

## The effect of oxidation on the iodine values of phospholipid in milk, butter and washed-cream serum

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**SUMMARY.** Phospholipid was isolated from milk, butter, and washed-cream serum by solvent extraction followed by simple counter-current distribution and thin-layer chromatography. Iodine values from fresh samples, determined by a micro-Wijs technique, ranged, for the cephalin fraction, from 70 to 86, for the lecithin fraction from 44 to 55, and for the sphingomyelin fraction from 36 to 44. In washed-cream serum, oxidation with copper and ascorbic acid led to reduction in extractable phosphorus, decreased chromatographic mobility of phospholipid and significant falls in the iodine values of the 3 phospholipid fractions. In milk, slight reductions in phospholipid iodine values were observed following copper-catalysed oxidation but they were not consistently significant. Iodine values of butter phospholipids remained unchanged even after gross oxidative quality deterioration.

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In studies on the origin of storage defects in butter, the need has been felt in the authors' laboratory for a chemical method suitable for routine application in assessing phospholipid oxidation. Such a method should be compatible with thin-layer chromatography (TLC), which is the most satisfactory technique for separating phospholipids from neutral lipids. This rules out the possibility of measuring peroxide development, which in early oxidation requires samples of about 100 mg, which is inconveniently large for TLC techniques.

Consideration has been given to the use, for this purpose, of the iodine value, which requires only 1 mg or less of sample for precise determination. The iodine value is a very crude index of lipid oxidation as it is affected normally only under severe conditions of oxidation such as dairy products do not usually experience. There is evidence in the literature, however, that the iodine value of milk phospholipids is sensitive to moderate oxidation. Thus, Swanson & Sommer (1940), Koops (1957) and Smith & Dunkley (1959) have reported substantial falls in phospholipid iodine values during copper-catalysed oxidation in milk and cream.

Swanson & Sommer's (1940) results in particular have been widely quoted as evidence that phospholipid oxidation is the cause of oxidized flavour in milk. The earlier workers examining this problem separated phospholipids from milk fat by precipitation with acetone, a procedure which gives little certainty of the nature and identity of the fraction, and has now been superseded by chromatographic techniques. Because of its significance in this respect, and because of its possible use in the investigation of butter deterioration, a further study has been made of the effect

of oxidation on milk phospholipid iodine values using counter-current distribution and TLC techniques for the isolation of phospholipids. These methods were first developed and applied to the examination of phospholipids in washed-cream serum as it was felt that any changes in iodine value could most readily be demonstrated in this easily oxidized product. The investigation was then extended to include milk, and both neutral and acid salted butter.

#### MATERIALS AND METHODS

Pasteurized non-homogenized market milk was used. Washed cream was prepared by separating the milk at 40 °C, diluting the cream to the original milk volume with water, and reseparatoring. This process was repeated to give 4 washings.

To simplify subsequent extraction and separative procedures, the bulk of the fat was removed from the washed cream as follows. The cream was churned and the buttermilk collected. The butter grains were melted at 45 °C and the butter serum obtained by centrifuging and decanting the fat. The butter serum was then combined with the buttermilk. This mixture of buttermilk and butter serum, representing the total aqueous phase of the washed cream, was used in the investigation and will be referred to as washed-cream serum. Analyses have shown that only a negligible loss of phospholipid is involved in the procedure.

Butter was made by churning 4-kg lots of commercially pasteurized sweet cream, salting to 2%, and working in a small sigma-blade blender. For acid butter, lactic acid was added to the cream before churning to give a pH of 5.0.

Copper was added as a solution of cupric chloride.

