sources of lactose were employed. A deproteinized casein whey containing 13.8% lactose was hydrolyzed at 35° C. with the 0.5% lactase preparation, but excluding the phosphate buffer. A 36.2% conversion of lactose to oligosaccharides was obtained in 5 hours, an increase of 11.6% over that obtained with a 15% solution of U.S.P. lactose.

The hydrolysis of a milk product with a lactose concentration above 25% was effected using 50% solids whey containing 35.5% lactose. The maximum conversion of lactose to oligosaccharides in the hourly fractions analyzed was 43.6% at 7 hours. This served as additional evidence that under the set of hydrolysis conditions employed in this study, a conversion of 44.6% (or approximately 45%) lactose to oligosaccharides was the limit.

Table II. Influence of Addition-Type Hydrolyses on Oligosaccharide Production

Lactose, %	Lactase, %	Time, Hr.	Hydrolyzate Components, Grams/100 Ml.				Lactose Converted to Oligosaccharides, %
			Lactose	Galactose	Glucose	Oligosaccharides	
15.0	0.5	8.0	1.4	4.4	5.5	3.7	24.6
15.0	0.5[a]	10.5	1.1	3.9	4.9	5.1	34.1
Add	0.25[b]						
25.0	0.5	8.0	3.4	5.5	7.5	8.6	34.4
25.0	0.5[a]	8.0	2.3	5.9	7.7	9.2	36.9
Add	0.25[b]						
35.0	0.58	5.0	2.3	6.8	10.3	15.6	44.6
35.0	0.58[a]	10.0	2.5	6.7	10.4	15.4	44.0
Add	0.29[b]						

[a] Starting concentration.
[b] Concentration at end of reaction.

Table III. Chromatographic Analysis of a 35% Lactose Addition-Type Hydrolysis

Time, Hr.	Hydrolyzate Components, Grams/100 Ml.				Lactose Converted to Oligosaccharides, %
	Lactose	Galactose	Glucose	Oligosaccharides	
5.0	8.1	4.5	7.5	14.9	42.6
6.0	5.9	4.7	9.4	15.0	42.9
8.0	3.7	5.7	10.5	15.1	43.1
10.0	2.5	6.7	10.4	15.4	44.0
12.0	1.8	6.9	11.1	15.3	43.7

Acknowledgment

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CAROTENE ASSAY

Determination of Carotene in Silages and Forages

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A rapid chromatographic method for determination of carotene in silages and forages is described. Necessary conditions for separation of carotene from impurities on magnesium oxide–Celite columns in the presence of small amounts of alcohol have been established. The analysis combines the advantages of splash-free blending extraction afforded by alcohol–Skellysolve B foaming mixtures and the elimination of epiphasic washing to remove alcohol. Additional advantages include faster and more compact elutions gained by the presence of alcohol on the column. Direct collections in smaller volumes eliminate concentration and transfer operations.

THE EXTRACTION OF CAROTENE from moist samples of forages and silages is usually effected by solvents which extract water as well. Methods in current use employ such extractants as 85% acetone (*1*), alcohol–petroleum ether mixtures (*6*), or acetone–petroleum ether mixtures (*9*). Generally, the extracts are made into two-phase systems by addition of water and petroleum ether—a hypophase of water plus acetone, or water plus alcohol, and an epiphase of petroleum ether. The two phases are allowed to settle before separation, carotene being extracted into the epiphase.

Because chromatographic separation of carotene from impurities is adversely affected by rather small amounts of alcohol, epiphases from alcohol–petroleum ether blendings are first made free of alcohol by water-washing, an operation often attended with troublesome emulsions. Zscheile (*9*) eliminated the need for washing by selecting acetone plus Skellysolve B as a blending liquid—acetone having much less effect than

alcohol on chromatographic separations of carotene from impurities. However, blender extractions made with the foaming mixture of alcohol and petroleum ether offer the advantage of relatively splash-free performance. A method combining these two desirable features—i.e., splash-free blending and elimination of washing to remove alcohol—takes advantage of the tolerance of the magnesium oxide–Celite column to a small amount of alcohol—the amount present in an aliquot of unwashed epiphase. This limited quantity of alcohol offers additional advantages of faster elutions and smaller volumes of eluent. The carotene, by virtue of small amounts of ethyl alcohol on the column, is eluted in a condensed volume into a volumetric flask without attendant concentration and transferral operations.

