The Determination of Vitamin A in Margarine[1]

J. BOLDINGH and J. R. DROST, Lever Brothers & Unilever N. V.,
Zwijndrecht, Netherlands

A METHOD has been developed in which irrelevant-absorbing substances originating from the unsaponifiable extract of margarine containing vitamin A are eliminated through the use of double chromatography, using two different adsorbents in series. It enables the potency to be determined by measuring the E max. 325 mμ and multiplying E 1% 1 cm. by the internationally established factor 1900. The method has been adapted to the use of only .60 μg of vitamin A alcohol per analysis and can be used as a highly accurate routine method.

Introduction

The development of the determination of vitamin A in liver concentrates during the last decade shows that there is a definite tendency to abandon the Carr-Price antimony chloride test. Nowadays it is generally agreed that the results obtained by this method of assay are often variable and furthermore that the low specificity may cause large errors, depending on the kind of concentrate being tested. This has been clearly demonstrated in the case of whale liver oil in which relatively high concentrations of kitol, and often anhydro-vitamin A, may give rise to erroneous results, up to 30% higher than those due to the actual vitamin A contents. The transient character of the chromophore may cause additional errors.

In the search for other means of determining vitamin A, attempts to use the ultra-violet light absorption at 325-328 mμ are certainly the most important. However it was only after pure vitamin A alcohol and its ester were made available that it became possible to avoid making errors of the same order as those involved in the Carr-Price test. It was then shown that most of the natural liver oils or concentrates derived from them have absorption curves which show more or less severe distortions, due to contaminants absorbing in the same region. This has limited the use of the direct measurement of E max. The most logical way to overcome this difficulty is to try to separate the vitamin A from the impurities; such a method has in fact been worked out by Gridgeman *et al.* (1) for the unsaponifiable fraction of whale liver oil and by Morton and Barua (2) for unsaponified liver oils, both using alumina as adsorbent. They succeeded in obtaining vitamin A fractions with a high optical purity and the determination of E max. ensured that there were no large errors in the potency calculations.

These procedures, of which the present authors prefer the Gridgeman modification, are superior to the planimetric correction procedure of Morton and Stubbs (3), which depends on the rather doubtful assumption that the irrelevant light absorption is linear between 334 and 311 mμ.

With the exception of liver oils and concentrates, up till now it has not yet been possible to follow the same route for other materials containing vitamin A, especially those with a low vitamin A content, such as butter or margarine (10-20 I.U. per gram). Here either the Carr-Price test has to be used or the method of Wilkie and de Witt (4) who use magnesium oxide combined with Celite as chromatographic adsorbent for the unsaponifiable extract. The latter investigators were not able to eliminate completely irrelevant-absorbing materials. They therefore proposed to determine the potency by measuring the absorption at 340 mμ and use a corresponding biological conversion factor. However the measuring of the absorption at another wave-length than at maximum absorption should be avoided, if possible, because small shifts of the curve or slight deviations of the wave-length dial of the instrument may cause relatively large errors in the final figure.

The present work describes a method in which the irrelevantly absorbing contaminants of the unsaponifiable extract are completely eliminated by double chromatography, using two different adsorbents in series, followed by selective elution of a pure vitamin A alcohol fraction. Although this method has been worked out especially for margarine, it has possbili-

