ESTIMATION OF THE VITAMIN A POTENCY OF BUTTER BY VARIOUS METHODS

by

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INTRODUCTION

Methods of estimating vitamin A and carotene content of food products have stimulated many enterprising investigations. Since the establishment of quantitative analysis by the absorption spectra and color tests, efforts have been made to supplant the more tedious method of biological assay. Research workers in different laboratories have been using the methods of determining vitamin A activity with varying success, due to lack of standardization of manipulation, type of assay, method of interpretation of data, or ways of expression of results.

Butter, one of the valuable sources of vitamin A in the diet, contains not only this vitamin but also its precursor β-carotene, which is converted into the vitamin in the animal organism. To these two substances butter owes the major part, if not all, of its vitamin A activity.

It is the purpose of this research to determine quantitatively, by the various methods developed during the last decade, the amount of vitamin A and carotene in certain samples of butter, and to compare the findings with results of the biological assay of butter by the single feeding method. Comparisons of the results obtained from the rela-

tively short processes of the physical and physico-chemical tests with those of the biological assay should be of value.

REVIEW OF LITERATURE

At present two methods for the quantitative estimation of vitamin A are: (1) the evaluation of the blue color intensity of the antimony trichloride test either by means of the tintometer or the spectrophotometer, (2) the measurement of the ultra-violet absorption at 328 mμ with a spectrograph. Biological assay measures total vitamin A activity, which includes vitamin A and carotene.

Primitive in the field is the biological method devised and used by Steenbock and his associates (1918) and by Osborne and Mendel (1920). Since then numerous methods have been put forth, and among the most used in this country is that of Sherman and Munsell (1925). Recently Sherman and Todhunter (1934) developed a modification styled the single feeding method, which is believed to give more nearly accurate results than most of the older procedures. Morton (1935) gives the pros and cons of the methods developed and used by the English workers.

With recent trends, the necessity of carrying on biological experiment is no longer essential in order to assay oils. Color reactions and absorption spectra have largely

superseded the older methods in this one phase. Initial in the last decade's progress was the observation of Rosenheim and Drummond (1925) that liver oil and extract gave a blue color with $AsCl_3$, the intensity varying with the vitamin content. Carr and Price (1926) found that a saturated solution of $SbCl_3$ in chloroform was more satisfactory. Estimations were first made by means of a tintometer. Later a more refined method employed the spectrophotometer, which when available provided a higher degree of accuracy. Booth (1933) in working with butter discovered that the blue color estimation was more reliable when the non-saponifiable residue of the butter, dissolved in chloroform, rather than the untreated butter, was used. When the proper precautions are taken, satisfactory estimates of the vitamin A in butter may be made.

Morton and Heilbron (1928) observed the presence of a specific absorption band at 328 mμ in oils, the intensity of the absorption being proportional to the vitamin A concentration. As a result, Drummond and Morton (1929) made an attempt to correlate the biological and spectroscopic tests on a quantitative basis. In 1930 Morton and Heilbron studied the absorption maxima in butter. Correlations were also made between the intensity of the blue color and the intensity of the absorption band at 328 mμ. A considerable

amount of vitamin assay has been carried on by these physical and chemical methods, especially since the discovery that vitamin A of butter passes quantitatively into the non-saponifiable fraction (Steenbock, 1918, and others) and most irrelevant absorption is eliminated in this way (MacWalter, 1934). Baumann and Steenbock (1933) found that sterols and fatty acids had no effect on the absorption at this point.

The ability to prepare or procure pure samples of vitamin A has led to the establishment of extinction coefficients which are essential in standardizing the procedure and making it universal for laboratories sufficiently equipped. Carr and Jewell (1933), in an investigation of the purest specimen they could prepare by vacuum distillation, found $E\ \frac{1\%}{1\ cm.}$ 328 mμ equal to 1600. In other words, a solution containing 1 per cent of pure vitamin A, adjusted to a depth of 1 cm. gave a value of 1600 for the density or log Io/I. Extinction coefficients at the maxima 583 and 620 mμ for the blue color spectrophotometric tests are 2600 and 5000, respectively, (Morton 1935). De (1935) developed a procedure for estimating vitamin A based on the fact that the vitamin is destroyed by ultra-violet light. He decolorized the ether extract of the foodstuff in question and then spectrographed immediately and after irradiation

for 3 hours. The difference between the absorption coefficient at 328 mμ was taken as representative of the vitamin A content.

The adoption of the spectroscopic methods of determining vitamin A has been hindered by the fact that the equipment necessary for this purpose, the quartz spectrograph, is expensive and its operation highly specialized. Irish (1936) described in detail a modified form of the spectrograph which has been placed on the market in recent years. The Vitameter, as it is called, is designed to measure the ultra-violet light near the region 328 mμ by means of the optical system which permits only light of this wave length to pass through the instrument. As in the spectrophotometer, the intensities of color, this time parallel green lines on a fluorescent screen, are matched. The author discussed the results of experiments testing the validity of quantitative measurements with this apparatus.

The problem of the relative reliability of the biological, tintometric, and spectrographic methods for the estimation of vitamin A in oils has been investigated by Coward et al. (1932) and Morgan et al. (1935). Baumann and Steenbock (1933) have made a similar, but less comprehensive, study on butter, involving also the determination of carotene.

Recently Moore (1930) proved by conclusive experiment that the plant pigment, carotene, was converted into vitamin A in vivo. This led to a search for more accurate methods of quantitative analysis not only in plants but also in butters. The quantitative method of determination outlined by Palmer (1922) assumes that carotene imparts the same color intensity to oils, such as butter, as an equal proportion of carotene gives to solvents such as petroleum ether. Goldblatt and Barnett (1932) disproved this and found that carotene in oils has a considerably higher color intensity.

The technique of Schertz (1923) for making spectrophotometric determinations of carotene has been used by various investigators, but with certain modifications. Although Barnett (1934) has shown that this method could not be accurately applied to butter fat because "the transmittancy of light at various wave lengths was different for carotene in oil solutions than for an equal concentration in alcohol or ethers", Shrewsbury and Kraybill (1933) used the procedure and from a series of experiments concluded that the carotene in petroleum ether and in butter fat dissolved in petroleum ether could be determined with considerable accuracy by means of the spectrophotometric method. These same investigators (1933) as well as others, Steenbock et

al. (1918), Baumann et al. (1931) and Cady and Luck (1930) found that butter fat contains natural antioxidants which protect the carotene from oxidation. These antioxidants are destroyed or removed by treatment with charcoal.