Reagents and Apparatus

Adsorbent. Magnesium oxide (Micron brand No. 2642, Westvaco Chlorine Products Co.) and Celite 535 (Johns-Manville Co.) are mixed 1 to 1 by weight and kept in a well-capped bottle.

Alcohol, absolute.

Skellysolve B and Skellysolve F (Skelly Oil Co.) are purified as described in an earlier publication (*8*).

Foaming mixture, 3 volumes of ethyl alcohol plus 1 volume of Skellysolve B.

Eluting solvent, 9 volumes of acetone plus 91 volumes of Skellysolve B.

Methanolic potassium hydroxide, 1 pound of potassium hydroxide dissolved in 1400 ml. of methanol (ACS grade).

Salt solution, 4% aqueous sodium chloride (ACS grade). Acetone (ACS grade).

Apparatus. Waring Blendor with variable transformer for speed control.

Separatory funnels, 1-liter Squibb type.

Sintered-glass funnel, coarse filter plate, 85-mm. diameter, 80-mm. depth, 400-ml. capacity.

Chromatographic tube, 25 mm. in inside diameter, 250 mm. in length, terminated with 24/40 standard taper joints—female at top, male at bottom. Sintered-glass filter disk, 25 mm. in diameter, sealed in immediately above bottom joint.

Collection bulb, 50 mm. in diameter, with 24/40 standard taper joint, terminated at the bottom with a stopcock and delivery tube. Side tube, 6 mm. in inside diameter, 25 mm. in length, attached to the side of the bulb at 45° angle for an air vent. The collection bulb was joined to the bottom of the chromatograph tube during filtrations.

Pressure source, a tank of nitrogen gas with a diaphragm pressure regulator with needle valve attached. Pressure is communicated through Tygon tubing from the regulator to the chromatographic tube.

Method

Gross samples are chopped with a paper cutter into approximately 1/4-inch lengths. Clumps present in the chopped mass are loosened and then handfuls of the material are held about 1 foot over a table surface and sprinkled over a circular area to effect an even distribution. Portions are taken from symmetrically disposed spots of the material for analytical samples. Twenty-gram samples are weighed for analysis of fresh green forage and grass or legume silages, 50 grams for corn silage. Ten-gram samples are taken in the case of alfalfa leaf meal or ground hays; the latter are softened before extraction by mixing well in a 100-ml. beaker with 30 to 35 ml. of warm water at 60° to 70° C.

The weighed sample is placed in a Waring Blendor, to which are added 275 ml. of foaming mixture and 25 ml. of methanolic potash. The blender is started, slowly at first, by means of a variable transformer while the contents are adjusted to maximum foaming condition by addition of either alcohol or a small amount of water; excess of either of these corrections is neutralized by addition of the other. The addition of alcohol is indicated when no foaming is shown. Maximum foaming and splash-free blending appear when barely enough alcohol is present to bring the water and Skellysolve B into a single phase. This condition is manifested by appearance of a light colored foam of inflated volume and by decreased blending noise. After adjustment the blender is operated at full voltage for 10 minutes. The contents of the blender are poured into a large sintered-glass filter funnel provided with a thin mat of Celite 535 and filtered directly by suction into a 1-liter separatory funnel. Alcoholic rinsings of the blender vessel are poured onto the residue, stirred, and filtered. Then 170 ml. of Skellysolve are added, stirred, and filtered, and the filter funnel is removed. Three hundred and fifty milliliters of sodium chloride solution are added to the separatory funnel, and the contents are given 50 vigorous shakes and allowed to settle. The lower layer is discarded; the top layer is poured from the mouth of the funnel into a 200-ml. volumetric flask containing 6 ml. of acetone. Skellysolve F is used to rinse the funnel and to bring the extract to volume. Ten-ml. aliquots are chromatographed.

