ACTA CHEMICA SCANDINAVICA 10 (1956) 1298—1302

The Separate Determination of Peroxide-containing Phospholipids and Other Lipoperoxides

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Methods for the separate determination of peroxide-containing triglycerides (TGPO) and phospholipids (PLPO) have been studied. Tentative methods based on the following principles are described: 1) Precipitation with acetone, 2) determination of TGPO in the filtrate from adsorption on alumina; calculation of PLPO as the difference between total lipoperoxide and TGPO, 3a) determination of TGPO in the filtrate from adsorption on alumina; determination of PLPO by carrying out the colorimetric thiocyanate reaction while the phospholipids are adsorbed on the column, 3b) as in 3a) but with the *indophenol* method. The separate determination of TGPO and PLPO is possible by any of the methods, but the best results are generally obtained by method 2).

In a previous paper[1] we described methods for the determination of peroxide groups in phospholipids and phospholipid-containing fats. In nature, phospholipids are generally found together with triglycerides and other lipids, and the processes whereby peroxide groups are formed most often take place in mixtures of different kinds of lipids. At the same time the phospholipids often have a fatty acid composition different from that of the triglycerides from the same source. The phospholipids are, as a rule, more unsaturated und must be assumed to have a larger tendency towards the formation of peroxide groups. Under such circumstances the problem arises of methods for the determination of how large a fraction of the peroxide groups is situated on the individual kinds of lipids, *e.g.*, methods for the separation of phospholipid peroxides (PLPO) from other lipoperoxides, mainly triglyceride peroxides (TGPO).

In order to carry out such a fractionation it was attempted to use methods similar to those used for the separation of non-peroxidized phospholipids from other lipids, *i.e.*, especially precipitation with acetone, and adsorption. As a result of our studies tentative methods based on different principles of separation were elaborated. They are described in the following together with the results of a comparison of the methods.

METHODS

Method 1. Precipitation with acetone

The presence of peroxide groups does not greatly alter the solubility properties of the lipids, *i.e.*, when acetone is added to a solution of lipids in petro-

leum ether, PLPO are precipitated together with other phospholipids while TGPO and other triglycerides etc. remain in solution.

The lipid is dissolved in 5 ml petroleum ether, and 20 ml acetone is added. After separation by centrifuging and evaporation of the solvents, the two fractions are dissolved in a small amount of chloroform. The peroxide contents are determined in each fraction, with the exclusion of atmospheric oxygen, by the thiocyanate or the indophenol method as described in our first paper.

Method 2. Adsorption on alumina. Determination of TGPO in the filtrate, and of PLPO by substraction from total lipoperoxide

The presence of peroxide groups increases the adsorption affinity of the lipids, but, in general, phosphoric acid groups make the lipids still more strongly adsorbed. By means of a suitable adsorbent, *e.g.* alumina, TGPO and PLPO can be separated. With petroleum ether as a solvent TGPO (and other peroxides) are adsorbed on the column while non-oxidized triglycerides and other substances like carotene are washed through. Petroleum ether is, however, a less suitable solvent for the separation of TGPO and PLPO. Better results are obtained with chloroform as a solvent.

When, accordingly, a solution of lipoperoxides in chloroform is sent through a column of alumina, a fractioning is obtained in such a way that TGPO can be determined in the filtrate by one of the known methods. The PLPO are very strongly adsorbed and difficult to elute. However, when the total amount of peroxide is also determined, the PLPO can be calculated by substracting the TGPO.

The chromatographic adsorption device consists of a 100×13 mm glass tube provided with a constriction below which the tube is drawn out. A cotton plug is placed above the constriction, and 5 g alumina (a good commercial preparation for chromatographic use or, preferably, the purified product used in method 3) is put into the tube and packed by gently tapping the tube on the bench. The tube is placed on a suction flask by means of a rubber stopper. The flask is connected to the pump, and by means of an interposed T-tube the pressure can be regulated. The suction flask is filled to about half its volume with glass beads for the convenient placement of a centrifuge tube to collect the filtrate.