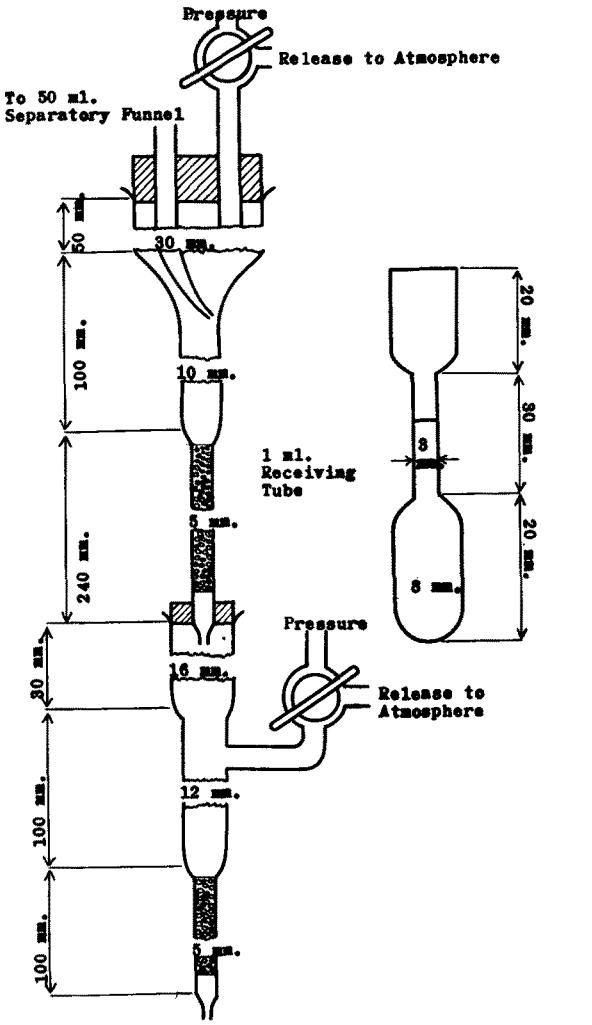


FIG. 1. Vitamin A chromatographic assembly.

[1]Presented by Lever Brothers Company, Research Department, Cambridge, Mass.

ties for the estimation of vitamin A at low potency in other materials.

Reagents

Ethanol: 95%, distilled from KOH.

Diethyl ether: Peroxide-free and freshly distilled.

Light petroleum (boiling range 40-60°C.): Purified by boiling with 1/5 part (v/v) concentrated sulfuric acid for 6 hours, separating from the acid layer, washing, drying over anhydrous sodium sulfate, and distilling.

KOH (analytical grade): 60% in water.

Hydroquinone: Pure.

Alumina: Crystalline alumina trihydrate heated at 750-800° C. for 6 hrs. (or commercial alumina at 600°C.) and sieved through a 180-mesh sieve; the portion passing the sieve should then be shaken with 2% water in an air-tight bottle and stored overnight before use.

Alkaline alumina: Mix equal parts (w/v) of the above-mentioned alumina and a 10% aqueous solution of caustic soda. Allow it to stand during 1 hour at room temperature. Then dry in a vacuum oven, first at 30-40°C., and after most of the water has been evaporated, for 1 hour at 100°C. (15-20 mm. Hg.). Fill into small bottles, each containing approximately 20 g., and seal with paraffin wax to prevent access of moisture.

Antimony trichloride: In chloroform, as used for the Carr-Price test.

Apparatus

The chromatographic apparatus consists of two separate tubes as shown in Figure 1. Both tubes or only the upper one can be put under pressure (1-2 cm. Hg.; nitrogen gas) by means of the taps.

The vessels for collecting the eluate fractions are also shown in the figure. They are carefully calibrated at 1 ml. and are labelled from 1 to 20. This number is quite sufficient for one run.

The liquid is withdrawn by means of a pipette with a fine tip which delivers exactly 0.5 ml. between two marks.

Another pipette not calibrated provided with a rubber sleeve is used to remove approximately 0.3 ml. from each eluate fraction.

Further apparatus necessary consists of 200-ml. flasks, a reflux condenser, and apparatus for distilling off the extraction solvent under a current of CO_2 (all provided with ground glass joints), 500-ml. separating funnels, a 10-ml. graduated flask, and 5-ml. test tubes.

The final measurement is made with a Beckman D.U. quartz spectrophotometer or similar instruments.

Procedure

Preparation of the chromatographic columns. Insert in both columns a small plug of cotton wool and fill the tubes with light petroleum to well within the widened part at the top. Add dry adsorbent to fill the narrow part of the upper column with activated alumina and that of the lower column with alkaline alumina, both to within 0.5 cm. of the top section (see Figure 1). Keep the columns disconnected and close them at the bottom by means of a glass tube and rubber sleeve. Before use, drain off the solvent until the meniscus is situated in the narrow section of the tube.

Sampling. Remove a slice 2-3 cm. thick from a piece of margarine. Then take a cross section of approximately 10 g., put it in the 200-ml. saponification flask, and weigh to within 50 mg.

Saponification. Add 40-50 mg. hydroquinone, 8 ml. 50% KOH, and 25 ml. 95% alcohol. Heat cautiously during ¼ hour by immersing the flask not more than ½ cm. deep in the water bath at 85-90°C. During the saponification a slow current of oxygen-free nitrogen must be passed into the flask while the gas is introduced by a tube through the reflux condenser.

