A STUDY OF THE PREPARATION OF THE UNSAPONIFIABLE MATTER OF FORTIFIED MILK SUITABLE FOR THE SPECTROGRAPHIC DETERMINATION OF VITAMIN D

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HISTORICAL AND INTRODUCTION

Among common natural food-stuffs Vitamin D occurs in some quantity only in eggs and milk. The amount they contain, however, is insufficient to meet the nutritional requirements of infants and children. Consequently cod liver oil was used until relatively recent years to assure normal development in infants and children.

Because of the ease of providing vitamin D to infants directly in milk and because it has been demonstrated that milk is a better menstruum than oily carriers, much work has been directed toward investigating the special role which the milk constituents might play in this connection. Supplee (1), and his associates reported that the vitamin D carried by the lactalbumin of milk showed greater effectiveness than that fed alone. They postulated that the vitamin D and the lactalbumin had formed a symplex, a system of a prosthetic group and a colloidal carrier, vitamin D being the prosthetic group and the lactalbumin the carrier. Thus the process of enriching fluid milk with vitamin D was started and so-called "Vitamin D Milk" is now widely sold throughout this country, the potency having been standardized at 400 U.S.P. units per quart.

There are several methods of enriching milk with Vitamin D. Most practical is that of the direct addition of a vitamin D concentrate. At first fish liver concentrates were used exclusively although in recent years activated ergosterol has come into extensive use. A solution of vitamin D in propylene glycol or some other suitable solvent, is simply added to yield milk of a standard potency.

A second method involves feeding irradiated material to the milk cow. Because of the high content of ergosterol in yeast, irradiated yeast has been fed to achieve a milk of standard vitamin D content. This method has not gained wide acceptance because of the inconvenience of adjusting the allowance of yeast to the level of milk production of each cow.

In a third method milk may be activated directly through the exposure of a thin film for a brief period to the active rays of ultra violet light from artificial sources. This type of milk was originally standardized at 135 units per quart, but for the last decade has attained the level of other milks, namely 400 units. This method, although still used by a few of the larger dairies, has failed to gain popularity because of the high initial cost of the equipment and the difficulty in maintaining production of milk of uniform potency.

It was found (2) that in general all these types of milk are effective to the extent of their unitage, for both the prevention and cure of rickets. However, the results on chicks (3) demonstrated that "irradiated milk" was approximately ten times more effective than the same number of rat units of "yeast milk". It is now well known that vitamin D_2 is much less effective in poultry than D_3, although there is very little difference in the case of mammals, including humans.

Milk has long been subjected to rigorous control by state and local health authorities. It was only natural then that when the fortification of milk was permitted and introduced, steps would have to be taken to periodically check the vitamin D content of such milk. The exercise

of this control work varies greatly. In the absence of a state law the communities fix the number of assays which are required yearly. In the State of Michigan this varies from none to twelve. In states which do have a law for this subject two or three assays per year are required.

The assaying of low potency materials like vitamin D milk is not an easy task especially if one wishes to do it routinely and economically. The biological method using either rats or chicks as test animals is still the official method. It is both expensive and time consuming inasmuch as each determination takes twenty-one days for the preparation of the rachitic rats and another ten days for feeding the test material. Consequently, a search for more practical and non-biological method was soon made.

Within the last ten or fifteen years, a number of physico-chemical methods have been developed, each claiming good agreement with the biological assay.

Brochmann and Chen (19, 20, 21) discovered that vitamin D on addition of a saturated solution of antimony trichloride in chloroform developed a pink color with a characteristic absorption maximum at 500 mu. By using the appropriate correction factors it was found possible to estimate vitamin D in products of high potency.

Milas, Heggie, and Raynolds (4) modified the above method by a preliminary treatment with maleic anhydride, thus destroying vitamin A, 7 - dehydro cholesterol and interfering carotenoids.

Nield, Russell, and Ziminerli (5) used a new reagent consisting of a solution of antimony trichloride and acetyl chloride in chloroform.

A yellowish pink color was produced with a maximum absorption at 500 mμ. They claimed greater sensitivity for their method. Later the same authors (6) improved their reagent by adding a reducing agent, zinc or tin or antimony to reduce the concentration of pentavalent antimony, thus making it satisfactory for the determination of cholesterol and other sterols as well as vitamin D_2 and D_3.