Twenty ml chloroform is sucked through the column, and the filtrate is discarded. The lipid dissolved in 5 ml chloroform is filtered through the column and washed with 25 ml chloroform (the pharmacopoea grade which contains a small amount of ethanol). The filtrate is collected in centrifuge tubes, an incidentally occurring precipitate removed by centrifuging, and the solvent evaporated to a small volume. The peroxide content is determined by the thiocyanate or the indophenol method; the result corresponds to the content of TGPO. An identical sample of lipid dissolved in 5 ml chloroform is used for the determination of total lipoperoxide. The content of PLPO is calculated as the difference between the two peroxide determinations.

Method 3. Adsorption on alumina. Determination of TGPO in filtrate, and of PLPO by color development on column

It is possible after chromatographic separation of TGPO and PLPO to carry out the determination of PLPO *in situ, i.e.*, by passing a color reagent through the column and measuring the color in the filtrate. Alumina is still a suitable adsorbent, but since commercial preparations are highly impure and would produce very strong colors with the color reagents, it is necessary to carefully purify the alumina in order to remove, as far as possible, all iron and other disturbing substances.

Preparation of purified alumina. Commercial alumina (*e.g.*, Aluminiumoxyd, reinst, Merck; Aluminiumoxyd, standardisiert nach Brockmann, Merck; or Aluminium Oxide for Chromatography, May & Baker) is repeatedly boiled with 5 % hydrochloric acid. The acid is removed by decantation and washing with redistilled water followed by ethanol, and the product is dried at 100 C *in vacuo.*

Method 3a. Thiocyanate procedure

Preparation of the thiocyanate reagent. In a graduated cylinder 50 ml 6 % ammonium thiocyanate in absolute ethanol (or methanol) is mixed with 50 ml chloroform. Oxygen-free nitrogen is bubbled through. After 15 minutes 1 ml ferrous chloride reagent prepared by the method of Loftus Hills and Thiel [2] is added. The reagent is protected from atmospheric oxygen by continuing the passing of nitrogen.

By means of the purified alumina a chromatographic column is prepared as described in method 2. Five ml thiocyanate reagent is filtered slowly through the column followed by absolute ethanol until a volume of about 10 ml is collected in the centrifuge tube. An incidentally occurring precipitate is removed by centrifuging, the volume adjusted to 10 ml, and light absorption at 500 mμ is measured. If the latter is high compared with the absorption of 5 ml thiocyanate reagent +5 ml ethanol, this indicates that the column does still contain traces of iron or other substances that will interfere with the PLPO determination. If that is the case, more portions of thiocyanate reagent followed by ethanol are filtered through until a constant low value is obtained.

With a well-purified alumina a low extinction is obtained in the first filtrate. In that case, the treatment of the column with thiocyanate reagent can be dispensed with, and reagent + ethanol can be used as a blank.

In the case the column has been treated with thiocyanate reagent, thiocyanate is removed by washing with about 20 ml ethanol, and the ethanol washed out with chloroform (about 20 ml). The lipoperoxide mixture containing both TGPO and PLPO dissolved in chloroform is filtered slowly through the column and eluted with about 25 ml chloroform. The filtrates, after incidental precipitates are removed by centrifuging, are evaporated, dissolved in ethanol-chloroform, and the amount of peroxide determined as usual, the result indicating the amount of TGPO present.

Suction is continued until no more chloroform drops from the tube. Then a new centrifuge tube is placed beneath the column and 5 ml thiocyanate reagent is filtered slowly through. When no more reagent is present on the

top of the adsorbent, ethanol is added, and filtration is continued until a total volume of little less than 10 ml is collected. After centrifuging and adjustment of the volume to 10 ml the intensity of the red color is measured, a reagent blank is substracted, and the peroxide content, corresponding to the PLPO content, is calculated.

Method 3b. Indophenol procedure

The method is carried out exactly as in 3a, but TGPO is determined by the indophenol method, and instead of the thiocyanate reagent the following reagent is used for preparation of the column and determination of PLPO.

Preparation of the indophenol reagent. In a graduated cylinder 50 ml 20 % $MgCl_2,6\ H_2O$ in absolute ethanol is mixed with 50 ml chloroform and oxygen-free nitrogen bubbled through. After 15 minutes 1 ml 2 % leuco-dichlorophenolindophenol and 1 ml 0.1 % ferric chloride, both in absolute ethanol, are added with continued bubbling.

RESULTS AND DISCUSSION

Table 1 illustrates results obtained by the methods described above. Mixtures of a triglyceride (lard) and a phospholipid (egg phosphatides repeatedly precipitated with acetone), both having a peroxide content of about 3 mequiv. per kg, were prepared and the recovery of TGPO and PLPO was determined by the different methods.