In a comparative study, Baumann and Steenbock (1933a) determined the carotene in butter fat directly in a Bausch and Lomb spectrophotometer. The butter was melted at 55°C. and then decanted from the curd. The absorption band was measured at 30°C. and pure carotene in refined cottonseed oil was used as a basis of comparison.

Less expensive apparatus, such as the tintometer and colorimeter are often used instead of the more costly spectrophotometer and are capable of giving a fair degree of accuracy. Potassium dichromate (Palmer, 1922) and bixin (Holmes and Bromund, 1936) have been used as standards in the colorimeter.

The study of the stability of carotene was considered imperative shortly after its significance was established as a standard of reference for vitamin A. Baumann and Steenbock (1933b) gave a review of the literature up to this point, and reported their results. They found that refined cotton seed oil (Wesson oil) is outstanding as a stabilizing solvent for carotene. Solutions in Wesson oil can be kept without deterioration for months if stored at

a low temperature and in a dark place.

PROCEDURE

The butters used in this experiment were obtained through the cooperation of the Department of Dairy Husbandry. They were churned from the sweet cream obtained from the secretions of the first, fifth and thirtieth days of lactation and stored at 0°C. until used for experiment. A generous quantity of one butter called a "Commercial sample", was purchased at the Kansas State College dairy sales counter in the early part of February 1936. Upon the latter, trial methods were carried out to ascertain the workable details of the procedures for this laboratory. Of the butters used, this commercial sample only contained added coloring matter, a vegetable compound which did not interfere with tests used, as shown by suitable checks.

Analysis of vitamin A was accomplished by use of the spectrograph and spectrophotometer. Carotene, the precursor of vitamin A, present in a significant and measurable amount, was determined by means of the spectrophotometer. Total vitamin A activity (vitamin A plus carotene) was determined biologically.

Spectroscopic Method

In order to evaluate the vitamin A by means of the spectrograph, duplicate samples of 5 gm. (colostrous butter) and 15 gm. (other butters) were prepared according to the method of Baumann and Steenbock (1933a) and Semb, Baumann, and Steenbock (1934).

The butter was saponified 30 minutes with 120 ml. of 12 per cent freshly prepared alcoholic potash in an atmosphere of water-pumped nitrogen. Immediately after saponification 125 ml. of distilled water were added and the solution allowed to stand in ice water until thoroughly chilled.

Commercial ether was freed from aldehydes and ketones by shaking it (1) with a saturated solution of $NaHSO_3$ and (2) with a 3 per cent solution of $KMnO_4$. The washed ether was distilled over KOH.

To the cold solution, 150 ml. of this purified ether and 500 ml. of cold distilled water were added. This mixture was shaken and the lower portion removed by means of a separatory funnel. Fifty ml. portions of ether were added to the aqueous alcoholic solution and extractions were made until no more color was obtained in the ether portion. The ethereal extracts were combined and washed with a total of about 2 liters of distilled water in 4 or 5 successive

washings. The washed portion was allowed to stand over Na_2SO_4 over night. Then it was decanted and concentrated to about 10 ml. by driving off the ether under reduced pressure in an atmosphere of nitrogen. About 15 ml. of hot methyl alcohol (synthetic absolute methyl alcohol free from aldehydes and ketones obtained from Mallinckrodt) were added as a solvent and the solution was chilled below -21°C. by a mixture of hydrated $CaCl_2$ and snow, then filtered and made up to 25 ml. Of this preparation 1 ml. was equivalent to 0.6 gm. of the original butter. Each sample was divided into two parts and sealed in test tubes in an atmosphere of nitrogen and kept in the dark until readings for vitamin A could be made.

The vitamin A content was determined by measuring the value of the extinction coefficient at 328 mμ visually from spectrograms photographed with a Bausch and Lomb quartz spectrograph equipped with a Hilger rotating sector disc and a quartz biprism. Equipment of this type now much used in vitamin A work is described by Philpot and Schuster (1933). The wave-length scale for the ultra-violet region was calibrated by using the emission lines of known wave-length given off by a mercury vapor lamp. The 2 cells with quartz ends, one containing the pure solvent and the other the solution, were 1 cm. deep, and had a capacity of approximate-

ly 1.5 ml. The light source was a Ni-Fe arc, with nickel as positive, drawing 1.6 amperes on direct current from 110 volts. Since the fluctations in the position of the arc would alter the spectrum produced, it was necessary to keep the position of the source absolutely constant. For this reason, a quartz plate about 15 mm. in diameter and about 1 mm. thick was used as a secondary source of light. Thus the plate was illuminated by the arc and from it, the light entered the cells, the source remaining perfectly constant in position even though the arc varied from second to second. To reduce these latter fluctations the electrodes were ground down into the form of a wedge.

The spectrograms used for this work were Wratten Panchromatic plates 4 in. by 10 in. A procedure specific for this type of work was followed in developing the plates, which were all photographed and developed in the laboratory of the Department of Physics, Iowa State College.

The value obtained by reading the spectrogram was Log Io/I or D where I was the intensity of light transmitted by the solvent and I was the intensity of light transmitted by the solution and D was the density. By use of the formula Log Io/I ÷ cl, where Log Io/I is the density read directly, c is the concentration of butter per 100 ml., and l the length of path or depth of the column taken in centi-

meters, the extinction coefficient $E^{1\%}_{1\ cm.}$ was computed. Then in order to obtain the vitamin A content the value was divided by the figure $E^{1\%}_{1\ cm.}$ 328 mu = 1600 (Carr and Jewell, 1933) for pure vitamin A. Since 1 γ is equal to one over one millionth of a gram the vitamin content was obtained by moving the decimal point over 6 places. The definition of an International Unit is the biological activity of 0.0006 mg. of pure β-carotene, (Nelson, 1935) and since 1 gm. β-carotene on hydrolysis is equal to 1.067 gm. vitamin A, this in turn is equal to 1.66×10^6 I. U. per gram. By calculation 1 gm. of vitamin A corresponds to 1.56×10^6 I. U. (Morgan, et al., 1935) and 1 γ vitamin A to 1.56 I. U. The number of γ obtained was converted into I. U. by multiplying by 1.56. The International Units were expressed "per gm. of butter" and "per gm. of butter fat".