Ewing, Kingsley, Brown and Emmett, (7) removed carotenoids, vitamin A and pigments by chromatographic adsorption. They used a two-step chromatographic treatment in which the $E\,^{1\%}_{1\ cm}$ was determined first for the combined vitamin D and sterols and then for the separated sterols. The value for the vitamin was obtained by difference. Recently Ewing and his associates (8) improved the above method by passing the unsaponifiable fraction through a column of superfiltrol, a mixed solvent composed of 50 parts of hexane, 10 parts of ethyl ether, and 1 part of absolute alcohol being used. The interfering substances were adsorbed, and vitamin D in the percolate measured by its extinction at 265 mμ.

DeWitt and Sullivan (9) modified the antimony trichloride method by using ethylene chloride as a solvent. This reagent was found to be more stable and it gave a salmon pink solution exhibiting an absorption maximum at 500 mμ.

Sobel, Mayer, and Kramer (10) developed a new colorimetric method by using glycerol dichlorohydrin in the presence of acetyl chloride or other acid halides. Different color changes were obtained with calciferol, ergosterol, and 7-dehydrocholesterol. They claimed the reagent to be

more stable and the absorption curves more specific.

Villar Palasi (11) devised another color reaction for vitamin D by using saccharose and absolute alcohol. When concentrated sulfuric acid was added, the color first became red and then blue. Upon shaking, the blue color increased in intensity. The final sky-blue color was stable and its intensity was found proportional to the amount of vitamin D.

Most of the above methods have been claimed to give satisfactory results for high potency materials. However, little success has been had with those of low potency, largely because of the difficulty in the extraction and concentration of the vitamin D and subsequently the removal of interfering substances.

Kon and Booth (12), (13), in studying the vitamin D activity of butter fat derived from ordinary milk, found that about 75% of the potency of autumn and winter butters was either destroyed or directed toward another fraction in the preparation of the non-saponifiable residue.

In a study of the seasonal variation of the vitamin D content of cow's milk, Bechtel (14) had to resort to the concentration of vitamin D by warm alcohol extraction of the milk fat. This made it possible to carry out the biological assay with a fair measure of success, and established that milk produced under common conditions of farm management varied in vitamin D potency from 4 to 45 units per quart. The potency paralleled the exposure of cows to sunshine. It is quite likely that the butter fat used in the studies of Kon and Booth (12), (13) was derived from milk of fairly low potency.

Although failure attended the attempts to quantitatively recover vitamin D from ordinary milk fat by the saponification procedure, it was nevertheless considered possible that in the case of fortified milk enough vitamin D was present to permit the method to be used. Accordingly it was the purpose of this investigation to apply a suitable method of extraction of the fat from vitamin D milks of various types, to saponify, and to test biologically for the vitamin D at various stages in the procedure. A quantitative study was also made of the amount of fat, total unsaponifiable matter, and cholesterol in the samples of milk which were obtained from various cities in Michigan.

An attempt was also made to prepare the unsaponifiable matter from a three quart sample of vitamin D milk containing approximately 1200 U.S.P. units of vitamin D. It was hoped that this would supply a large enough amount of vitamin D for the spectrographic analysis of this vitamin.

EXPERIMENTAL PROCEDURE

I. Preparation of the unsaponifiable matter

A. Extraction of the milk fat

100 ml. of the milk was delivered into a 2-liter separatory funnel, 15 ml. of concentrated NH_4OH added and the mixture well shaken to keep the protein in solution. 100 ml. 95% ethyl alcohol was added and the mixture again well shaken. Then 200 ml. of peroxide-free ether was added, and the mixture carefully shaken. 200 ml. of Skelly-solve B was now added and the mixture again well shaken. After the two layers had clearly separated, the aqueous layer was removed and the extraction repeated twice. The combined extracts were transferred to a beaker and evaporated to dryness on a steam bath. This residue was used both for the determination of total fat and for the subsequent preparation of the unsaponifiable matter.

