CLXXXVIII. THE FLUORESCENCE OF SOME VITAMIN A-CONTAINING FATS.

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WHEN a beam of light from a quartz mercury vapour lamp, completely enclosed so that the only light emitted has to pass through a filter cutting out nearly the whole of the visible portion of the spectrum, is allowed to fall on to any object in a darkened room, a more or less characteristic fluorescence is usually observed. Metals, such as gold, silver, iron, etc., are quite non-fluorescent and take on the appearance of black velvet when observed in this light, but many other substances emit quite a bright coloured light. Several references are to be found in the literature suggesting the application of this phenomenon in various branches of analysis (*e.g.* Gerngross and Schulz [1927]; Popp [1927]; Volmar [1927]), and perhaps the most striking observation is that butter can be distinguished from margarine by the quite dissimilar fluorescence. Butter shows a fairly bright yellow colour, but margarine in this light is definitely blue.

I. APPARATUS FOR THE MEASUREMENT OF FLUORESCENCE.

Work on the use of fluorescence in analysis has been hindered by the difficulty of describing, without the help of a demonstration, the phenomena that have been observed. This difficulty can be overcome by giving some sort of measurement to the fluorescence, and in the present work we have used an adaptation of the colorimeter described by Guild [1927] and manufactured by Adam Hilger & Co., Ltd. This instrument is used for the standardisation of colours in terms of the three additive primaries: red, green and blue. For that purpose the coloured sample is illuminated by a standard lamp, and the light from it is viewed in an eyepiece and matched by the light from the primaries, which fills a rectangle with one side adjacent to the rectangle of light from the coloured sample. The direct reading obtained gives a measure of the colour in units (up to 100 each) of red, green and blue, but this reading is dependent on the sensitivity of the observer's eye to the different primaries, and fairly wide variations are obtained between the readings of different individuals. These can be eliminated by referring all the readings to the particular observer's reading on a standard white disc supplied with the instrument. The final expression obtained gives (i) the "shade," which is the measure of the total light reflected from the sample when the total light

reflected from the standard white disc illuminated similarly = 100, and (ii) the "quality," which is the analysis of the total light reflected into percentages of red, green and blue, assuming that the analysis of standard white is 33.3 % red, 33.3 % green and 33.3 % blue.

The colorimeter was housed in a dark room, and in making fluorescence readings the sample of fat was illuminated by filtered ultra-violet light instead of by white light from the standard lamp. The fluorescence was viewed in the eyepiece and matched by the additive primaries exactly as are the colours illuminated by white light, and expressions of "shade" and "quality" were obtained by referring the readings to the same observer's readings on "standard white" illuminated by the ordinary light. The "shade" figure is a measure of the brightness of the fluorescence, in purely arbitrary units. The "quality" figure has exactly the same significance as in ordinary colour readings.

Fig. 1. *a*, eyepiece; *bbb*, knobs controlling colour filter apertures; *c*, stand carrying skip with fat sample; *d*, mirror reflecting sample; *e*, watch-shaped quartz flask filled with water; *f*, filter cutting out most of visible light from mercury vapour lamp; *g*, mercury vapour lamp.

Fig. 2. Detail of stand and skip for fat sample.

Throughout the work recorded in this paper, all the readings were carried out by two observers, and unless good agreement was obtained in the analysis, the result was rejected.

In our adaptation of the colorimeter to the measurement of fluorescence, the apparatus was arranged as shown in the diagram, Fig. 1.