Blue Color Spectrophotometric Test

As a second method for estimating vitamin A, the spectrophotometric determination of the blue color with antimony trichloride, $SbCl_3$, was employed. The unsaponifiable portion was prepared essentially as described by Morgan and associates (1935), who used the recommendations of the Society of Public Analysts for the determination of unsaponifiable matter (1933).

Duplicate samples of 5 gm. of colostrous butter or 15 gm. of the other butters were saponified with 100 ml. of alcoholic potash (5 gm. KOH to 100 ml. of alcohol) for 10 minutes. Three volumes of distilled water (Gillam, et al., 1933) were added to the soaps and the composite mixture cooled to 0°C. The unsaponified portion was extracted with freshly distilled ether -- one 100 ml. and three 50 ml. portions -- or until no more color was removed. The ether solutions were bulked and carefully washed, once with water, once with dilute alkali (KOH) and then twice with water. The amount was approximately 200 ml. in each case. Care was taken not to shake but to swirl gently in the washing process. The ether portion was dried over anhydrous Na_2SO_4 over night. In the preliminary experiment this was omitted but it was found necessary to insert this step in order to obtain a dry residue more easily and more quickly. After 24-48 hours the ether portion was filtered and freed from the ether by evaporation in an atmosphere of nitrogen and under reduced pressure, with the temperature maintained at 70-80°C. Twice the residue was treated with a few drops of absolute alcohol and blown dry. Just before use the residue was dissolved in chloroform and made up to a suitable volume. All materials were kept moisture-free to obtain

clear solutions essential for spectrophotometric reading.

In preparing the solution for the spectrophotometer a proportion of 1:10 was maintained. (Gillam, 1935). One-half ml. of solution and 4.5 ml. of anhydrous $SbCl_3$ solution, prepared according to Wokes and Willimott, (1927), were mixed and read against pure chloroform in less than 30 seconds at 583 and 620, $E^{1\%}_{1\ cm.}$ being equal to 2600 and 5000 respectively. The $SbCl_3$ was purified by recrystallization from anhydrous chloroform. That absorption was due to vitamin A rather than to carotene was shown by running a control using an equivalent amount of carotene dissolved in Wesson oil in the above procedure in which case no color developed. Strain (1935) and others stated that carotene gave a color reaction with $SbCl_3$, but with different maxima. This, however, was for concentrated solutions. The computations used were similar to those described under the spectrographic method except that the extinction coefficients taken from the literature (Morton, 1935) were as previously stated.

Here, it may be stated that the new reagent, guaiacol, was tried in the color tests, using the modified method introduced by Rosenthal and coworker (1935). The technique was successful but the drawback was that no extinction coefficients could be found in literature. This would be

more satisfactory in point of technique, because the color was less transient than the blue color tests but further investigation must establish its value as a quantitative method.

Determination of Carotene

Samples of 5 or 10 gm. of butter were used for the carotene determination. The butter was dissolved in 25 ml. of petroleum ether (Skelly solve) as suggested by Shrewsbury and Kraybill (1933). The solution was filtered, made up to 100 ml., and read in a visual spectrophotometer at 455, 470 and 480 mu. The extinction coefficients, 2380, 2000, and 2120 respectively, determined by Peterson (1936) for this solvent, were used. With this data, computations were made as explained in the blue color test procedure. Equivalent weights of butter were decolorized with Norit charcoal and treated as in the above procedure in order to correct for any possible effect of the butter fat dissolved in the Skelly solve.

An attempt was made to dry the solution with Na_2SO_4 over night, and then make the filtered solution up to volume. Here difficulties were encountered in extracting the traces of carotene from the curd and Na_2SO_4, causing a suspension of fine particles which would settle out on standing. This

step did not prove satisfactory or necessary and was eliminated.

Biological Assay

The single feeding method of Sherman and Todhunter (1934) was employed in making the biological assay because previous work, in which its technique was used, had proved satisfactory.

Rats from Wistar Institute stock and weighing from 38 to 40 gm. were depleted of their vitamin A stores by the following diet:

Vitamin A-free casein	18 per cent
Dried brewer's yeast	10 per cent
Osborne and Mendel salt mixture	4 per cent
Cornstarch	67 per cent
Sodium chloride	1 per cent

Squibbs' viosterol, 0.9 gm. per 1000 gm. of feed, as recommended by the American Drug Manufacturer's committee (1931) was fed to furnish the vitamin D.

The general technique was that outlined by Bair (1936). When the rats were considered ready for experiment, they were divided into groups comparable as to weight, sex, and litter. A single feeding of butter, estimated to furnish 15 to 20 γ of vitamin A was fed to each rat and weekly weighings made during the experimental period which lasted from the time of depletion until the death of the animal.

For negative control, no source of vitamin A food was given after the depletion period.

Carotene (International Standard for vitamin A held for the Health Section of the League of Nations by the National Institute for Medical Research, Hampstead, London, N.W.3) was secured through the Bureau of Chemistry and Soils, United States Department of Agriculture. The carotene, used for positive control, was dissolved in cottonseed oil (Wesson oil) and stored in the dark below 0°C. until needed; 0.68 ml., of this solution, equivalent to 17γ pure β-carotene, was given as a single feeding to each of a group of rats.

Eleven groups of 10 or more rats each were used. The average depletion period was 40 days and the average weight for rats at the beginning of the experimental period was 93 gm.

Composite growth curves were plotted on cross section paper, 10 squares to the inch, and a planimeter used for measuring the areas. For each area the base line was the horizontal line representing the average weight loss of the negative controls at the end of the first week and the remainder of the boundary was the average weight curve of the group of rats in question. The area of the carotene curve was used as a standard of reference and the vitamin A activity was calculated by direct proportion.