B. Hydrolysis

The extracted milk fat was transferred to an Erlenmeyer flask and 30 ml. ethyl alcohol 95% and 5 ml. 50% aqueous KOH were added. The mixture was boiled for one hour under a reflux condenser.

C. Extraction of the unsaponifiable matter

After the hydrolyzed mixture had cooled down, it was transferred to a separatory funnel. The Erlenmeyer flask used in the hydrolysis was washed first with warm water, then with cold water, and finally with ethyl ether. The washings were combined with the main hydrolyzed mixture. The cooled solution was then extracted with seven 50 ml. portions of ethyl ether. Each time after the addition of the ether the

separatory funnel was slightly tilted to mix the two layers but avoid the formation of an emulsion. In case an emulsion did form, one or two ml. of ethyl alcohol was added, and the emulsion broke within 2 or 3 minutes.

The combined ether extracts were washed with distilled water until free from alkali as shown by testing with phenolphthalein. The washed ether extract was dried by pouring through a filter containing anhydrous sodium sulfate. The solvent was then removed by evaporation and the residue weighed.

II. Removal of Interfering Substances and Spectrographic Study of the Prepared Unsaponified Matter

The unsaponifiable matter from three quarts of M.S.C. vitamin D milk prepared as above was dissolved in 10 ml. of a mixture of 50 parts of hexane, 10 parts of ethyl ether, and 1 part of purified ethyl alcohol. The solution was passed through a 9 cm. column of superfiltrol prewashed with 15 ml. of the same solvent mixture. Vitamin D was eluted with the 50-10-1 solvent, and the eluant put under reduced pressure at 60°C. to remove the solvent. The residue was then taken into alcohol and its absorption measured at 265 mμ with a Beckman quartz spectrophotometer.

III. Biological test at various stages

In order to test the procedure at each stage a check was made using the biological line test as a criterion. In each case these assays were made on the original milk, on the extracted fat and on the unsaponifiable matter. In the case of the chromatographic separation of interfering

substances biological assays were made on the solution before and after passing through the adsorption column.

A. Preparation of test animals

Albino rats weighing 45-55 g. (about 21 days of age) were made rachitic by feeding them a basal ration of the following:

Cornmeal	72
Wheat gluten	20
Yeast	4
Ca CO_3	3
NaCL	1

The animals were put in separate cages and fed the rachitogenic diet for 21 days. Water and the basal ration were given ad libitum during this period. The animals usually gained from 20 to 30 gms. Those that gained less than 15 gms. were considered unsuitable and were discarded.

B. Test period

In each series the vitamin D milk was compared with equivalent amounts of the extracted fat and of the unsaponifiable matter. 10 ml. of milk containing 4 U.S.P. units of vitamin D was mixed with 40 g. of the basal diet, dried, and ground. All rats used throughout these tests were fed on this basis during the supplementary period.

The extracted fat samples and the unsaponifiable matter were dissolved in ethyl ether and a portion equivalent to 4 units was evaporated on 40 g. of the basal ration containing 3% milk powder. The milk was included in the assay of the extracts in order to simulate the supplements of the rats receiving the milk.

The animals usually consumed the supplemented ration in 7 or 8 days and then were fed the basal ration for the remainder of the ten-day period.

At least 7 animals were used for the assay of each sample and for each group of assays 4 negative controls were included.

C. Line test

On the tenth day of the supplementary feeding the animals were etherized and the wrist bones removed and cleaned of adhering tissue. The bones were kept in 95% alcohol for at least two days and then split longitudinally with a clean, sharp blade to expose a plane surface through the junction of the epiphysis and diaphysis. The sections were then immersed in 2% $AgNO_3$ solution for two minutes. Then they were put in distilled water, and exposed to light until the calcified areas were clearly distinguishable. The average response of the various groups was then evaluated. The extent of calcification reflects the amount of vitamin D contained in the samples.

IV. Determination of fat, unsaponifiable matter, and cholesterol of some vitamin D milks.

A. Fat

Fat was extracted as previously described, followed by drying at 90°C. in an oven until constant weight was obtained. The drying was done at half hour intervals to minimize thermal decomposition.

B. Unsaponifiable matter

The hydrolysis and extraction were as previously described. The

unsaponifiable matter was also dried at 90°C. for half hour intervals until constant weight was obtained.