Extraction. Add 50 ml. distilled water through the reflux condenser and cool the soap solution to room temperature. Transfer the solution to a 500-ml. separating funnel and rinse the flask with 50 ml. distilled water. Extract with successive portions of 100, 50, 50, and 50 ml. ether by gentle shaking. Wash the combined extracts with 4 x 50-ml. portions of distilled water, the first time only by gentle swirling, the following times by gentle shaking.

Evaporation. With the flask only ½ cm. deep in the water-bath (80-90°C.) drive off the solvent by means of a current of oxygen-free carbon dioxide or nitrogen. When approximately 5 ml. are left, transfer the extract to a 50-ml. Erlenmeyer flask with some ether and drive off the solvent with the stream of gas. If traces of moisture are still detectable, add a few ml. of acetone or ethanol and again remove the solvent. Take up the residue immediately in a little light petroleum and transfer it to the upper chromatographic column, using as little solvent as possible (5 ml.).

Chromotographic separation. Insert the 50-ml. funnel and the pressure line with the rubber stopper and develop only the upper column with 5 ml. light petroleum and subsequently with 5-ml. lots of light petroleum containing 4, 8, and 12% ether (v/v), respectively. The eluate contains those substances which move down the column faster than vitamin A; it is discarded.

Now connect the second column and develop with 5-ml. portions of light petroleum containing 16, 20, and 24% ether (v/v) respectively, and finally with light petroleum containing 36% ether (v/v), ad libitum. The first 5 ml. of eluate after the columns are running in series can be discarded as it does not yet contain the vitamin A. The next eluate is collected in the 1-ml. calibrated tubes. The eluants are introduced through the top funnel one after the other, and mixing above the column should be avoided as far as possible. Furthermore only small amounts of eluate from the upper column should be collected on the lower column and then drained through the lower one before introducing new eluate onto the lower column. This is easily regulated by means of the pressure line taps.

Test for vitamin A. When the elution is finished, starting at tube No. 1, remove from each one approximately 0.3 ml. solution, after having made the contents homogeneous by blowing air bubbles through by means of the rubber-sleeved pipette.

Transfer the samples to 5-ml. test tubes, add 1 drop of acetic anhydride, and 0.5-1 ml. antimony chloride reagent. Note the numbers of the tubes which show a positive Carr-Price reaction (use a white background to judge the color). From these tubes make up the final solution to be measured.

The final solution. Take from each of the tubes showing a positive reaction exactly 0.5 ml. and collect them in a 10-ml. graduated flask. Make up to the mark with light petroleum. Usually 7 to 9 tubes, starting at the 4th or 5th tube, will cover the vitamin A fraction in the eluate.

Measurement and potency calculation. Measure the light absorption in a 1-cm. cell with a Beckman D.U.

quartz spectrophotometer or similar instrument between 280 mμ and 360 mμ and compare the curve obtained with the curve for pure vitamin A alcohol, set at the same extinction at 325 mμ. The curves must show a close similarity.

Calculate the potency by multiplying $E^{1\%}_{1\,cm.}$ 325 mμ with the internationally established biological conversion factor 1900,[1] taking into account that only a 50% aliquot of the total vitamin A from the margarine is present in the final solution.

Results

In factory margarines containing 20 I.U. per gm., added in the form of varying amounts of a natural vitamin A ester concentrate, whose potency was determined by the use of the chromatographic method of Gridgeman *et al.* (1), values were found which were much higher than those estimated by the older Carr-Price method (5). The latter method gave values ranging from 14 to 17.5 I.U. per gram in spite of the fact that the same concentrate was used in constructing the calibration curve for the colorimeter ("Unicam").

The results obtained with the present method are reproduced in Table I.

TABLE I

Multiple Determinations on Three Margarine Samples[a]

Margarine	Potency found after double chromatography I.U. per gram
1	19.2; 19.3; 19.4; 19.3; 19.4; 20.0; 20.2; 19.7; 19.4; 19.3; 19.5
2	20.6; 20.8; 20.2; 20.4; 20.6; 20.2
3	19.3; 19.7; 19.3

[a] Each series made from one piece of margarine in the course of not more than 4 days.