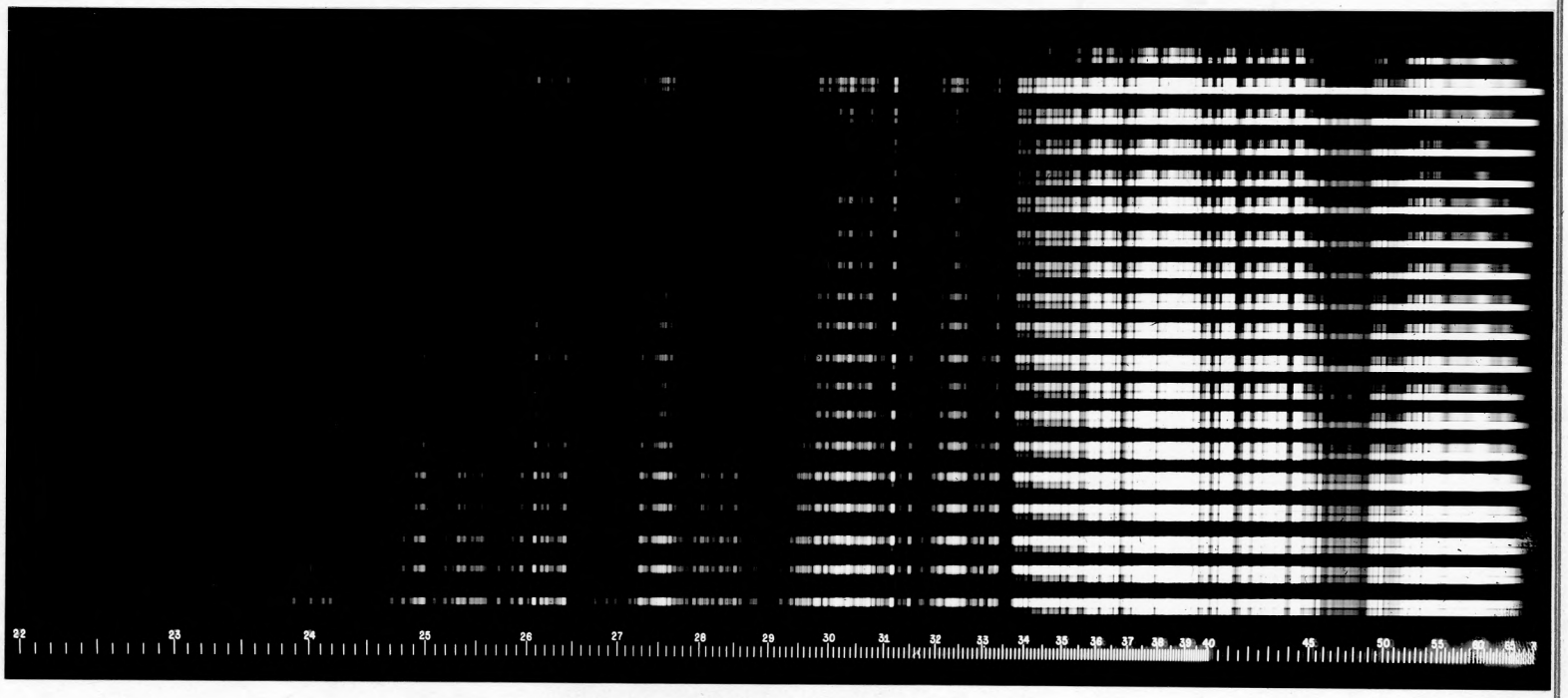
Table 1. Record of cows used in experiment.

Cow	Date	Fat	Total solids	Total yield milk	Total yield fat	Total yield solids	Fat in butter samples
		per cent	per cent	pounds	pounds	pounds	per cent
Emily Emperor	10-27-35	5.40	26.171	17.7	.955	4.632	89.5
born 8-12-19	11- 1-35	5.33	14.486	45.3	2.414	6.562	83.5
4th calf - Holstein	11-27-35	4.43	12.870	63.5	2.813	8.172	84.0
F.B. Titan	10-30-35	4.200	17.319	6.5	.273	1.125	84.5
born 9-15-32	11- 4-35	6.398	15.872	24.0	1.535	3.809	91.0
2nd calf - Jersey	11-30-35	6.706	16.556	28.8	1.931	4.768	86.0
J.R. Tipsy	7-26-35	7.150	17.430	13.7	.872	2.388	79.5
born 10-16-23	7-31-35	3.700	12.960	28.3	1.047	3.667	90.0
10th calf - Jersey							
Commercial sample	Purchased Feburary, 1936						80.0

Table 2. Vitamin A determinations on the unsaponfiable fraction of butter made with quartz spectrograph at 328 mμ.

Butter	Sample	D Sector reading	C gm. butter per 100 ml.	l	$E\frac{1\%}{1\text{ cm.}}$ (D ÷ cl)	$\frac{E\frac{1\%}{1\text{ cm.}}}{E\frac{1\%}{1\text{ cm}}\text{ pure}} \times 100\%$	Vitamin A per gram butter: $1\gamma = 10^{-6}$ g.	Average	$\gamma \times 1.56$	Vitamin A per gram butter fat: γ	I. U.
		as read		cm.		% vitamin	γ	γ	I. U.	γ	I. U.
Commercial	A	0.80	60	1	0.0133	0.000831	8.31				
Sample	B	0.34	30	1	0.0113	0.000706	7.06	7.69	12.00	10	16
Emily	A	1.20	10	1	0.1200	0.007500	75.00	75.00	117.00	84	131
Colostrum	B	-	-	-	-	-	-	-	-		
Emily	A	0.34	30	1	0.0113	0.000706	7.06				
5th day	B	0.40	30	1	0.0133	0.000831	8.31	7.69	12.00	9	14
Emily	A	0.50	60	1	0.0083	0.000519	5.19				
30th day	B	0.60	60	1	0.0100	0.000625	6.25	5.72	8.92	7	11
Tipsy	A	0.50	6.25	1	0.0800	0.005000	50.00				
Colostrum	B	0.40	5	1	0.0800	0.005000	50.00	50.00	78.00	63	98
Tipsy	A	-	-	-	-	-	-				
5th day	B	0.60	30	1	0.0200	0.001250	12.50	12.50	19.50	14	22
Titan	A	0.70	10	1	0.0700	0.004375	43.75				
Colostrum	B	0.68	10	1	0.6800	0.004250	42.50	43.13	67.28	51	80
Titan	A	0.26	30	1	0.0087	0.000544	5.44				
5th day	B	0.33	30	1	0.0110	0.000688	6.88	6.16	9.61	7	11
Titan	A	0.25	30	1	0.0083	0.000519	5.19				
30th day	B	0.35	60	1	0.0058	0.000363	3.63	4.41	6.88	5	8

Plate I. Absorption spectrum of vitamin A of the unsaponifiable fraction of Emily colostrum (E1A) butter.



328 mμ

Table 3. Vitamin A determinations on unsaponifiable fraction of butter by the blue color test.