The results show that the separate determination of TGPO and PLPO may be carried out by using any of the principles mentioned in the introduction of this paper.

Precipitation with acetone has given the least satisfactory separation. Especially, too little PLPO is found in the precipitate when only a small amount of phospholipid is present. This is in accordance with the well-known fact that acetone does not give a complete precipitation of phospholipids; phospholipids, and probably also PLPO, are somewhat soluble in acetone. In other circumstances, by the examination of phospholipids of other origin than eggs, precipitation with acetone may be more advantageous in comparison with adsorption.

From the TGPO-values found by the different adsorption methods it is seen that the phospholipid preparation used has probably contained a small amount of TGPO. Taking this into consideration, it is seen that the recovery of TGPO has been almost quantitative in methods 1, 2, and 3 a. Pre-treatment of the column with indophenol reagent has resulted in loss of TGPO (method 3b).

PLPO values calculated as the difference between total lipoperoxide and TGPO are in quite good agreement with the "calculated values". In the two adsorption methods where PLPO is determined by carrying out the colorimetric reactions on the columns although oxygen could not be completely excluded, too low PLPO values are regularly found. Generally, a recovery of about 60 % PLPO is found. In most cases, therefore, a determination of PLPO as the difference between total lipoperoxide and TGPO will be the best method. In certain circumstances, the determination of PLPO by the colori-

Table 1. Comparison of different methods for the separate determination of peroxide-containing triglycerides (TGPO) and phospholipids (PLPO).

Mixture	Separation method	Thiocyanate method				Indophenol method	
		"Calculated value"	Method 1	Method 2	Method 3a	"Calculated value"	Method 3b
A	TGPO	0.60	0.61	0.65	0.58	0.36	0.165
	PLPO	0	0.00	(0)	0.00	0	0.02
	Total	0.60	(0.61)	0.60	(0.58)	0.36	(0.185)
B	TGPO	0.54	0.61	0.58	0.56	0.32	0.19
	PLPO	0.06	0.04	(0.02)	0.08	0.04	0.065
	Total	0.60	(0.65)	0.60	(0.64)	0.36	(0.255)
C	TGPO	0.30	0.47	0.36	0.36	0.18	0.06
	PLPO	0.31	0.22	(0.24)	0.24	0.19	0.16
	Total	0.61	(0.69)	0.60	(0.60)	0.37	(0.22)
D	TGPO	0.06	0.33	0.10	0.10	0.04	0.025
	PLPO	0.56	0.33	(0.53)	0.40	0.34	0.16
	Total	0.62	(0.66)	0.63	(0.50)	0.38	(0.185)
E	TGPO	0	0.38	0.05	0.05	0	0.015
	PLPO	0.62	0.28	(0.57)	0.44	0.38	0.265
	Total	0.62	(0.66)	0.62	(0.49)	0.38	0.28

Method 1: Precipitation of PLPO with acetone, TGPO remaining in solution.
,, 2: Adsorption of PLPO on alumina, determination of TGPO in filtrate, calculation of PLPO as difference between total lipoperoxide and TGPO.
,, 3a: Adsorption of PLPO on alumina, determination of TGPO in filtrate, determination of PLPO by carrying out the colorimetric reaction while the phospholipids are adsorbed on the column.
,, 3b: As method 3a, but with the indophenol method instead of the thiocyanate method.

The figures give the extinctions read in the photometer after blanks have been subtracted. The mixtures B, C and D were prepared from the TGPO-solution A, and the PLPO-solution E in the proportions 9:1, 1:1, and 1:9, respectively. "Calculated values" are values calculated from A and E. Values between parentheses (total lipoperoxide in methods 1, 3a, and 3b, and PLPO in method 2) are calculated from the other, directly observed, values presented in the table.

metric reaction on the adsorption column may be preferable, *e.g.*, when the amount of PLPO is very small compared with TGPO. Certain products may contain phospholipids that are not completely adsorbed on alumina. In such cases other adsorbents or the precipitation with acetone may be an advantage.

Acknowledgement. This work was supported by grants from *P. Carl Petersens Fond* and *Laurits Andersen's Fond.*

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Received June 23, 1956.