C. Cholesterol

Cholesterol was determined colorimetrically. The unsaponifiable matter was dissolved in 25 ml. $CHCl_3$ (C.P. grade) and 5 ml. of this solution was taken for analysis. Further dilution was made if necessary.

2 ml. of redistilled acetic anhydried was added and the tube well shaken. In a similar way 0.1 ml. of concentrated H_2SO_4 was added. Exactly 15 minutes later the green color developed was measured in a photelometer using a red filter. From the percent of transmission the amount of cholesterol could be read from a standard curve obtained similarly by using known concentrations of cholesterol.

The addition of the concentrated H_2SO_4 was usually made to a series of solutions at 2 minute intervals so that successive readings on the photelometer could be completed at uniform times.

RESULTS

A. The similarity in response of the various groups as judged by the line tests indicates that the extraction, as well as the saponification and extraction of the unsaponifiable matter were quantitative within the limits of accuracy of the method employed.

B. Complete recovery of vitamin D was also observed in the solution passed through the adsorption column and used for the spectrographic determination of vitamin D.

Three fractions were collected and examined. None showed a distinctive absorption maximum at 265 mu. This failure may have been due to a combination of factors of which the presence of impurities in the solvents and the presence of excessive amounts of interfering substances in the unsaponifiable matter from the large sample of milk were perhaps the most serious. Time did not permit a solution of this part of the problem.

C. The results of the determination of fat, unsaponifiable matter, and cholesterol are shown in Table I. The fat content of the vitamin D milks examined varied from 3.21 g/100 ml. for the Central Creamery, Detroit, to 4.70 g/100 ml. for the Michigan State College Creamery. Most of the samples had a fat content between 3.50 g. and 3.80 g.

The range for the unsaponifiable matter was from 40.4 mg./100 ml. for the Lansing Dairy Company, Lansing, to 75.0 mg./100 ml. for the Rebel Creamery, Detroit. When calculated on the basis of fat the variation was from 1.06% for the Lansing Creamery, to 1.97% for the Heatherwood Company, Lansing, as shown in Table I. The majority of the samples

examined had unsaponifiable matter values between 53.0 and 60.0 mg./ 100 ml.

The cholesterol contents ranged from the 8.50 mg. per 100 ml. for the United Dairies to 16.0 mg. per 100 ml. for the M.S.C. Creamery. When calculated on the basis of fat and of the unsaponifiable matter, the values were 0.222-0.436% and 14.4 - 35.7% respectively.

Table I. Fat, Unsaponifiable Matter, and Cholesterol Contents of Vitamin D Milks

Sample			Fat	Unsap. Matter		Cholesterol		
No.	Place	Dairy	gm/ 100 ml.	mg/ 100 ml.	o/o in fat	mg/ 100 ml.	o/o in fat	o/o in unsap. matter
1	Detroit	Rebel	3.99	75.0	1.88	12.8	0.321	17.1
2	Detroit	Central	3.21	55.1	1.71	11.0	0.343	20.0
3	Detroit	United	3.60	59.0	1.64	8.5	0.236	14.4
4	Detroit	Detroit	3.56	68.6	1.93	13.3	0.374	19.4
5	Detroit	Rosebud	3.55	57.8	1.63	12.0	0.338	20.8
6	Marquette	Northern	3.98	56.7	1.43	10.4	0.261	18.4
7	Marquette	Bencroft	4.28	62.8	1.47	9.5	0.222	15.1
8	Battle Creek	Ashley	3.54	53.0	1.50	12.3	0.348	23.2
9	Grand Rapids	Borden's	3.73	47.7	1.28	11.7	0.314	24.5
10	Lansing	Lansing	3.81	40.4	1.06	12.6	0.331	31.2
11	Lansing	Heatherwood	3.52	69.4	1.97	15.3	0.435	22.1
12	Lansing	Quality	3.57	55.8	1.56	15.2	0.426	27.2
13	Jackson	Lakeside	3.51	42.3	1.21	15.1	0.430	35.7
14	Jackson	Loud and Jackson	3.51	53.8	1.53	15.3	0.436	28.4
15	Jackson	Servall Jersey	3.75	57.3	1.53	14.5	0.387	25.3
16	Midland	Midland	3.68	44.6	1.21	15.4	0.419	34.5
17	Pontiac	Arctic	3.62	60.3	1.70	12.5	0.345	20.7
18	East Lansing	M.S.C.	4.70	52.5	1.12	16.0	0.341	29.8