A chromatographic column is made by pouring a slurry of 15 grams of adsorbent mixture in Skellysolve B into the chromatographic tube. Pressure, 10 pounds per square inch, is applied from a tank of nitrogen to the top of the chromatographic tube. The adsorbent is compressed to a compact form in the solvent stream. The solvent is expressed nearly to the top level of the column, and the pressure is removed and reduced to 0.5 pound per square inch. A 10-ml. aliquot of carotene extract is added and also forced to the adsorbent level. A small rinsing volume, about 5 ml. of eluting solvent, is also forced down to the level of the column. Approximately 70 ml. of the eluting solvent are added to the tube and pressure is restored. Carotene travels as a fine band on the top portion of the column and widens somewhat as it approaches the bottom. Eluent may be examined in the collection bulb with its stopcock adjusted so that approximately 2 cm. of liquid is maintained above the stopcock. After the first appearance of yellow at the top of this liquid column, the stopcock is closed and a 50-ml. volumetric flask is placed below the delivery tube. The stopcock is opened to the volumetric flask and the eluent is collected until clear. The filtrate is made to volume with the eluting solvent and spectrophotometric readings are made at 450 mμ with a Beckman Model DU, or equivalent, spectrophotometer. With the absorbance, α, of 1 γ per ml. in a 1-cm. cell taken as 0.25, the carotene in 1 gram of forage or silage may be calculated from the spectrophotometer reading, A (1-cm. cell), by means of the formula

$$\gamma/\text{gram} = \frac{A \times \text{reading volume (ml.)}}{\alpha \times \text{aliquot wt.}} = \frac{A \times 50}{0.25 \times \text{aliquot wt.}}$$

Experimental Results

The effect of methanolic potassium hydroxide as an additional blending component was tested in efforts to obtain epiphases from which aliquots could be directly chromatographed.

Carotene extracts were obtained by blending 20-gram samples of various types of silages with and without the addition of methanolic potassium hydroxide in the foaming mixture. The extracts—epiphasic layers—were made to 200 ml. with Skellysolve F without washing, 6 ml. of acetone being included in each volumetric flask. Two 10-ml. aliquots of each extract were chromatographed—one directly, the other after being freed of alcohol. The removal of alcohol was conveniently effected by bringing the aliquots to dryness in vacuo, the dry residues being dissolved and rinsed onto the column with the eluting solvent. Directly chromatographed aliquots were eluted by less than 50 ml. of the eluting solvent into 50-ml. volumetric flasks; alcohol-free aliquots required considerably larger volumes of eluting solvent of approximately 200 ml., and a longer time for elution. The latter eluates, collected in 500-ml. flasks, were brought nearly

to dryness in vacuo at approximately room temperature and transferred to 50-ml. volumetric flasks with the eluting solvent. Filtrates by both procedures were adjusted to 50 ml. volumes and the absorbances at 450 mμ were compared as shown in Table I.

When 0.10 ml. of ethyl alcohol was added to dealcoholated aliquots which had been redissolved in the eluting solvent, subsequent chromatographic elution was rapid and required less than 50 ml. of the eluting solvent. Analyses of these aliquots with adjusted alcoholic content, 0.10 ml., are compared in Table II with those obtained by direct chromatography.

Discussion

Skellysolve B rather than Skellysolve F is used in the foaming mixture because of lower volatility and chance of ignition by vapors passing over the motor brushes of the blender. However, Skellysolve F constitutes the greater portion of the final extract, as it gives quicker clearing and settling of phases than Skellysolve B and is more easily removed by vacuum when concentration or alcohol removal is desired.

As the destructive action of Celite on carotene in Skellysolve B is suppressed by small percentages of acetone (*8*), 6 ml. of the solvent are included in the 200-ml. volume of carotene extract.

The addition of methanolic potassium hydroxide to the foaming mixture serves several purposes. Foaming action is improved, less adjustment being required to secure stable foaming with various samples. The removal of chlorophyll into the hypophase by action of the potash leaves the carotene pigment in a more stable environment (*7*). Epiphases clarified by potash give considerably less fouling of glassware. The main function of the methanolic potash treatment, however, is to make possible the direct chromatography of epiphasic aliquots with rapid and clean separation of carotene from other pigments. This purpose is possibly accomplished by a more complete removal of alcohol into the hypophase when methanolic potash is present—alcohol left in the epiphase being reduced to a safe level.

In Table I the analysis of the alcohol-free aliquot is used as a valid reference method differing little in detail from the Association of Official Agricultural Chemists' method (*1*). Results in this table indicate that potash-treated aliquots agree consistently well with the reference method; analyses on unsaponified aliquots often show sizable differences.

Data in Table II indicate that separation of carotene from other pigments can safely be performed when 0.10 ml. of ethyl alcohol is present under the prescribed conditions of the method. A few tests in this laboratory have indicated that higher amounts of alcohol—i.e., 0.20 ml.—will sometimes permit small amounts of noncarotene pigments to elute with the carotene.

When use of 10-ml. aliquots results in undesirably low absorbance values, larger aliquots may be dealcoholated in vacuo, and upon addition of 0.10 ml. of alcohol, be rapidly chromatographed with a small volume of the eluting solvent.