#### *Isolation of lipids*

Lipids were isolated by solvent extraction with chloroform and methanol. Milk and washed-cream serum were extracted directly, but with butter, extraction was carried out on the serum obtained by melting the butter at 45 °C and centrifuging it. Chloroform and methanol were used in the proportions stipulated by Bligh & Dyer (1959). The final emulsion in solvent containing chloroform, 20 parts; methanol, 20 parts and water, 18 parts was centrifuged in separating funnels, and the lipids removed with the chloroform layer. It was found an advantage to add 2% NaCl to the milk samples beforehand as this gave a much more compact casein coagulum and facilitated separation of the chloroform layer.

The chloroform extracts were evaporated to dryness at 45 °C in a rotary-film evaporator under reduced pressure and in an atmosphere of nitrogen. In butter serum and washed-cream serum the ratio of neutral lipids to phospholipids is usually 5:1 or less and separation of the 2 groups can be effected conveniently on the thin-layer plate. The extracts from these products, therefore, were taken up immediately in chloroform containing 5% (v/v) methanol, to give a phosphorus concentration of about 1 mg/ml, suitable for application to the thin-layer plate. Usually the volume of the final solution was from 3 to 5 ml, and to assist in the preparation and collection of this small volume of solution the flask used in the evaporator was fitted at its bottom with a small tube of about 2-ml capacity.

In milk the ratio of neutral lipid to phospholipid is about 100:1 and a preliminary

fractionation is necessary before application of the phospholipid fraction to the thin-layer plate. This was done by partitioning the extract between light petroleum (B.P. 40–60 °C) and 87% (v/v) ethanol in the simple counter-current distribution system described by Galanos & Kapoulas (1962). The final ethanolic solution containing the phospholipids was evaporated under nitrogen and reduced pressure at 45 °C until only about 1 ml remained; this was taken up in a mixture of equal parts of chloroform and methanol, and 2% sodium chloride solution was then added to adjust the composition of the fluid to: chloroform, 20 parts; methanol, 20 parts; sodium chloride solution, 18 parts (Bligh & Dyer, 1959). After separation, the chloroform layer was usually transferred to an open 11 × 2.5 cm tube and held overnight, packed in solid CO<sub>2</sub> in a Dewar flask (with butter serum and washed-cream serum, extraction and duplicate analyses could be completed in one day). Next morning the chloroform solution was evaporated to dryness and the crude phospholipids dissolved, as with the serum extracts, in chloroform containing 5% (v/v) methanol to give a phosphorus concentration of about 1 mg/ml.

#### *Thin-layer chromatography*

Thin-layer plates, 20 × 20 cm, were coated with 0.5 mm silica gel G (Merck), air-dried and heated for 1 h at 110 °C 24 h before use. The lipid extract (0.5 ml, containing approximately 0.5 mg phosphorus or 12.5 mg phospholipid) was applied to the plate by spotting. The developing solution was: chloroform, 65 parts; methanol, 25 parts, and water, 4 parts. With this system, the neutral lipids run with the solvent front and the phospholipids divide into 3 well-defined bands corresponding, in descending order of  $R_F$  value, to the main phospholipid classes cephalin, lecithin and sphingomyelin. The bands were made visible by spraying the plate with a solution of bromthymol blue and were scraped off and transferred by suction to filter tubes made by letting 5 × 0.5 cm (I.D.) glass tubing into the bottom of a 12 × 1.5 cm-test tube and placing at the junction a small filter of cellulose powder supported on cotton wool.

Considerable difficulty was experienced in obtaining satisfactory recoveries of phospholipid from the thin-layer plates. The method finally adopted was as follows. The eluting solvent was: chloroform, 10 parts; methanol, 20 parts, and water, 8 parts—as stipulated by Bligh & Dyer (1959) for the first stage of their lipid extraction procedure. This solvent mixture was saturated with CaCl<sub>2</sub>. It was added in 3 successive 5-ml portions to filter tubes containing the silica gel scraped from the plates. Each was gently stirred and passed through the filter under mild pressure. The filtrates were combined, made up to 19 ml with the solvent and further diluted in a separating funnel with 5 ml chloroform and 5 ml of 10% aqueous NaCl. These additions brought the solvent ratio to: chloroform, 20 parts; methanol, 20 parts, and water, 18 parts—as in the second stage of the Bligh & Dyer (1959) extraction, and on shaking a chloroform layer of approximately 10 ml, containing the phospholipid, rapidly settled out. It was made up to 10.5 ml and duplicate 2- and 3-ml portions were taken from it for phosphorus and iodine value determinations, respectively.

