# CCXVIII. THE REACTION OF ANTIMONY TRICHLORIDE WITH COD-LIVER OIL AND ITS UNSAPONIFIABLE FRACTION.

By ERNEST LESTER SMITH and VIOLET HAZLEY.

*From the Glaxo Research Laboratory, 56 Osnaburgh Street, London, N.W. 1.*

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During the last two years we have had occasion to carry out the antimony trichloride colour test on a number of batches of unsaponifiable matter extracted from fish-liver oils. The oils were saponified by the cold process, or sometimes by boiling with alcoholic sodium or potassium hydroxide, and the unsaponifiable matter was extracted from the soap solution, usually with ether. Although no special precautions (such as working in an atmosphere of nitrogen) were taken to conserve the chromogen[1], it frequently happened that extracts, both those prepared on a small laboratory scale and those prepared on a factory scale, gave values of well over 100 for the percentage extraction, as judged by the colour test. We were aware from published work and from our own observations that the intensity of the blue colour was not a linear function of the concentration of cod-liver oil in the test solution. We had also found that the unsaponifiable fraction gave a linear relationship. This suggested the presence of some saponifiable substance in the oil which inhibited the production of the blue colour—a suggestion confirmed by the observation that the addition of olive oil to the solution of unsaponifiable matter reduced the intensity of the blue colour[2]. Lack of time however prevented until recently a detailed investigation of these phenomena. Meanwhile Norris and Church [1930, 1], working along similar lines to us, reached the same conclusion. They found that oleic acid and unsaturated oils produced the inhibitory effect, and they showed that unsaponifiable matter extracted from cod-liver oil gave an intensity of blue proportional to concentration in chloroform solution, but exhibited the "inhibition" curve typical of cod-liver oil on addition of oleic acid. Norris and Church [1930, 2] conclude that the colour test on cod-liver oil only gives a valid assay if the slope of the tangent to the dilution curve at the origin is measured or calculated. This tangent should coincide with the dilution curve for the unsaponifiable fraction of the corresponding oil (con-

[1] Throughout this paper, the substance present in cod-liver and similar oils, giving a blue colour with antimony trichloride, is referred to as "the chromogen," without prejudice to the question of its identity with, or relation to, vitamin A.

[2] Incidentally, some olive oils, which we have examined, themselves slowly develop a blue colour with antimony trichloride in chloroform.

centrations being expressed in terms of the equivalent amount of oil), but Norris and Church did not secure complete extraction without loss of chromogen, so that their dilution curves for the unsaponifiable fractions cross the curves for the corresponding oils.

Norris and Church used light petroleum for some of their extractions; this solvent is notoriously inefficient for the extraction of unsaponifiable matter from soap solutions in the case of fish-liver oil soaps [bibliography, Smith, 1928, 1]. Most of the data are given for one of their extractions with this solvent [Norris and Church, 1930, 1]; if it may be assumed that the same volume of solvent was used for the second, third, and fourth extractions as for the first (for which it is given), then we are confident that they can only have extracted less than half the total unsaponifiable matter. Very large volumes of solvent and repeated extraction are necessary to secure even approximately complete extraction with light petroleum. In the experiment quoted, Norris and Church used (presumably) 600 cc. of this solvent to extract the soap derived from 100 g. of cod-liver oil; in one of our experiments the same volume of solvent (in 7 portions) was used to extract the soap from only 5 g. of cod-liver oil, and, even so, calculations indicated only 94 % extraction.

The other solvent used by Norris and Church was ethylene dichloride, following the method of Marcus [1928]. Our own experiments have shown that the partition coefficient for unsaponifiable matter between ethylene dichloride and potassium soap magma saturated with ethylene dichloride is very low; calculations showed that to extract about 96 % of the unsaponifiable matter, the soap from 25 cc. of cod-liver oil would need to be treated with about 2500 cc. of ethylene dichloride in four portions. We learn that Norris and Church [private communication] used even larger proportions of solvent than this, but they add, "We were thoroughly aware that the extraction was not complete."

It is true that most of our measurements of partition coefficients were carried out on the total unsaponifiable fraction and not on the chromogen, for which the value is not necessarily the same. So far as our experiments have gone however they have revealed no significant differences between the partition coefficients for the solid (sterol) and liquid fractions of the unsaponifiable matter and for the chromogen, although we have found different values for substances of quite different compositions which have been examined, such as aniline and an azo-dye. It is hoped to extend and publish this work at a later date.