Apparently a much higher recovery than with the Carr-Price test is possible, and the figures show a remarkably high degree of reproducibility.

The deviations between the series may be caused by inaccurate dosage on a factory scale. In order to eliminate this effect, analyses were made with groundnut oil, to which was added pure crystalline vitamin A acetate (Hoffman-La Roche) at a level of 18.1 I.U. per gram prior to analysis. The recoveries were: 17.6, 17.5, 17.9, and 18.2 respectively. We may thus safely conclude that in general the recovery is better than 95%.

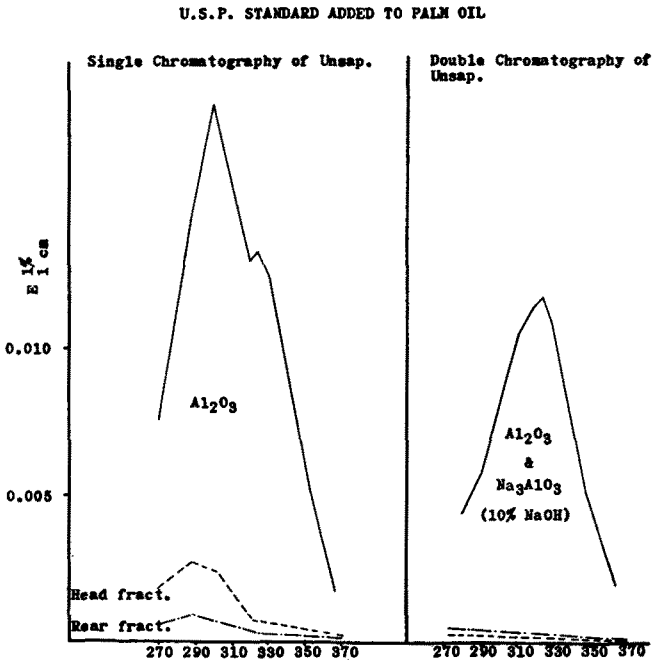
In addition, the method has been examined by the use of fortified coconut oil, soyabean oil, palm oil, groundnut oil, and palm kernel oil. No anomalies were encountered.

The shape of the absorption curve of the vitamin A alcohol fraction after double chromatography is shown in Graph 1. In the same graph is given the curve obtained after the eluate from the same extract has only passed through the upper column. This shows the action of the alkaline alumina column quite clearly.

Discussion

To obtain the high reproducibility indicated in the preceding section, it must be emphasized that

[1] The factor 1900 is recommended for a solution in isopropanol and may be incorrect when light petroleum is used, as tests carried out in collaboration with several laboratories have proved. The effect of the solvent on the factor however has not been established with certainty so that for the time being the factor 1900 is used. This difficulty can be avoided by evaporating off the light petroleum and dissolving the residue in isopropanol for the final determination.



GRAPH 1.

the directions given for the saponification and the extraction should be carefully followed and that the absence of direct sunlight must be ensured during the whole analysis.

If, during the saponification, heating is done on a steam-bath, large deviations within a series of controls may occur occasionally. This seems to depend on the sort of margarine used because not all the samples that were assayed showed this tendency. This fact may be related to the properties of the fat incorporated in the margarine, e.g., traces of metal which may render the saponification at temperatures higher than 85°C. somewhat risky.

With regard to the nature of the irrelevant-absorbing compounds, Wilkie[4] has already pointed out that tocopherols play a part. Since tocopherols are practically non-absorbing at 325 mμ, their elimination should not be necessary. However it is quite possible that along with tocopherols quinone derivatives are present which absorb at much higher wave-lengths than their reduced forms. The activated alumina column in fact cannot separate these compounds from vitamin A alcohol while the alkaline alumina has a high retaining capacity for them. The substances become visible as a brown-yellow zone in the upper part of this column but cannot be eluted as such. With ethanol a yellow eluate is obtained, the color of which rapidly fades on contact with carbon-dioxide. The absorption maximum at 290-295 mμ however disappears so that it may be concluded that tocopherols and related compounds are decomposed by the alkaline alumina. Probably there is a chemisorption with formation of a so-called color lake. A case in point is the behavior of phenols in the presence of alumina and caustic soda (6).