*Prevention of oxidation*

To minimize lipid oxidation, all the chloroform used for extraction contained 0.005% butylated hydroxytoluene (Wren & Szczepanowska, 1964). This antioxidant could not be used during development as it contaminated the phospholipid and, since it had an appreciable iodine uptake, it resulted in slightly higher iodine values. No significant differences in iodine values were observed when development was carried out with de-aerated solvents and in an atmosphere of commercial oxygen-free nitrogen, in equipment described by Badings (1964). It was concluded, therefore, that any oxidation during normal development in air could, for the purposes of the investigation, be disregarded.

*Analytical procedures*

To determine phosphorus, extracts were wet-ashed with  $H_2SO_4$  and  $H_2O_2$  and the phosphomolybdate blue colour developed with amidol (Allen, 1940). For very small quantities of phosphorus as in blanks from TLC plates, the method of Bartlett (1959) was used. Phospholipid values were calculated on the assumption that the cephalin band was oleyl-stearyl-phosphatidyl ethanolamine, m.w. 745, the lecithin band oleyl-stearyl-phosphatidyl choline, m.w. 788, and the third band sphingomyelin, with a  $C_{22}$  fatty acid and a m.w. of 787 (Sprecher, 1964).

Iodine values were determined by a micro-modification of the Wijs technique. The halogenating solution was standard Wijs solution, prepared from iodine monochloride according to the official method of the American Oil Chemists' Society (1956). It was diluted with acetic acid, to slightly weaker than  $N/50$ , so that 1 ml reacted with 9.5–10.0 ml  $N/500 Na_2S_2O_3$ . The 3-ml portions of chloroform solution, containing 1–1.5 mg phospholipid, were transferred to 50-ml glass-stoppered flasks, 1 ml halogenating solution added, and the mixture held for 1 h in the dark. After adding 5 ml 2% iodine-free KI solution, the mixture was titrated with freshly prepared  $N/500 Na_2S_2O_3$  solution using starch as an indicator. Flasks containing only 3 ml chloroform were treated similarly as blanks and the iodine values were calculated by difference in the usual way. This method was found to give results which did not differ significantly from those obtained with the macro-method for samples of butterfat, and of soyabean, peanut, maize and safflower oils. It was established by experiment that traces of bromthymol blue indicator accompanying the phospholipid fractions, and variations in the methanol and water contents of the chloroform solution, did not affect the iodine value determination.

Fat peroxide values were determined by the method of Loftus Hills & Thiel (1946). Thiobarbituric acid (TBA) tests were made on milk by the method of Dunkley & Jennings (1951) and on butter serum and washed-cream serum as described by Pont & Birtwistle (1966).

## RESULTS

*Treatments of samples*

Milk, to which 5 ppm. copper had been added, was dispensed in 1-l quantities into clear glass flasks, which were then exposed to direct sunlight for 10 min and held for 7 days at 5 °C. At the end of this time the milk had developed an intense tallowy flavour.

Copper was added, at 0.6 ppm., to the cream used for making the butter. After 3 months' storage at 2 °C, the neutral butter had a strong tallowy flavour. The acid butter was extremely oxidized and had an intense fishy flavour. In both the milk and the butter the degrees of off-flavour and the chemical indices of oxidation were much higher than are encountered even in extremes of commercial practice. The products were clearly inedible.

Washed-cream serum had 2.0 ppm. copper and 50 ppm. ascorbic acid added to it, the latter as sodium ascorbate. The characteristic fishy flavour induced by this treatment developed within a few hours and was intense after 2 days.