It is important that the sample whose fluorescence is to be examined should be illuminated by filtered ultra-violet light at fairly close range, otherwise the fluorescence as seen in the eyepiece is only feeble, and cannot be matched with accuracy. The housing of our ultra-violet lamp (the Hanovia analytical quartz

lamp) did not make this easy, but the difficulty was overcome by reflecting the fluorescent light into the eyepiece by means of an ordinary mirror. The sample of fat was pressed into a small metal skip $1'' \times 3\frac{3}{4}''$, and smoothed to give a plane surface. The skip was fixed into a slot on the end of a wooden stand which could always be placed in exactly the same position on top of the case of the colorimeter, in a line parallel with the axis of the eyepiece, and in the same vertical plane. The arrangement of the skip and the stand is seen in detail in the diagram, Fig. 2. The mirror also was held in a thick wooden stand (to protect it from the heat of the lamp), and a watch-shaped quartz flask (8 mm. thick), filled with water, was interposed between the lamp and the fat sample, so that the latter should not become unduly heated by the radiation from the lamp. The whole apparatus could be dismantled and reassembled exactly as in the original positions.

This arrangement is admittedly susceptible of considerable improvement, but it has enabled us to make several measurements of interest, and to express our results in an understandable form. The standard lamp and the light source to the primaries are fitted with variable resistances, and in our colour readings we have used 112 volts and 120 volts respectively across the two lamps. In the fluorescence readings we did not at first control the voltage across the mercury vapour lamp, but later it appeared to be necessary because of fluctuations in the current supply, and the voltage across the lamp and fixed resistance attached was set at 112.

II. THE FLUORESCENCE OF THE UNSAPONIFIABLE MATTER FROM COD-LIVER OIL.

Peacock [1926] reported that cod-liver oil exhibits a brilliant "golden" fluorescence in filtered ultra-violet light, and that exposure to white light of sufficient intensity results in the gradual disappearance of the fluorescence. From the observations that the fluorescence persists some time after the oil has ceased to give the arsenic trichloride test for vitamin A, and that, after the fluorescence has been entirely destroyed, if the oil is stored for several months in the dark, partial recovery of fluorescence takes place, he concluded that the destruction of vitamin A and the destruction of fluorescence are not identical phenomena. We have carried out many further experiments, not reported in the present paper, and we find that though there is a very close association between vitamin A and a characteristic fluorescence, certain discrepancies, such as those observed by Peacock, do occur. We have not yet observed any fat containing vitamin A that does not also show the fluorescence associated with it, and it seems that an explanation of the discrepancies may be that, while vitamin A is actually a brightly fluorescent substance, in certain circumstances other substances may be formed in oils, giving a fluorescence somewhat similar to that due to the vitamin. There does not appear to be any connection between fluorescence and vitamin D.

When a little unsaponifiable matter from cod-liver oil is added to a solid fat, the brightness of the fluorescence is increased, and the colour of the fluorescence is made less bluish. In Table I and Figs. 3 and 4, the effect of the addition of unsaponifiable matter from cod-liver oil upon the fluorescence of two fats is shown. The two fats used were hardened coconut oil and jus. For margarine manufacture, jus (pure rendered beef fat) is separated into two fractions of different melting point range. The lower melting fraction is "oleo," the higher is "oleo stearine." Jus normally gives only a dull fluorescence. It is seen how with successive additions of unsaponifiable matter the brightness is increased and the percentage of blue in the analysis

Fig. 3.

Fig. 4.

of the emitted light is diminished. As would be expected, the brightness reaches a limit after a certain amount of unsaponifiable matter has been added, and then begins to fall off slowly. If the fat were perfectly transparent to the exciting rays and the emitted light, it would be observed, when regarded from the side, that with successive additions of unsaponifiable matter the brightness would increase for a while through the whole depth of fat. Then a distinct zone of fluorescence would appear and the fat below this would not fluoresce, because the whole of the fluorescence-exciting rays had been absorbed in the depth of the zone. With further additions the zone would become more and more shallow until confined to a layer at the surface. Regarded from the surface, the limit of brightness would be reached as soon as the fluorescent

zone appeared, and after a while further additions of unsaponifiable matter would slightly depress the fluorescence, because of the opacity of the added material to the emitted light. The unsaponifiable matter from cod-liver oil is dark in colour, and, as will be seen later, the addition of a pigment depresses the brightness of the fluorescence of a fat.