Butter	Sample	Setting of instrument	D	C	l	$E^{1\%}_{1cm}$	$\frac{E^{1\%}_{1cm}}{E^{1\%}_{1cm}}$ x 100% pure	Vitamin A per gram butter: 1 γ = 10^{6} g.	Vitamin A per gram butter: Average	Vitamin A per gram butter: γ x 1.56	Vitamin A per gram butter fat: γ	Vitamin A per gram butter fat: I. U.
		mμ	as read	gm. butter per 100 ml.	cm.	(D ÷ cl)	% vitamin A	γ	γ	I. U.	γ	I. U.
Commercial Sample	A	583	0.66	37.50	1.5	0.0117	0.000450	4.50				
		620	1.05	37.50	1.5	0.0186	0.000372	3.72				
	B	583	0.42	25.00	1.5	0.0112	0.000431	4.31				
		620	0.47	25.00	1.5	0.0126	0.000252	2.52	3.76	5.87	5	8
Emily Colostrum	A	583	1.12	14.28	1.5	0.0522	0.002009	20.09				
		620	1.48	14.28	1.5	0.0690	0.001380	13.80				
	B	583	0.52	7.14	1.5	0.0486	0.001869	18.69				
		620	0.85	7.14	1.5	0.0794	0.001588	15.88	17.12	26.71	19	29
Emily 5th day	A	583	0.56	24.19	1.5	0.0154	0.000592	5.92				
		620	0.68	24.19	1.5	0.0187	0.000374	3.74				
	B	583	0.50	20.83	1.5	0.0160	0.000615	6.15				
		620	0.76	20.83	1.5	0.0243	0.000486	4.86	5.17	8.07	6	9
Emily 30th day	A	583	0.24	21.42	1.5	0.0074	0.000284	2.84				
		620	0.40	21.42	1.5	0.0124	0.000248	2.48				
	B	583	0.36	30.00	1.5	0.0080	0.000307	3.07				
		620	0.59	30.00	1.5	0.0131	0.000262	2.62	2.75	4.29	3	5
Tipsy Colostrum	A	583	0.24	6.58	1.5	0.0243	0.000934	9.34				
		620	0.43	6.58	1.5	0.0435	0.000870	8.70				
	B	583	0.34	7.81	1.5	0.0290	0.001115	11.15				
		620	0.45	7.81	1.5	0.0384	0.000894	8.94	9.53	14.87	12	19
Tipsy 5th day	A	583	0.66	30.00	1.5	0.0146	0.000561	5.61				
		620	1.07	30.00	1.5	0.0237	0.000474	4.74				
	B	583	0.67	30.00	1.5	0.0148	0.000596	5.69				
		620	1.04	30.00	1.5	0.0231	0.000462	4.62	5.17	8.07	6	9
Titan Colostrum	A	583	0.61	10.00	1.5	0.0406	0.001561	15.61				
		620	0.93	10.00	1.5	0.0620	0.001240	12.40				
	B	583	0.62	10.00	1.5	0.0413	0.001588	15.88				
		620	0.86	10.00	1.5	0.0573	0.001146	11.46	13.84	21.59	16	25
Titan 5th day	A	583	0.40	24.19	1.5	0.0110	0.000423	4.23				
		620	0.61	24.19	1.5	0.0168	0.000336	3.36				
	B	583	0.52	25.86	1.5	0.0134	0.000515	5.15				
		620	0.74	25.86	1.5	0.0190	0.000380	3.80	4.14	6.46	5	8
Titan 30th day	A	583	0.27	20.60	2.5	0.0052	0.000200	2.00				
		620	0.40	20.60	2.5	0.0077	0.000154	1.54				
	B	583	0.27	45.45	1.5	0.0039	0.000150	1.50				
		620	0.39	45.45	1.5	0.0057	0.000114	1.14	1.55	2.42	2	3

Table 4. Carotene determinations on butter in petroleum ether with spectrophotometer.

Butter	Setting of instrument	D	C gms. butter per 100 ml.	l	E 1% 1 cm. (D ÷ cl)	E 1% 1 cm. / E 1% 1 cm. x 100% pure	Carotene per gram butter: Average	Carotene per gram butter: γ x 1.66	Carotene per gram butter fat: γ	Carotene per gram butter fat: I. U.
	mμ	as read		cm.		% vitamin A	γ	I. U.	γ	I. U.
Commercial Sample	455	0.34	27	3	0.0042	0.000176				
	470	0.29	27	3	0.0036	0.000180				
	480	0.30	27	3	0.0037	0.000174	1.77	2.94	2	3
Emily Colostrum	455	0.90	5	4	0.0450	0.001890				
	470	0.76	5	4	0.0380	0.001900				
	480	0.76	5	4	0.0380	0.001792	18.60	30.88	21	35
Emily 5th day	455	0.22	10	4	0.0055	0.000231				
	470	0.20	10	4	0.0050	0.000250				
	480	0.18	10	4	0.0045	0.000212	2.31	3.83	3	5
Emily 30th day	455	0.26	10	4	0.0065	0.000273				
	470	0.22	10	4	0.0055	0.000275				
	480	0.22	10	4	0.0055	0.000259	2.69	4.47	3	5
Tipsy Colostrum	455	1.38	2.5	4	0.1380	0.005676				
	470	1.19	2.5	4	0.1190	0.005850				
	480	1.20	2.5	4	0.1200	0.005660	57.69	95.77	73	121
Tipsy 5th day	455	0.53	5	4	0.0265	0.001113				
	470	0.46	5	4	0.0230	0.001150				
	480	0.46	5	4	0.0230	0.001084	11.16	18.53	12	20
Titan Colostrum	455	1.61	10	3	0.0536	0.002252				
	470	1.37	10	3	0.0456	0.002280				
	480	1.35	10	3	0.0450	0.002122	22.18	36.65	26	43
Titan 5th day	455	0.49	10	4	0.0122	0.000512				
	470	0.43	10	4	0.0107	0.000535				
	480	0.41	10	4	0.0102	0.000481	5.09	8.45	6	10
Titan 30th day	455	0.33	10	4	0.0082	0.000344				
	470	0.30	10	4	0.0075	0.000375				
	480	0.30	10	4	0.0075	0.000353	3.57	5.93	4	7
Decolorized Butter	455	0.06	10	4	0.0015	0.000063				
	470	0.04	10	4	0.0010	0.000050				
	480	0.05	10	4	0.0012	0.000051	.56	.93	1	2
Decolorized Butter	455	0.03	5	4	0.0015	0.000063				
	470	0.02	5	4	0.0010	0.000050				
	480	0.02	5	4	0.0010	0.000033	.53	.88	1	2

Table 5. Summary of data from not feeding experiments.