#### Recoveries

The amounts of lipid-soluble phosphorus extracted from washed-cream serum dropped by 5-7% following oxidation. There were no significant differences between fresh and oxidized samples in the amounts of phospholipid extracted from milk and butter.

Recoveries of phosphorus from thin-layer plates ranged from 95 to 100%. No differences were observed between fresh and oxidized samples.

Table 1. *Effect of oxidation on the iodine values of phospholipid fractions from milk, butter and washed-cream serum*

|   | Washed-cream serum                    |                  | Salted butter       |                  |                     |                  | Milk              |                  |
|---|---------------------------------------|------------------|---------------------|------------------|---------------------|------------------|-------------------|------------------|
|   | 2.0 ppm. plus<br>50 ppm ascorbic acid |                  | Serum pH 7.0        |                  | Serum pH 5.0        |                  | 5.0 ppm.          |                  |
|   | 2 days<br>at 5 °C                     |                  | 3 months<br>at 2 °C |                  | 3 months<br>at 2 °C |                  | 7 days<br>at 5 °C |                  |
|   | Initial                               | After<br>storage | Initial             | After<br>storage | Initial             | After<br>storage | Initial           | After<br>storage |
| Fat peroxide value                              | —                                     | —                | 0.07                | 0.92             | 0.06                | 7.02             | 0.06              | 1.11             |
| TBA value                                       | 0.08                                  | 0.70             | 0.21                | 0.83             | 0.20                | 15.80            | 0.06              | 0.13             |
| Phospholipid fractions, percentage distribution |                                       |                  |                     |                  |                     |                  |                   |                  |
| Cephalin  | 33.8                                  | 29.1             | 29.1                | 28.8             | 26.0                | 25.2             | 32.6              | 29.1             |
| Lecithin  | 42.0                                  | 42.3             | 43.0                | 43.1             | 41.7                | 41.5             | 33.2              | 37.0             |
| Sphingomyelin                                   | 24.2                                  | 28.6             | 27.9                | 28.1             | 32.3                | 33.3             | 34.2              | 33.9             |
| Iodine values                                   |                                       |                  |                     |                  |                     |                  |                   |                  |
| Cephalin  | 86.6                                  | 73.0             | 82.6                | 83.2             | 70.5                | 71.6             | 82.3              | 80.0             |
| Lecithin  | 54.9                                  | 44.2             | 49.9                | 47.8             | 44.8                | 44.7             | 47.2              | 45.3             |
| Sphingomyelin                                   | 44.2                                  | 41.7             | 43.0                | 44.1             | 36.0                | 36.1             | 42.5              | 39.5             |
| Combined iodine value                           | 61.9                                  | 53.0             | 58.5                | 58.4             | 50.4                | 50.8             | 58.3              | 54.8             |

#### Phospholipid iodine values

The results are summarized in Table 1. The iodine values of the individual phospholipid fractions are the means of duplicate analyses on each of duplicate thin-layer plates. The values have been corrected also for a tendency, apparent from the statistical analysis, for the values to decrease slightly with increasing amounts of phospholipid taken for analysis.

As examination of the data suggested that the variability did not differ significantly from experiment to experiment, the results were combined to give a single

estimate of variability. The overall standard deviation for individual fractions was  $\pm 0.73$  and for the iodine values of the combined fractions it was  $\pm 0.74$ . The least significant differences before and after storage, for the individual fractions, were 2.15 and 2.93 for the 0.05 and 0.01 probability levels, respectively. For the combined values, the least significant differences were 2.33 and 3.32 at the 0.05 and 0.01 probability levels.

In washed-cream serum there were substantial falls in phospholipid iodine values after oxidation, the decreases for the cephalin and lecithin fractions and in the combined values being significant at the 0.01 level. Similar results were obtained in other experiments with washed-cream serum.