To check the purity of carotene eluted from magnesium oxide–Celite columns, carotene extracts from alfalfa and orchard grass plus clover silages were passed through lime-Celite columns (*4*) from which the cis-trans isomers were individually eluted (*8*). These isomers may be identified from well established absorbance curves (*2*). Neo-β-carotene-U, the fourth isomer eluted in significant amounts, was used to mark the completion of carotene removal.

Summation of the absorbances of the four isomers at 450 mμ agreed within

Table I. Effect of Methanolic Potassium Hydroxide in Foaming Mixture on Carotene Analysis

Sample No.	Sample Description	Carotene Analysis, γ/G. Dry Matter: Foaming Mixture A[a]	Foaming Mixture B[b]	Foaming Mixture + KOH A[a]	Foaming Mixture + KOH B[b]
236	Silage, 1st cutting alfalfa + clover	175.6	154.6	154.6	151.9
263	Silage, 2nd cutting alfalfa + clover	135.1	134.5	127.2	127.2
264	Silage, 2nd cutting alfalfa + clover	184.6	184.6	184.1	183.5
267	Silage, 2nd cutting alfalfa + clover	119.3	120.9	121.6	122.0
268	Silage, 2nd cutting alfalfa + clover	171.4	168.2	175.2	175.2
270	Silage, 2nd cutting alfalfa + clover	185.8	180.8	182.6	182.1
237	Silage, 2nd cutting alfalfa + clover	151.0	133.8	132.3	127.2
238	Silage, 2nd cutting alfalfa + clover	211.8	186.6	174.7	170.4
241	Silage, 2nd cutting alfalfa + clover	173.3	161.9	169.5	166.3
247	Silage, 2nd cutting alfalfa + clover	133.1	120.9	122.7	117.8
255	Silage, 2nd cutting alfalfa + clover	129.3	117.1	115.3	114.8
256	Silage, 2nd cutting alfalfa + clover	149.7	133.2	141.1	141.6
248	Silage, 3rd cutting alfalfa	204.2	200.8	181.7	180.8
252	Silage, 3rd cutting alfalfa	200.9	163.8	186.2	186.2
253	Silage, 3rd cutting alfalfa	222.9	211.9	219.5	215.2
254	Silage, 3rd cutting alfalfa	216.9	214.9	183.2	183.2
257	Silage, 3rd cutting alfalfa	211.9	198.3	205.6	203.6
260	Silage, 3rd cutting alfalfa	53.5	52.6	53.5	51.7
276	Silage, 3rd cutting alfalfa	66.4	63.8	63.8	63.8
273	Silage, 1st cutting, oats + clover	155.1	143.2	157.1	157.1
469	Silage, 1st cutting orchard grass + clover	230.3	229.5	220.3	218.1
470	Silage, 1st cutting orchard grass + clover	339.3	311.0	302.4	304.4
473	Silage, 1st cutting orchard grass + clover	242.0	226.2	208.8	211.8
474	Silage, 1st cutting orchard grass + clover	309.9	294.2	294.2	295.8
471	Silage, 1st cutting orchard grass	139.8	137.8	139.8	138.8
472	Silage, 1st cutting orchard grass	151.2	155.7	155.7	155.7
186	Silage, 1st cutting orchard grass + clover			166.9	165.8
187				249.7	249.7
188				214.2	213.3
189				200.0	197.4
190				271.5	269.0
191				321.0	319.4
...	Alfalfa leaf meal			110.0	110.4
	Corn silage			30.63[c]	30.53[c]

[a] Epiphasic aliquot not dealcoholated before chromatography.
[b] Epiphasic aliquot dealcoholated before chromatography.
[c] 30-ml. aliquots dealcoholated; 0.10 ml. ethyl alcohol added to A.

Table II. Carotene by Chromatography

Sample No.	Sample Description, 7 Months Ensiled	Carotene, γ/G., Dry Basis A[a]	B[b]
192	1st cutting, fresh orchard grass and clover + corn meal (10 + 1)	171.9	170.7
197	1st cutting, fresh orchard grass and clover + corn meal (10 + 1)	108.2	106.3
193	Fresh orchard grass control	268.8	268.0
194	Fresh orchard grass + kylage preservative, 5 lb./ton	233.2	231.6
199	Fresh orchard grass + kylage preservative, 5 lb./ton	283.2	282.4
195	2nd cutting, fresh orchard grass unchopped	108.4	108.4
196	2nd cutting, fresh orchard grass chopped	221.2	221.2

[a] Untreated aliquots of epiphase.
[b] Adjusted aliquots containing 0.10 ml. ethyl alcohol.