With an imperfectly transparent fat, the fluorescence-exciting rays are absorbed, not only by the fluorescent substance, but by the fat, and the fat also absorbs part of the emitted light. The limit of brightness will be reached when a fairly large increase in the amount of unsaponifiable matter present will make so small a difference to the depth of the fluorescent zone that the opacity of the fat ceases to be an important factor.

Table I. *Effect of the addition of unsaponifiable matter upon the fluorescence of a fat.*

	Antimony trichloride "blue" figure*	Shade	Quality		
			Red	Green	Blue
Jus	—	29	14	33	53
Jus + unsap. matter from c.-l.o. ...	0.66	52	19	39	42
Ditto	1.33	55	20	39	41
Ditto	1.90	57	20	40	40
Ditto	3.33	60	21	41	38
Ditto	10.0	58	22	40	38
Hardened coconut oil	—	50	5	14	81
Hardened coconut oil + unsap. matter from c.-l.o.	0.33	72	11	25	64
Ditto	1.67	80	15	32	53
Ditto	3.33	77	17	38	45
Ditto	6.66	71	19	39	42
Ditto	9.0	60	20	42	38

* The "antimony trichloride blue figures" are determined in a Lovibond tintometer according to the method described by Carr and Price [1926]. 0.2 cc. of a wt./vol. solution of the oil or fat in chloroform is run into an 8 mm. test-tube, and 2 cc. of a saturated solution of antimony trichloride in chloroform are added. The blue colour obtained is matched by the Lovibond glasses, and the result calculated to a 20 % solution of oil. The blue figure of 0.3 may be taken as that of good butter-fat (determined from readings on the unsaponifiable matter), and the average for a large number of cod-liver oils purchased in various parts of England this year was 6.5.

It is seen clearly from Figs. 3 and 4 (plotted from Table I) how sensitive the fluorescence of jus is to small additions of the unsaponifiable matter from cod-liver oil. Jus is an animal fat and it is probable that it contains a small amount of vitamin A. Though the fluorescence of the sample of jus used was quite considerable, it is possible that it was due entirely to the small amount of vitamin A present, for it seems probable that, if all the vitamin A were withdrawn, the fluorescence would sink nearly to zero. A sample of jus that had become rancid gave the fluorescence:

shade = 6,

quality = 8 (*R*), 17 (*G*), 75 (*B*).

When examined in the melted state, it appeared to be practically non-fluorescent.

The actual composition of the emitted light from jus is changed very considerably by the addition of unsaponifiable matter up to 0.66 Lovibond

blue units (calc.), but it is seen that very little change takes place on further addition, until the effect of the colouring matter of the unsaponifiable matter becomes apparent.

Hardened coconut oil has a bright blue fluorescence, and this exerts a strong effect on the fluorescence of the added unsaponifiable matter. Though the fluorescence of hardened coconut oil is very bright, if examined in the liquid condition, it is seen to extend through a deep zone, and thus a sufficient excess of unsaponifiable matter from cod-liver oil is able to cut out most of the blue fluorescence, and the quality becomes similar to that of jus with unsaponifiable matter.

III. THE FLUORESCENCE OF BUTTER, AND THE EFFECT OF THE ADDITION OF UNSAPONIFIABLE MATTER FROM COD-LIVER OIL ON THE FLUORESCENCE OF MARGARINE.

The fluorescence of butter is yellow, varying somewhat with different samples. It is due almost entirely to the fat, and the separation of the aqueous phase does not make a great difference, as shown in Table II.

Table II. *Fluorescence readings on butters, butter-fats and margarines.*

			Quality			
			Shade	Red	Green	Blue
Irish butter	...	...	32	27	44	29
Danish butter I	...	...	34	24	41	35
Danish butter II	...	...	30	22	37	41
Irish butter-fat	...	...	31	31	46	23
New Zealand butter-fat			31	27	48	25
Danish butter-fat I	...	...	30	25	42	33
Danish butter-fat II	...	...	29	22	44	34
Margarine I	...	...	24	10	24	66
Margarine II	...	...	35	9	22	69

Table II also shows the fluorescence of two samples of margarine. Both of these gave blue fluorescences quite different from the fluorescence of butter, but one was considerably brighter than the other. The brighter sample contained vegetable fats only, but the fat of the duller sample contained 35 % oleo.