It thus seems evident that Norris and Church did not extract the whole of the chromogen from their saponified oil. This is apart from any destruction which may have occurred—and in this connection it may be mentioned that their use of calcium chloride for drying some of the extracts is open to criticism, since Jones [1928] has shown that calcium chloride may accelerate the destruction of the chromogen by light and oxygen. Norris and Church were unfortunate in their choice of solvents, since with some other solvents, particularly

with ether and ethyl acetate, there is no difficulty in securing practically complete extraction of unsaponifiable matter. We were interested therefore to see whether it would be possible to extract the chromogen without loss by oxidation or thermal destruction during the manipulations involved.

In our first experiment, an elaborate apparatus was set up to enable the whole series of operations to be carried out in an atmosphere of nitrogen. Saponification was effected by boiling for 5 minutes only with strong alcoholic potassium hydroxide, since one of us [Smith, 1928, 1] had previously shown that this was enough for complete saponification; four extractions were made with ether, the ethereal solution was washed, and the bulk of the ether was distilled off in an atmosphere of nitrogen; chloroform was then added, and the distillation was continued at reduced pressure, to remove the remainder of the ether and to dry the extract, traces of water distilling over with the chloroform. When this extract was submitted to the antimony trichloride test, the dilution effect was represented by a straight line, tangential at the origin to the dilution curve for the corresponding oil, as nearly as could be estimated graphically (see Fig. 1).

Oil A (unsap. matter)
Oil C (unsap. matter)
Oil A
Oil D (unsap. matter)
Oil C
Oil D
Units of blue, Lovibond
Mg. cod-liver oil in 2·2 cc. reaction mixture

Fig. 1.

× —— × Cod-liver oil.
○ —— ○ Unsaponifiable matter extracted by chloroform.
+ —— + „ „ „ ether (in nitrogen).
△ —— △ „ „ „ ether (5 mins. saponification).
□ —— □ „ „ „ ether (1 hour saponification).

The experiment was repeated without special precautions to exclude air and gave results identical within the limits of experimental error. Another repetition under the same conditions, except that the time of saponification

was increased to 1 hour, again gave complete recovery of chromogen—despite the fact that the ether extract was distilled to dryness and allowed to remain on the water-bath for about 5 minutes before the chloroform was added.

The unsaponifiable matter from 2 g. of cod-liver oil was allowed to form a thin film covering the bottom of a flat-bottomed flask, by evaporation of its solution in chloroform; after solvent vapour had been blown off, the film was heated in an electric oven at 102° for 40 minutes. Even this drastic treatment destroyed only about half of the chromogen originally present.

It appears that the chromogen is more stable than has sometimes been supposed, and that there is no particular difficulty in obtaining practically complete extraction of the total unsaponifiable matter from cod-liver oil. It is nevertheless preferable to avoid exposure of the hot extract to the air, and we always fit the distilling flask with a tap-funnel, so that chloroform may be added without allowing access of air.

Extraction of four different samples of saponified cod-liver oil, with ether, ethyl acetate and chloroform, gave similar results in each case; these are presented in graphic form in Fig. 1. In no case is there any tendency for the dilution curve of the extract to cut that of the oil, indicating that extraction is quantitative within the limits of error of the method. Oil *A* was exceptionally rich in chromogen, oils *B* and *C* were good, while oil *D* was rather poor in this respect, so that these oils are representative of any that are likely to be encountered.

In order to find out whether complete extraction could be secured with light petroleum the following experiment was carried out. 5 g. of cod-liver oil *E* were saponified with 2·5 cc. of 10*N* aqueous potassium hydroxide and 20 cc. of alcohol; 10 cc. of water were added, and alcohol to 50 cc.; the soap solution was purposely made strongly alcoholic, since experiment has shown that this tends to increase the partition coefficient for unsaponifiable matter between light petroleum and soap solution; on the addition of light petroleum, the volume increased to 60 cc. The mixture was extracted with two 50 cc. portions of light petroleum (B.P. 40–60°), the extracts were washed with water, the solvent was distilled off, and a chloroform solution of the residue was tested in the usual way with antimony trichloride. The soap solution was then further extracted with five 100 cc. portions of light petroleum, and the extract was treated in the same way. A greater amount was extracted by this further treatment than by the first, and the sum of the two sets of values yielded a line tangential at the origin to the dilution curve for the original oil (see Fig. 3). Only about 40 % of the chromogen was recovered in the first two extractions, indicating a partition coefficient of about 0·35. From this figure it can be calculated that the whole seven extractions should have removed approximately 94 % of the chromogen originally present in the soap solution. This experiment was repeated using oil *B*, the only difference being that 10 cc. more of water and a correspondingly smaller amount of alcohol were used in preparing the soap solution for extraction. The results, which are included in