1% with the absorbance values obtained at 450 mμ by use of magnesium oxide–Celite columns.

Concerning the selection of wave length for absorbance measurement, other wave lengths and appropriate coefficients may be used to estimate the carotene with greater stoichiometric accuracy since the cis isomers have lower coefficients than the all-trans isomer at 450 mμ. Results calculated at this wave length as all-*trans*-β-carotene are low. Biological activities of the cis isomers relative to absorbancies are even lower than for the all-trans form (*3*, *5*). Hence, calculation as suggested at 450 mμ may reflect the biological potency with less error than calculations at other wave lengths which indicate higher carotene content.

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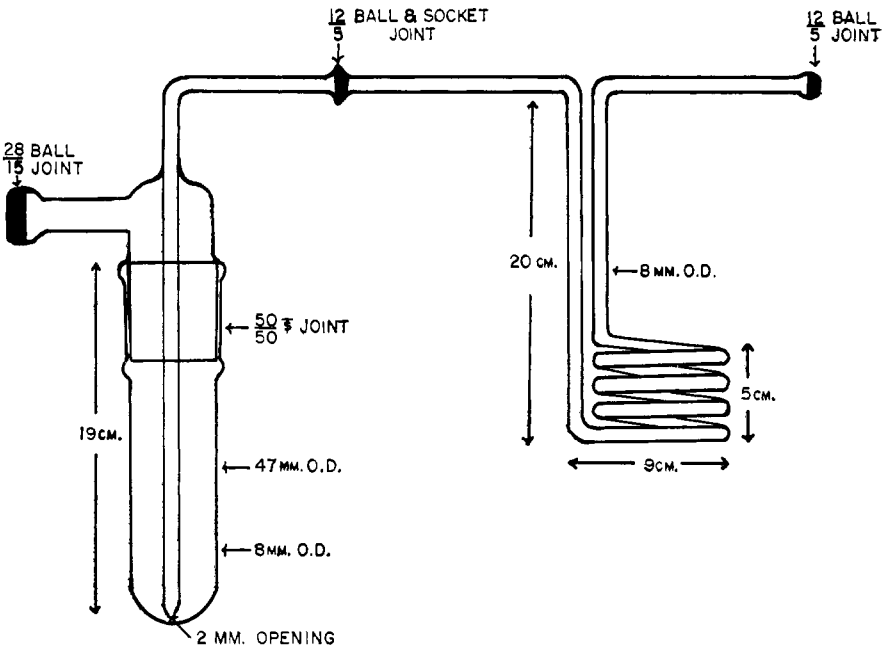
Determination of Antioxidants in Edible Fats—Correction

Figure 4 was omitted from the article on Determination of Antioxidants in Edible Fats [Constance Anglin, J. H. Mahon, and R. A. Chapman, J. Agr. Food Chem. **4,** 1018 (1956)]. It is printed below.

▼ Figure 4. Distilling flask and superheater coil



NUTRITIVE VALUE OF BEANS

Nutrients in Central American Beans

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Because of the special nutritional importance of beans in Central America and Panama, the factors influencing protein, methionine, lysine, and tryptophan content of 25 varieties were studied. Niacin, thiamine, and riboflavin were also determined. Over-all differences in nitrogen and tryptophan content among varieties and between localities were highly significant. The fertility of the land significantly alters the yield and riboflavin content of the kidney bean, but the content of nitrogen, methionine, lysine, tryptophan, niacin, and thiamine is not detectably affected by fertility differences.

The kidney bean, *Phaseolus vulgaris*, is second only to maize in importance in the diets of the people throughout most of Central America. It is not unusual for these beans to account for 20 to 30% of the protein in the diet (*3*, *5*, *20*). Under these circumstances the quality of their protein becomes of crucial importance. However, data on the variations in nutritive value of the kidney bean, especially in essential amino acids, are surprisingly limited. The most complete report is that of Jaffé (*16*), who described the chemical composition, digestibility, protein efficiency, and limiting essential amino acids in a group of bean samples from Venezuela.

Large differences in thamine and riboflavin content of beans grown at different locations have been reported by Eheart and associates (*4*). Guyer, Kramer, and Ide (*10*) observed that as harvest was delayed the yield increased but the