Butter	Rats used: No.	Rats used: Ave. depletion period	Rats used: Ave. wt. when depleted	Amount fed	Ave. length of survival	Area	Sq. in. per gm. fed	Value: per gm. butter: β-carotene: γ	Value: per gm. butter: β-carotene: I. U.	Value: per gm. butter fat: γ	Value: per gm. butter fat: I. U.
		da.	gm.	gm.	da.	sq.in.	sq. in.				
Commercial Sample	7	39	91	1.2	39	12.15	10.13	16.40	27.30	21	34
Emily Colostrum	12	41	93	0.3	40	20.28	67.60	109.45	182.20	122	204
Emily 5th day	11	46	96	1.2	34	8.00	6.67	10.80	17.98	13	22
Emily 30th day	10	44	93	1.5	29	6.28	4.19	6.78	11.29	8	13
Tipsy Colostrum	11	44	92	0.5	43	27.74	55.48	89.82	149.53	113	188
Tipsy 5th day	11	39	91	1.0	31	9.75	9.75	15.79	26.28	18	29
Titan Colostrum	11	38	94	0.5	34	14.80	29.60	47.92	79.77	57	94
Titan 5th day	8	42	94	1.5	29	5.65	3.77	6.10	10.16	7	11
Titan 30th day	8	39	95	2.0	29	4.35	2.18	3.53	5.88	4	7
Carotene	11	37	91	(17 γ)	33	10.50	-				
Negative	17	40	93	-	15	-	-				

Fig. 1. Average gain curves for animals from single feeding method.

FIG. 1.

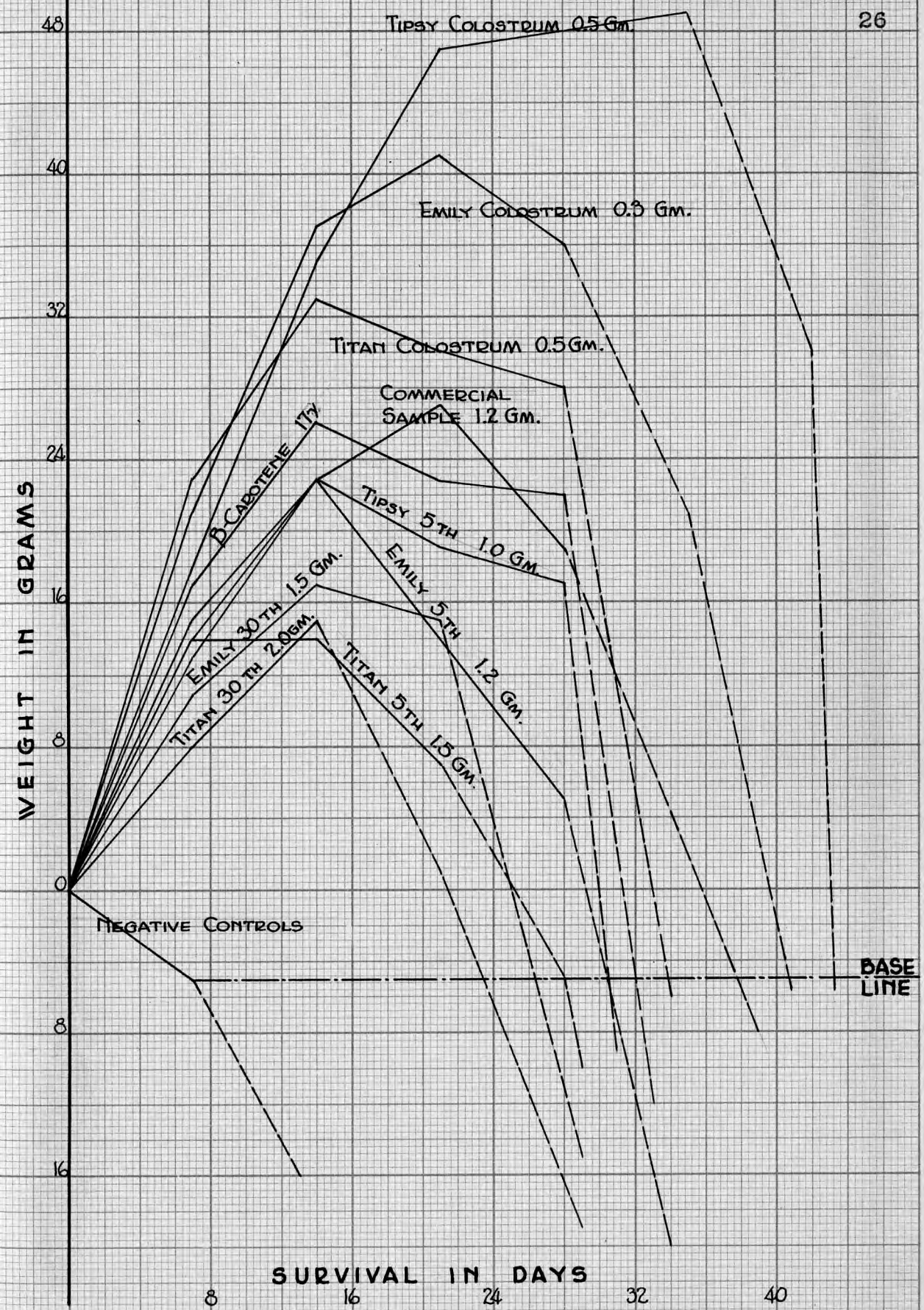

TIPSY COLOSTRUM 0.5 GM.
EMILY COLOSTRUM 0.3 GM.
TITAN COLOSTRUM 0.5 GM.
COMMERCIAL SAMPLE 1.2 GM.
B CAROTENE 17γ
TIPSY 5TH 1.0 GM.
EMILY 30TH 1.5 GM.
EMILY 5TH 1.2 GM.
TITAN 30TH 2.0 GM.
TITAN 5TH 1.5 GM.
NEGATIVE CONTROLS
BASE LINE
WEIGHT IN GRAMS
48
40
32
24
16
8
0
8
16
SURVIVAL IN DAYS
8
16
24
32
40

Table 6. Summary of results expressed in International Units per gm. butter fat.