In Table 1, the results for the milk sample showed small but significant falls after oxidation, both in the fractional and in the combined iodine values. This result contains an anomaly in that the sphingomyelin, which was the least unsaturated of the 3 fractions, showed a highly significant ( $P = 0.01$ ) lowering of iodine value. This could be an artifact due to overlapping of the lecithin and sphingomyelin fractions which on some plates could not be distinguished with certainty from one another. A discrepancy, probably arising also from irregularities of distribution between the bands, was observed in another sample of milk treated similarly. The iodine values for the 3 fractions were initially: cephalin 77.5, lecithin 48.8 and sphingomyelin 37.9. After storage, the values were 75.1, 43.1 and 42.0, respectively, there being an apparent rise in the value of the sphingomyelin fraction. After storage there was a slight but non-significant fall from 54.7 to 53.4 in the combined values for the sample. It appears that changes in phospholipid iodine values for milk oxidized under these conditions are at the limit of detectability by this technique.

Both in neutral and acid salted butters, only negligible changes in iodine value were observed after storage.

#### *Distribution of phospholipid fractions*

The relative proportions of the phospholipid fractions from each sample are shown in Table 1. Significant falls in phospholipid iodine values during storage were accompanied by alterations in the distribution of phospholipid between the 3 bands on the TLC plates, the amount of material in the more polar fractions increasing at the expense of the less polar. In washed-cream serum a substantial decrease in the apparent cephalin and an increase in the apparent sphingomyelin fractions after oxidation was evident in this and other experiments; in some instances there was a decrease also in the apparent lecithin fraction. These changes in distribution could be due, in part at least, to the formation of lyso-compounds from cephalin and lecithin. Lyso-lecithin would be expected to run with, or near, sphingomyelin on TLC plates. Changes in the distribution of the fractions from milk followed the same pattern though the differences generally were less marked. In butter also, even though the phospholipid iodine values were unaltered, there was a tendency for the less polar fractions to decrease after storage. The differences were slight however.

## DISCUSSION

In the present investigation care was taken to ensure that, as far as possible, results were not affected by failure to recover phospholipid from thin-layer plates or by oxidation during extraction, manipulation and isolation procedures. The only serious discrepancies in this respect were in the experiments with washed-cream serum in which, after oxidation, a fall in extractable phosphorus was observed, and an alteration in distribution due to increased polarity of the phospholipid fractions. Similar changes in the distribution on thin-layer plates of phospholipid from blood cells were observed by Dodge & Phillips (1966) who attributed them to oxidation of their lipid extracts. These changes are consistent with the significant decreases in the iodine values of the 2 more highly unsaturated phospholipid fractions, and taken together demonstrate the marked oxidative changes produced in the membrane phospholipids of washed-cream under the influence of the powerful copper-ascorbic acid catalyst system (Olson & Brown, 1942; Forss, Dunstone & Stark, 1960.)

In both milk and butter, there were no significant differences between fresh and oxidized samples in the amounts of phosphorus extracted and in the recoveries from thin-layer plates, though there was a tendency in the oxidized samples for the more polar fractions to increase and the cephalin fraction to decrease. These observations increase the significance of the main finding that phospholipid iodine values for milk were only slightly affected and those for butter showed no sign of change when oxidative deterioration had reached levels far beyond those experienced in practice. The phospholipid iodine value, therefore, appears to have little or no relevance for the study of phospholipid oxidation in these products.

The large alterations in phospholipid iodine values reported by Swanson & Sommer (1940) and Koops (1957) in oxidized milk and cream remain to be explained. It appears most likely that their results were influenced by difficulties in separating phospholipid from neutral lipid by precipitation with acetone. It is now recognized that with this technique fractionation may not be complete, since it is difficult to separate phospholipid from some of the higher melting-point neutral lipids. A significant proportion of the phospholipids may also remain in solution (Hanahan, 1960).

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