (NEX) xanthophyll determination. The rapid method here presented is simple and

Table IV. Comparison of Methods of Analysis for Pigmenting Xanthophyll^a

Sample	Method	WU-	WU-	Tentative
		Rapid	1971	AOAC
		NEX ^b	NEX ^b	DHPE ^c
Dehy whole alfalfa		158.7	160.5	162.6
		122.6	125.7	119.4
Dehy leaf alfalfa		152.8	156.6	155.0
Freeze-dried alfalfa		189.6 ^d	182.9	199.3
Dehy turf grass		298.3	297.6	285.8
Dehy cauliflower leaf		237.0	236.7	238.0
PRO-XAN		243.7 ^e	240.8	245.0

^a Values are averages, mg/lb, of duplicate analyses. ^b Nonepoxide xanthophyll. ^c Dihydroxy pigment equivalent xanthophyll. ^d Extracted by stirring for 15 min at room temperature. ^e Extracted by stirring for 30 min at room temperature.

reproducible. The carotenoid separations are clearly defined and can therefore be carried out properly by personnel with relatively little training or experience in chromatography, while yielding reliable results.

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