DISCUSSION

The procedure used for preparing the unsaponifiable matter from vitamin D milk appears to allow complete recovery of the vitamin D as shown by the line tests at end of each stage. Kon and Booth (12),(13), did not get satisfactory results from their extraction probably due to the fact that they worked with materials of much lower potency. It may also be that the ethyl ether used in their extractions was not peroxide-free and might have contributed to loss of vitamin D. In the present study care was taken to use peroxide-free ethyl ether. Fritz, Halpin, Hooper, and Kramke (15) reported that the various forms of vitamin D, including crystalline activated ergosterol and 7 - dehydro-cholesterol as well as natural sources, are all susceptible to destruction. Avocado oil, which is being used increasingly in human diets, has been studied for its vitamin D content recently. Weatherby (16) concluded that this oil contains appreciable quantities of vitamin D. However, Lassen Bacon, and Sutherland (17) obtained negative results from the extraction of the unsaponifiable matter of the avocado. In view of these contradictory reports it would be of interest to apply the procedure used in this study to the avocado.

The result of testing the unsaponifiable matter with the Beckman quartz spectrophotometer after chromatographic separation of interfering substances proved to be disappointing. A number of reasons may be offered for the failure to find the characteristic absorption at 265 mu. The application of this method to vitamin D concentrates involves much smaller quantities of the sample and consequently less possible interfering substances. In the case of concentrates, considerably less than one gram

of the sample is needed. The use of 3 quarts of milk involved the processing of approximately 100 grams of milk fat. Obviously the quantity and nature of the interfering substances might be such as to make the chromatographic separation incomplete. Moreover, the interfering substances introduced by the use of the large volumes of solvents needed to process a three-quart sample of milk would be considerable. It is also quite likely that the amount of vitamin D supplied, namely about 1200 U.S.P. units, was insufficient for the spectrographic determination in its present state of development. It may be added that unless much smaller amounts of vitamin D could be determined spectrophotometrically, the cost of the purified solvents alone would make this method prohibitive.

The analysis of vitamin D milks for fat content shows that the variation was from 3.21 to 4.69 g./100 ml. The average was about 3.60 g./100 ml. The extraction of fat by this method of solvents gave reliable results for the duplicates checked quite well with each other.

In the case of the unsaponifiable matter greater differences between duplicate determinations were observed. No satisfactory explanation is forthcoming for these results. There was little correlation between the fat content and the unsaponifiable matter.

The cholesterol values ranged from 8.5 - 16 mg. per 100 ml. These agree very well with those found by Nataf, Mickelsen, and Keys (18) who reported values of 9 mg. to 17 mg. per 100 ml. Some correlation was observed with the fat content of the milk as was reported by the above workers.

SUMMARY

1. The unsaponifiable matter of fortified milk was prepared by extracting the milk fat with ethyl alcohol, ethyl ether, and Skelly solve B, followed by hydrolyzing with alcoholic potash, and final extraction with ethyl ether.
2. The line tests performed with rats fed equivalent amounts of vitamin D in the form of fortified milk, extracted fat, and unsaponifiable matter indicated complete recovery of vitamin D.
3. The unsaponifiable matter from a three-quart sample of vitamin D was also prepared. The spectrographic examination made after the attempted removal of interfering substances by chromatographic absorption did not show an absorption maximum at 265 mμ. The failure was probably due to excessive amounts of interfering substances introduced by the use of large volumes of solvents and the large quantity of sample.
4. Eighteen samples of vitamin D milk from different dairies in various cities of Michigan were analyzed for the fat content, the unsaponifiable matter, and cholesterol. Results obtained were from 3.21 g. to 4.70 g./100 ml. for the fat content, 40.4 mg. to 75.0 mg./100 ml. for the unsaponifiable matter, and 8.50 mg. to 16.0 mg./100 ml. for cholesterol.

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