For comparison with Table II it may be of interest to give the colour readings on some of the same samples, *i.e.* when the samples are illuminated by white light from a standard source instead of by ultra-violet light (Table III).

Table III. *Colour readings on butters, butter-fats and margarines.*

			Quality			
			Shade	Red	Green	Blue
Irish butter	...	...	70	45	37	18
Danish butter I	...	...	65	43	37	20
New Zealand butter-fat	...	...	45	51	40	9
Danish butter-fat I	...	...	58	47	40	13
Margarine I	...	...	67	45	38	17
Margarine II	...	...	67	45	37	18

An outstanding difference between butter and margarine is that butter contains considerable amounts of vitamins A and D. This deficiency of margarine may be made good by the addition of a sufficient amount of the unsaponifiable matter from cod-liver oil (the amount is calculated to give an antimony trichloride blue figure for the margarine of 0.27), and the addition produces a considerable change in the fluorescence. The margarines so treated can always be readily distinguished by means of filtered ultra-violet light from similar margarines without the vitamin fraction (Table IV).

Table IV. *Effect of the addition of unsaponifiable matter from cod-liver oil upon the fluorescence of margarine.*

	Shade	Quality		
		Red	Green	Blue
Margarine I	24	10	24	66
Margarine I v (i.e. margarine I with the addition of cod-liver oil unsap. to antimony trichloride blue figure of 0.27)	28	16	32	52
Margarine II	35	9	22	69
Margarine II v (i.e. margarine II with cod-liver oil unsap. as above)	41	13	26	61
Danish butter I	34	24	41	35

It is seen that the change effected by the addition of unsaponifiable matter is towards butter fluorescence. The fluorescence of margarine I with the unsaponifiable matter is definitely yellowish, but as is readily seen in the analysis, it does not equal the yellowness of butter fluorescence. As more and more unsaponifiable matter is added, the fluorescence becomes brighter and more nearly yellow, but about five times the amount necessary to bring margarine to the same vitamin A content as butter must be added before the fluorescence approximates in quality to that of butter. It appears probable that this is due in part to the masking effect of the blue fluorescence of the vegetable fats; for the margarine I v (with 35 % oleo in the fat) approaches butter in its fluorescence much more closely than does the margarine II v (containing only vegetable fats). This, however, does not furnish a complete explanation, for a margarine made entirely with animal fat, and with the normal amount of cod-liver oil unsaponifiable matter added, is still not a match in fluorescence for butter.

Two differences between butter and ordinary margarine have so far been considered—the comparative richness of butter in vitamins, and the presence in margarine of certain vegetable fats—and both have been shown to be concerned in accounting for the difference observed between the fluorescences of butter and margarine. The only other factor that would seem likely to be of importance is a possible difference between the colouring matters present. The mixture of colouring matters usually added to margarine is non-fluorescent, and when an excess of it is added, it is seen that the fluorescence is very much depressed in brightness. Butter frequently contains added colouring matter but a large proportion of its colour, at least in spring and summer, is derived

from the pigments in the cow's food, and it might be expected that these pigments would be fluorescent. Moreover, annatto, the colouring matter commonly added to butter, is fluorescent. Margarine normally contains only a small proportion of natural colouring matter, derived from the beef-fat and the vegetable oils. Red palm oil is slightly fluorescent and very strongly coloured, and its pigment is closely related to the pigment in butter. In some countries it has been used for colouring margarine. 0.8 % of red palm oil added to oleo brings the colour to that of Danish butter-fat, and when unsaponifiable matter from cod-liver oil to the vitamin potency of butter-fat has been added, the fluorescence also is a match for that of Danish butter-fat (Table V).