Apart from the adsorption of tocopherol-like compounds, the hitherto unknown adsorption properties of alkaline alumina may be demonstrated by the fact that kitol, if present in the sample to be analyzed, is firmly adsorbed while vitamin A alcohol passes rap-

idly out of the column; kitol can be eluted without decomposition by an ethanol-ether mixture 1:1. Synthetic dyes which are sometimes used may, depending on their chemical composition, either be eluted from the upper column before putting the two columns in series, or they are adsorbed at the very top of the second column without showing any sign of moving down during subsequent elution.

Carotene can be recovered from the upper column eluate with the first 15 ml. of eluant and can accordingly be determined separately by colorimetry.

It is intended to check the method for the analysis of other materials which have a low content of vitamin A, e.g. butter, cattle feeds, blood, and faeces.

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Phase Relations Pertaining to the Solvent Winterization of Cottonseed Oil in Hexane and in Acetone-Hexane Mixtures

RICHARD E. BOUCHER and EVALD L. SKAU, Southern Regional Research Laboratory,[1] New Orleans, Louisiana

COMPLETE phase relation data on the solvent winterization of cottonseed oil in acetone (1) showed that commercial C. P. acetone could be used as a winterization solvent. It was pointed out however that for certain oil-solvent ratios at a temperature about 5°C. below that required for adequate winterization, two liquid phases were present in addition to the solid phase. It was also indicated that moisture in the acetone might cause this separation into two liquid layers to take place at higher temperatures, thus causing interference with the efficient separation of the solid from the liquid. It has been found that this effect of moisture can be effectively counteracted by the presence of a small proportion of a hydrocarbon, such as hexane, to the acetone. Fifteen percent by weight of hexane was found sufficient to prevent liquid-liquid phase separation from interfering with the solvent winterization when less than about 1.4% by weight of moisture is present in the acetone as in the case of commercial grades of acetone.

The present report deals with the pertinent phase behavior in the solvent winterization of cottonseed oil in two additional solvents; namely, commercial hexane and a solvent mixture consisting of 85% by weight of acetone and 15% of hexane. With these additional data it is now possible to compare the relative advantages and disadvantages of acetone, hexane, and the acetone-hexane mixture for use as the winterization solvent for cottonseed oil.

Materials. Cottonseed oil No. 2, for which the complete data with acetone have already been published (1), was used in this investigation in order to permit direct comparison of the relative applicability of the different solvents. It was a commercial refined and bleached oil having an iodine value of 108.2. The commercial hexane used was Skellysolve B.[2] A commercial C. P. acetone was used containing 0.5% of moisture as measured by a special Karl Fischer reagent for ketones.[3]

Effect of Moisture in Acetone. To determine the approximate magnitude of the effect of moisture upon the formation of two liquid layers in the cottonseed oil-acetone system, 40% oil solutions were made up gravimetrically, using commercial acetone containing various amounts of added water. The solutions were slowly chilled to find the temperature at which two liquid layers appeared. The presence of 1.4% by weight of moisture in the acetone caused the break to take place at +5°C. instead of at −12°C., as previously reported for commercial acetone.

It was found that the addition of hexane counteracts the tendency of moisture to cause two liquid layers. Using as solvent a mixture of 15 parts by weight of hexane and 85 parts of acetone containing 1.4 weight percentage of water, a 40% cottonseed oil solution can be winterized at a chilling temperature of −8°C. without the appearance of two liquid phases. This suggests the use of an acetone-hexane mixture as a winterization solvent for cottonseed oil as a safety measure against the presence of moisture in the oil or the acetone. Fifteen weight percentage of hexane in the mixture should be more than adequate to counteract the amounts of moisture which would be expected to be present in commercial acetone.

Winterization Procedure. Laboratory bench-scale winterization tests were carried out by the same procedure as previously described except that the rubber stoppers for the centrifuge bottles were wrapped in tin foil to prevent the sorption of hexane from the samples. In brief, duplicate weighed samples of the oil in definite oil-solvent ratios were chilled in a bath of the desired temperature for a definite holding-time. They were then centrifuged for 10 minutes. The clear supernatant liquid was quickly decanted into a tared flask and the residual solid fraction weighed. From the weights and oil contents of the solid and super-

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] The mention of trade products does not imply that they are endorsed or recommended by the Department of Agriculture over similar products not mentioned.

[3] Mitchell, J., Jr., and Smith, D. M., "Aquametry," Interscience Publishers, New York, 1948, pp. 146-151.