Fig. 2, were almost identical with those previously obtained in the proportions extracted by the two treatments with solvent. In addition, the sum of the two sets of values agreed closely with those obtained by extraction with chloroform and with ethyl acetate. Equal volumes of solvent were used for successive extractions, since one of us [Smith, 1928, 2] has shown that with a given volume of solvent and a given number of extractions, the highest degree of extraction is obtained by subdivision of the extracting solvent into equal portions.

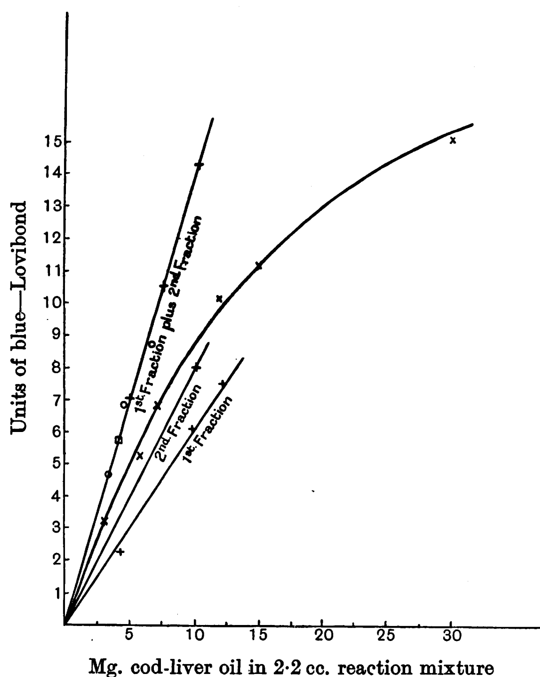


Fig. 2.

× Cod-liver oil.
○ Unsaponifiable matter extracted by chloroform.
+ ,, ,, ,, light petroleum.
□ ,, ,, ,, ethyl acetate.

*A new technique for the antimony trichloride colour test.*

Recent workers, particularly Norris and Danielson [1929] and Norris and Church [1930, 1 and 2], have shown that the dilution curves for cod-liver oils are non-linear and vary in shape, so that a true comparison of two oils is not possible when the colour test is carried out in the usual manner. Norris and his colleagues have suggested taking readings at low colour values (below 4 Lovibond units) in which region the dilution curves are approximately linear, or, better, plotting the dilution curves, and comparing the slopes of the tangents at the origin. The tangents are drawn by inspection, or can be calculated in cases where the dilution curve fits a formula of the type $y = ax^2 + bx + c$.

Both these proposed modifications are open to the objection that readings must be taken at very low colour intensities for which the degree of accuracy attainable is, at any rate for the unpractised, considerably less than for readings taken in the neighbourhood of 10 Lovibond units; the second method moreover requires considerable time.

Since we have shown that the chromogen can be readily and quantitatively extracted in the unsaponifiable fraction, which gives a linear dilution curve, an obvious solution of the problem is to carry out the colour test on this fraction.

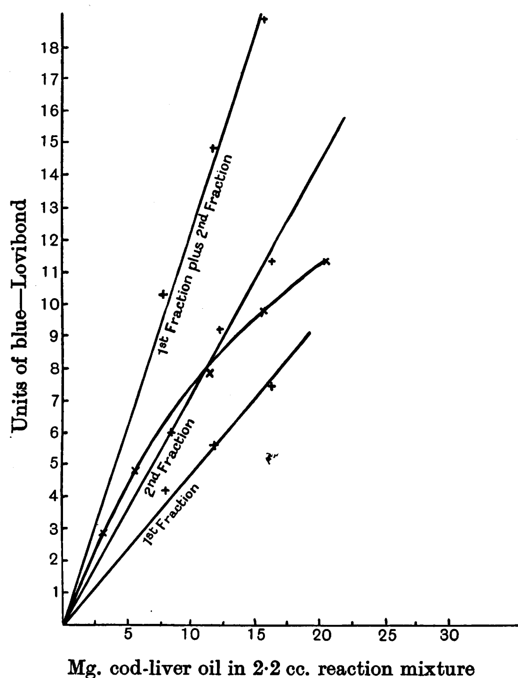


Fig. 3. Cod-liver oil *E*.