Table V. *Effect of pigments on the fluorescence of fats.*

	Shade	Quality		
		Red	Green	Blue
Jus	29	14	33	53
Jus + 0.8 % red palm oil	20	22	43	35
Jus + 0.8 % red palm oil + unsap. to vitamin potency of butter-fat	25	26	42	32
Danish butter-fat I	30	25	42	33
Danish butter-fat II	29	22	44	34
Oleo + 0.8 % red palm oil + unsap.	31	24	43	33
Jus + 1.6 % red palm oil	16	27	45	28
Jus + 1.6 % red palm oil + unsap.	24	29	47	24
New Zealand butter-fat	31	27	48	25
Irish butter-fat	31	31	46	23
Oleo	44	11	34	55
Oleo + synthetic butter colour	14	15	34	51

It is observed that the addition of red palm oil to jus causes the blue component in the analysis to drop, and the red and green components to increase. At the same time the intensity of the fluorescence (shade) is depressed somewhat. The addition of unsaponifiable matter to the coloured fat increases the intensity of the fluorescence again, and further diminishes the blue component. The quality of the fluorescence from jus with unsaponifiable matter and 0.8 % red palm oil is a match for that of Danish butter-fat, and doubling the amount of pigment brings the quality to a match with that of New Zealand or Irish butter-fat. The shade figure for jus with unsaponifiable matter and 0.8 % red palm oil is lower than the shade figure for oleo with the same additions, but this is to be attributed to the greater opacity of jus. The greater opacity to ordinary light is obvious. The greater opacity to fluorescence-exciting rays can be demonstrated by shielding a crystal of uranium nitrate from the direct rays of the lamp by a thin layer of jus or oleo in a Petri dish. The crystal of uranium nitrate fluoresces much more strongly under the screen of oleo than under the screen of jus of the same thickness.

The samples that respectively match Danish butter-fat and New Zealand or Irish butter-fat in the quality of their fluorescence, also match them in the actual colour by ordinary illumination (Table VI). Here, as would be expected, the greater opacity of the jus increases the brightness by reflected light.

Table VI. *Actual colour by reflected light of samples that match in fluorescence.*

	Shade	Quality		
		Red	Green	Blue
Danish butter-fat I	58	47	40	13
Oleo + 0.8 % red palm oil + unsap. to vitamin potency of butter-fat	57	48	41	11
Jus + 0.8 % red palm oil + unsap.	69	47	39	14
New Zealand butter-fat	45	51	40	9
Jus + 1.6 % red palm oil + unsap.	53	52	39	9

SUMMARY.

1. A method has been devised by which the actual brightness of the fluorescence of a solid fat, illuminated by ultra-violet light filtered practically free from visible light, may be determined, and the colour expressed in terms of three additive primaries: red, green and blue.

2. There is present in the unsaponifiable matter from cod-liver oil a brightly fluorescent substance. Curves and tables are given showing the effect of the addition of unsaponifiable matter from cod-liver oil on the fluorescence of two fats, one already slightly fluorescent (jus), and the other brightly fluorescent (hardened coconut oil).

3. The fluorescence of butter or butter-fat is yellow in colour, but the normal fluorescence of margarine is blue. This difference cannot be accounted for solely by the known differences in vitamin content.

4. The blue fluorescence of margarine can be modified as follows:

(a) by varying the fat mixture: certain vegetable fats fluoresce bright blue, while the fluorescence of oleo and jus is pale greenish;

(b) by the addition of unsaponifiable matter from cod-liver oil: the first small additions markedly increase the brightness and diminish the blueness of the fluorescence;

(c) by varying the nature of the pigment present: some pigments depress the fluorescence more markedly than others.

5. A sample of oleo coloured with sufficient red palm oil to match it with butter-fat, and with sufficient unsaponifiable matter from cod-liver oil to bring it up to butter-fat in vitamin A potency, exactly matches butter-fat in fluorescence.

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