Butter	Vitamin A		Carotene	Vitamin A potency		
	(1) absorption at 328 mμ	(2) blue color test	(3) absorption at 455,470,480 mμ	Physico-chemical methods (1+3)	Physico-chemical methods (2+3)	Biological assay
Commercial Sample	16	8	3	19	11	34
Emily Colostrum	131	29	35	166	64	204
Emily 5th day	14	9	5	19	14	22
Emily 30th day	11	5	5	16	10	13
Tipsy Colostrum	98	19	121	219	140	188
Tipsy 5th day	22	9	20	42	29	29
Titan Colostrum	80	25	43	123	68	94
Titan 5th day	11	8	10	21	18	11
Titan 30th day	8	3	7	15	10	7

Table 7. Total daily yield of vitamin A expressed in International Units.

Cow	Date	Butter fat		Vitamin A	
		pounds	grams	Physico-Chemical Assay (1+3)	Biological Assay
Emily	10-27-35	0.955	433.2	71,911.2	88,372.8
	11- 1-35	2.414	1095.0	20,805.0	24,090.0
	11-27-35	2.813	1276.0	20,416.0	16,588.0
Tipsy	7-26-35	0.872	395.5	86,614.5	74,354.0
	7-31-35	1.047	474.9	19,945.8	13,772.1
Titan	10-30-35	0.272	123.4	15,178.2	11,599.6
	11- 4-35	1.535	696.3	14,622.3	7,659.3
	11-30-35	1.931	875.9	13,138.5	6,131.3

DISCUSSION

Table 1 gives in brief the history of the cows and of the butter produced and used in this experiment. The original data for this table were obtained from the Department of Dairy Husbandry. The percentage of fat (column 3) in colostrum may be higher or lower than in later milk but the total daily yield of fat (column 6) increased during the course of lactation for the cows studied. It is also noted that the percentage of total solids (column 4) was much higher for colostrum but that the total solids (column 7) per 24 hours increased with progress of lactation. The percentage of fat in each butter (column 8) was essential for later computation of vitamin content per gram of butter fat. Tipsy (8-31-35) 30th day butter was prepared but must have been contaminated for it molded during refrigeration and was unsatisfactory for experimental use.

The results of the vitamin A determinations with the quartz spectrograph are recorded in table 2. D (column 3) is the sector reading which corresponds to the strip matched on the spectrogram (e.g. Plate I). The extinction coefficient was computed, using the formula $E^{1\%}_{1\text{ cm.}} = D/cl$, $E^{1\%}_{1\text{ cm.}}$ being the extinction coefficient of a 1 per cent solution of butter read at a depth of 1 cm. D, C, and 1 are as pre-

viously explained. In order to find the percentage of vitamin A in the butter it was necessary to use the extinction coefficient of a 1 per cent pure vitamin A solution recorded by Carr and Jewell (1933). To differentiate between these two extinction coefficients, the one found in the literature was designated as $E\ \frac{1\ \%}{1\ cm.}$ pure. The headings of the columns indicate the steps in the computations. The last column gives the International Units of vitamin A per gram of butter fat, expressed as the nearest whole numbers.

The high vitamin A content of colostrous butters which is 4 to 9 times as much as on the fifth day and 10 to 12 times that of the thirtieth day is in keeping with findings recorded in the literature. Dann (1933) reported that the vitamin A concentration in colostrum may be from 10 to 100 times as high as in later milk from the same cow independently of season. Holstein colostrous butter (Emily) was considerably richer in vitamin A than the Jersey colostrous butters (Tipsy and Titan) examined. Sutton and Krauss (1936) have suggested that the butter fat of Holstein will be richer in vitamin A than that of Jersey cows.

Table 3 contains a record of the results of the blue color tests as read in a Bausch and Lomb visual spectrophotometer. The column headings for this table are similar to those of table 2 and use the same symbols. It will be noted

that the results obtained from the 583 mμ readings are in every case higher than those from 620 mμ. Similar results are reported by other workers (Morgan et al., 1935). Variations in results may be due to several factors. In the first place the transient blue color made checks difficult to obtain even with rapid work. Great exactness was necessarily sacrificed to speed in mixing the solutions for reading. The concentration of vitamin A in the butters as obtained by this method are lower than those recorded in the previous table but the relationships at the various stages of lactation are similar. The blue color test has often been described as an easy one for vitamin A work. This may be true when the test is applied directly to the raw material, for example cod liver oil, but is not so true in cases where saponification, concentration and drying are necessary in the preparation of the samples for reading. The results yield information regarding the relative richness in vitamin A of the various samples. These determinations definitely distinguish the butters of high vitamin A content from those of low vitamin A content.

Table 4 gives data from the carotene determination, obtained by use of one of the methods recommended as suitable for material like butter. The commercial butter contained added coloring matter which suggested special pre-

cautions. A sample of this coloring matter was obtained and used for a blank saponification determination in 10 times the amount apt to appear in a butter sample. None of the color appeared in the ether-soluble portion. Therefore the commercial butter was first saponified and the carotene determined on the unsaponifiable portion dissolved in Skelly solve. Other samples of butter were not saponified but were dissolved directly in Skelly solve. To correct for the butter fat in the solvent, equivalent portions of butter were decolorized with Norit charcoal, dissolved in Skelly solve and made up to volume. When read in the spectrophotometer, the values obtained were scarcely more than could be due to error of reading or to the accuracy of the machine. This indicates that the butter fat in amounts used did not interfere with the carotene readings. Gillam and Heilbron (1935) suggest that the xanthophyll be separated from the carotene by partition between 90 per cent methyl alcohol and light petroleum ether. Gillam (1934) found the proportion of xanthophyll to carotene to be fairly constant and to represent a ratio of 1:14 by weight, or approximately 6 per cent of the total yellow coloring matter. In later routine work he did not preform the separation but simply corrects for the xanthophyll. Other authors feel that it

is unnecessary to correct for xanthophyll, and carotene figures in this study are presented uncorrected.

Data secured from the rat feeding experiments are summarized in table 5 and figure 1. In every group, the number of rats started on experiment was larger than reported but some were dropped from final records because of apparent over or under depletion. The areas under the curves as shown in figure 1 were measured with a planimeter and entered into table 5. The area under the carotene curve was used as a standard of reference and the total vitamin A activity calculated per gram of butter and per gram of butter fat.