+ Unsaponifiable matter extracted by light petroleum.
× Cod-liver oil.

A just comparison of two samples is then possible from a single determination on each, made near the colour values at which the eye is most sensitive to small variations in blue.

Since the extract is finally required in chloroform solution, chloroform suggests itself for extraction of the soap solution; it is a fairly good solvent for the purpose and does not form emulsions with soap solutions if these contain sufficient alcohol. More reliable results can thus be obtained than by any other method, and certainly in no more time than is required to determine the dilution curve for an oil. The method is described in the experimental section.

Bertram [1929, and private communication], has suggested that it may not be justifiable to evaluate the chromogen content of cod-liver oil by carrying

out the colour test on its unsaponifiable fraction, since any "oxycholesterol" which may be present develops a blue colour with the reagent, though not in the presence of oil. To investigate this point, we prepared "oxycholesterol" by the method of Lifschütz [1919]. The colour given in the test by 0·2 cc. of a 0·4 % chloroform solution of this product (0·8 mg.) was less than 2 Lovibond units, while Rosenheim [1927] obtained for "oxycholesterol" prepared by the action of benzoyl peroxide on cholesterol, 12 units for 1 mg. of the product. Since the unsaponifiable matter from cod-liver oil is known to be composed largely of other substances [Drummond, Channon and Coward, 1925; Drummond and Baker, 1929], it is evident that any trace of "oxycholesterol" which may be present will have a negligible effect on the blue colour developed with antimony trichloride.

The colour developed with cod-liver oil unsaponifiable matter is somewhat difficult to match against Lovibond glasses. Instead of the purplish blue given by cod-liver oil, the colour is almost invariably, in our experience, greenish blue[1] and is matched by a combination of blue and yellow glasses in the ratio of 10 to 4, very approximately. In this observation we are in agreement with Norris and Church [1930, 1]. Moreover, the colour test solution is brighter than the combination of glasses, so that neutral tint glasses have to be used in conjunction with the test solution. In view of the constancy of the colour, we considered the possibility of matching against a permanent colour standard in a colorimeter, as it seemed that this might well give greater accuracy than the Rosenheim-Schuster tintometer. The colour can be imitated by bromocresol green at about $p_H$ 5·4, or by a solution of cobalt chloride in a 0·2 $N$ solution of hydrochloric acid in 95 % alcohol. The latter is probably preferable to the solution of a somewhat unstable dye-stuff. Preliminary experiments indicate that colour test solutions could be matched against the cobalt chloride solution in a Hellige type colorimeter with an accuracy at least as great as that attainable with the Rosenheim-Schuster colorimeter.

## Experimental.

### *Ether extraction in nitrogen.*

A 350 cc. conical flask was fitted with a reflux condenser and a right-angled tube with stopcock, dipping well into the flask. 10 g. of cod-liver oil were weighed into the flask, which was then flushed out with a rapid current of nitrogen *via* this tube. 5 cc. of 10 $N$ aqueous potassium hydroxide and 20 cc. of alcohol were added *via* the condenser, and the mixture was heated on a water-bath for 5 minutes and shaken continuously until the oil globules disappeared. The flask was cooled and 70 cc. of water and 150 cc. of ether were poured down the condenser; after the solution had been shaken and allowed

[1] The only exception was the extract from a sample of oil stored for several years in a half-filled bottle; this, like the oil itself, gave a muddy purple colour with the reagent; the chromogen had been largely destroyed.

to separate, the right-angled tube was adjusted so that its lower end came just above the soap layer, and the ether layer was transferred through this tube to a separating funnel by blowing nitrogen into the flask through the condenser. The separating funnel was equipped with a cork carrying a small tap-funnel for wash liquors and a right-angled tube with stopcock for delivery of the ether extract; it had previously been filled with nitrogen. The extraction was continued with three further portions each of 125 cc. of ether, and the three ethereal solutions were transferred to the same separating funnel. In order to remove soap the combined extracts were washed successively with 10 cc. portions of water, $2N$ sodium hydroxide, water, and dilute hydrochloric acid, and then finally with 200 cc. of water to remove alcohol [Smith, 1928, 1], and transferred to a bolt-head flask previously flushed with nitrogen; most of the ether was distilled off, and chloroform was then added, distillation being continued under diminished pressure until all traces of water had disappeared and the extract was reduced to small bulk. It was then cooled in the atmosphere of nitrogen, and finally rinsed out with chloroform into a 50 cc. graduated flask.