A summary of the findings obtained from the preceding tables is presented in table 6. Absorption at 328 mμ is at present considered one of the best methods to use in vitamin A work. The results so obtained were in every case higher than those secured from the blue color test. Either method distinguishes the samples which contain largest and smallest amounts of vitamin A. Corrections for the readings at 328 mμ have been suggested by several authors. De (1935) developed a method which required the making of spectrograms before and after 3 hours exposure to ultra violet light. The difference of the two absorptions was taken to calculate

the vitamin A value. Morton (1935) described methods of correction and pointed out the interference of carotene and xanthophyll when chloroform is the solvent. Readings obtained when methyl alcohol is used as a solvent are usually accepted without correction as here presented.

Total vitamin A potency is recorded in three ways; (a) the summation of results obtained from the intensity of absorption at 328 mμ and from the carotene determinations, (b) the summation of results from the blue color tests and from the carotene determinations and (c) the biological assay finding. The three sets of figures show interesting correspondence for results obtained in such diverse ways. The first summation most often includes the highest figures for total vitamin A activity whereas the second summation often includes the lowest of the three figures for any one sample. By either of the three methods, the samples of colostrum are shown to contain butter fat of highest vitamin A potency. The results obtained from the carotene determination of the colostrous butter of Emily are open to question since the sample had molded before this determination could be made. The carotene reading was low in comparison to other colostrous butters although the sample had a good yellow color. The butter fat produced on the thirtieth day of lactation gave lowest results for vitamin A content. The

commercial butter was at least as rich as these thirtieth day samples. Inspection shows that the proportions of the total vitamin A potency contributed by the carotene vary widely among the samples studied. In one instance far more than one half, and in several others approximately one half of the total vitamin A value of the butter fat appeared to be due to the carotene. The commercial butter had the lowest carotene content, accounting for about 19 per cent of the total vitamin A value shown in (1 + 3) column (table 6). This butter was churned from milk delivered at the dairy farm from neighboring farms, whereas the other samples were made from milk of cows on specially balanced rations. Bauman and Steenbock (1933) found that the carotene contained in butter accounts for about 15 per cent of the vitamin A activity.

Items from tables 1 and 6 were used to compile table 7. Variation may be noted in the amounts of butter fat reported for the first day of lactation, the amount for Titan being particularly low. This may be due to some of the problems which made complete collection of first day samples difficult and perhaps accounted for the apparently low total production of vitamin A on the first day of lactation. Other cows furnishing samples for this experiment produced between 70 and 90 thousand I. U. of vitamin A on the first

day of lactation. As lactation progressed the total daily yield of butter fat increased but the vitamin A potency of this butter fat dropped so that the total vitamin A output on the thirtieth day of lactation was distinctly lower. Dann (1933) studied colostrum by the blue color method and concluded that a calf receives on the first day of life supplies of vitamin A greater than later milk could give in 20 to 50 days. The present investigation of butter shows that the vitamin A production on the first day of lactation may be 4 or 5 times as great as on the thirtieth day for the same cow.

SUMMARY AND CONCLUSIONS

Samples of butter made from composite 24-hour samples of the secretions of the first, fifth and thirtieth days of lactation of three individual cows, a Holstein and 2 Jerseys, were obtained from the Department of Dairy Husbandry. Other butter, called the "commercial sample" was purchased at the college dairy sales counter. For all samples, the vitamin A content was estimated by measuring the absorption spectra at 328 mμ with a quartz spectrograph (courtesy Physics Department, Iowa State College) and the blue color intensity, obtained with the antimony trichloride reagent added to the unsaponfiable fraction, in a visual

spectrophotometer. The amount of carotene was measured spectrophotometrically with petroleum ether (Skellysolve) as the solvent.

The vitamin A potency, or combined vitamin A and carotene value, was determined by the single feeding method of biological assay, according to Sherman and Todhunter. Albino rats, depleted of vitamin A stores, were fed a single sample of butter along with vitamin A-free diet and distilled water ad libitum. The time of experiment lasted from the day of butter-feeding until the death of the rat. Composite growth curves were compiled for comparisons.

Results of all methods used were computed and expressed as International Units of vitamin A per gram of butter fat and compared in various ways.

Results of the antimony trichloride tests yielded information regarding the relative amounts of vitamin A in the various samples. These blue values distinguished the butters of high vitamin A content from those of low vitamin A content. Determinations made with the quartz spectrograph showed that the butters made from colostrum are rich in vitamin A, with some cows yielding products more potent than others. The butter fat produced on the fifth day of lactation contained less vitamin A, from one-fifth to one-ninth the amount per gram produced by the same cow on the first

day. On the thirtieth day of lactation, the butter fats showed small decreases in vitamin A content. The sample of commercial butter had a vitamin A content similar to that of the later butters of the individual cows. Readings with the visual spectrophotometer showed that the carotene contents of the colostrous butters were definitely superior to those of the other butters. The carotene content dropped considerably during the course of lactation, on the fifth and thirtieth days the amount being about one-fifth to one-seventh as much as on the first day. The carotene content of the commercial butter was slightly lower than that of any other sample studied; however, this sample was subjected to further manipulation for it was saponified to remove the butter coloring before reading.

Biological assays conducted by the single feeding method showed the butter fat of colostrum to be richest in total vitamin A value. The butter fat produced on the fifth day of lactation was only one-sixth to one-ninth as potent while that produced on the thirtieth day of lactation was even lower in vitamin A value. Total vitamin A values per gram of butter fat determined by the single feeding method showed similarity to the summations of the vitamin A (quartz spectrograph) and the carotene (spectrophotometer) determinations.

Results from these methods show that two samples of butter fat from colostrum contained about 200 International Units of vitamin A per gram. Samples produced by the same cows on the fifth day of lactation may contain only one-tenth this amount of vitamin A. By either of the three methods of measurement, butter fat of the thirtieth day of lactation was still lower in vitamin A content.

Total daily yields of vitamin A were calculated for the individual cows, two of these produced between 70 and 90 thousand International Units on the first day of lactation. As lactation progressed the total daily yield of butter fat dropped so that the total vitamin A output on the thirtieth day was distinctly lower - about one-fourth to one-fifth as great as on the first day.

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