*Ethyl acetate extraction.*

10 g. of cod-liver oil were mixed with 5 cc. of $10N$ potassium hydroxide and 5 cc. of alcohol, and the mixture was heated on a water-bath for about 5 minutes; 100 cc. of water were added, and the soap was dissolved by gentle heat. Dilute hydrochloric acid was added drop by drop, with stirring, until the soap solution was only faintly alkaline to phenolphthalein. This procedure was adopted in order to avoid hydrolysis of the ethyl acetate by excess of alkali. Four extractions were given, each with 125 cc. of ethyl acetate. The solvent was distilled from the combined extracts under reduced pressure; the residue was then dissolved in about 100 cc. of ether, fatty acid was removed by washing with alkali and water, and the final chloroform solution was prepared as described above.

*Chloroform extraction.*

*The modified colour test.* 2 g. of the oil are weighed into a boiling tube; 1 cc. of $10N$ aqueous potassium hydroxide and 5 cc. of 95 % alcohol are added and the mixture is heated in a boiling water-bath for 5 minutes, being shaken until oil globules disappear. The tube is immediately cooled under a tap, and the soap solution is rinsed into a small separating funnel with 25 cc. of water and 10 cc. of alcohol and shaken vigorously with 40 cc. of chloroform. When separation is complete (in about 5 minutes), the lower layer is run into a second separator, and the soap solution is extracted with two further 30 cc. portions of chloroform. The combined chloroform extracts are washed, first with 5 cc. of water to remove most of the soap (shaking must be very gentle, to avoid emulsification), then with two 100 cc. portions of water (the first slightly acidified) to remove alcohol. The chloroform layer is transferred to a small flask, care being taken to prevent globules of water running into the flask,

and the solution is distilled to small bulk; the residue is rinsed with chloroform into a 10 cc. or 25 cc. graduated flask, according to the potency of the oil. The antimony trichloride colour test is carried out on this solution.

In the earlier experiments 35 cc. of chloroform were used for the first extraction and 30 cc. for the second and third. In one case the soap solution was then extracted with three further 25 cc. portions of chloroform. This treatment suffices to recover any chromogen which remains in the soap solution after the first three extractions; the amount recovered was 1/40 of the total. The volumes of chloroform used for extraction were then increased to the values given above, which give a degree of extraction as near 100 % as the method justifies.

Our procedure with the actual colour test differs slightly from that described by others. A suitable weight of the substance to be tested is diluted with chloroform in a small graduated flask. 2 cc. of a solution of antimony trichloride in dry, alcohol-free chloroform saturated at 20° is measured from a burette (fitted with a calcium chloride tube) into a ½″ all-glass cell, and 0·2 cc. of the above solution is run in from a 1 cc. graduated pipette. If added carefully, this floats on top of the denser antimony trichloride solution and the two do not mix appreciably until stirred with a glass rod. Thirty seconds after stirring, the colour is matched by means of a Rosenheim-Schuster tintometer fitted with a daylight lamp. Readings are taken at approximately 20°. Since with unsaponifiable matter the colour intensity-concentration relationship is linear, the value obtained for the chromogen content is not affected by the colour range in which the reading falls; after a preliminary test the concentration of the test solution may be altered if necessary to bring the reading within the range which the observer prefers for colour matching. To compute the units of chromogen per g., we calculate the Lovibond blue units which should be given by 0·2 cc. of a 10 % solution (*i.e.* 20 mg.) of the original substance under the above conditions; for these units we use the symbol $\beta$. Thus if the unsaponifiable matter extracted from 2 g. of cod-liver oil is made up to 25 cc. and this solution gives a reading of 6·0 Lovibond units, the chromogen content of the oil is said to be

$$\frac{6 \cdot 0 \times 10 \times 25}{2 \times 100} = 7 \cdot 5\beta \text{ per g.}$$

SUMMARY.

The unsaponifiable fraction of cod-liver oil gives with antimony trichloride in chloroform a blue colour proportional to its concentration.

The line representing the dilution effect for the total unsaponifiable fraction is tangential at the origin to the dilution curve for the corresponding cod-liver oil.

The unsaponifiable fraction can be extracted almost without loss with ether, ethyl acetate, chloroform or light petroleum, under suitable conditions.

A method is described for carrying out the colour test on the unsaponifiable fraction extracted with chloroform.

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