paper on methodology

A simple technique for the analysis of positional distribution of fatty acids on di- and triacylglycerols using lipase and phospholipase A_2

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Abstract A simple technique is described for the analysis of positional distribution of fatty acids on di- and triacylglycerols using lipase and phospholipase A_2 that de-esterify fatty acids from specific *sn* positions. The technique makes use of the fact that methanolic-NaOH methylates only fatty acids esterified to glycerol, while methanolic-HCl methylates both free and esterified fatty acids. After lipase action it is possible to determine the fatty acid released by lipase activity by comparing the fatty acid contents of the two methylation reactions. A computer program has been written to calculate enzyme activity and positional distribution from the results. The new technique is easier to use as it eliminates thin-layer chromatography used in the standard technique and can be performed on smaller samples using less lipase.—**Williams, J. P., M. U. Khan, and D. Wong.** A simple technique for the analysis of positional distribution of fatty acids on di- and triacylglycerols using lipase and phospholipase A_2. *J. Lipid Res.* 1995. **36:** 1407–1412.

Supplementary key words plant galactolipid • sulfolipid • phospholipid

The determination of positional distribution of fatty acids on glycolipids (GL) and triacylglycerols (TAG) from plants has been performed for some time using pancreatic lipase (1) and lipase from *Rhizopus arrhizus delemar* (2) which specifically remove esterified fatty acids from the *sn*-1 and *sn*-3 positions of the glycerol backbone. Analyses of phospholipids (PL) in bacteria, plants, and animals have used phospholipase A_2 (3) which specifically removes fatty acids from the *sn*-2 position of PL but is less effective on GL or TAG.

The techniques used for positional distribution analyses generally involve incubation of the purified lipid with an aqueous suspension of the enzyme, separation of the products (free fatty acid and lysolipid) by column and/or thin-layer chromatography (TLC), and analysis of fatty acid methylesters (FAME) in each fraction by gas–liquid chromatography (GLC) (1, 2, 3). The method is time consuming, and it is often impossible to quantify the extent of the reaction. The results can be affected by contamination by other lipases and/or nonspecificity of the lipase which may result in fatty acids being removed from the 'wrong' *sn* position of the glycerol. Fatty acid migration may also occur between *sn* positions during lipase-catalyzed reactions (4). In addition, the variation in lipase activity and the high quantity of lipids needed for TLC require considerable preliminary preparation and analysis of lipids.

To simplify these analyses we have developed a simple, rapid technique that determines the total enzyme activity, eliminates the need for TLC, and can be performed on relatively small samples using small quantities of the lipases. The technique uses the fact that methanolic-NaOH methylates only fatty acids that are esterified to the glycerol backbone and converts free fatty acid to aqueously soluble sodium salts; methanolic-HCl methylates both esterified and free fatty acids (see review by Liu, ref. 5). After lipase action the difference in fatty acid composition of the two methylation reactions is a quantitative measure of the fatty acid released by the enzyme. From these determinations it is possible to calculate the positional distribution of each fatty acid. As these calculations can be time-consuming, we have developed a computer program to compute the % enzyme reaction and the % distribution (mol% or weight %) of the fatty acids at each *sn* position.

We present data using this technique on standard triacylglycerol (TAG) and plant diacylglycerols (monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulphoquinovosyldiacylglycerol (SQDG))

Abbreviations: DGDG, digalactosyldiacylglycerol; FAME, fatty acid methylester; GL, glycolipid; MGDG, monogalactosyldiacylglycerol; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; TLC, thin-layer chromatography; 16:0, palmitic acid; 16:3, *cis*-7,10,13-hexadecatrienoic acid; 18:0, stearic acid; 18:1, octadecenoic acid (*cis*-9-oleic acid and *cis*-11-vaccenic acid); 18:2, *cis*-9,12-linoleic acid; 18:3, *cis*-9,12,15-linolenic acid.

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with lipase from *Rhizopus arrhizus* and phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) with phospholipase A_2 from bee venom. This technique can be used on a wide range of acylglycerols from plants, bacteria, and animals.

MATERIALS AND METHODS

Sources of lipids and enzymes

Standard TAG (*rac*-glyceryl-1-palmitate-2-oleate-3-stearate (POS)) was obtained from Matreya (Pleasant Gap, PA. cat. no. 1142). Lipids were extracted from *Brassica napus* leaves and separated into classes by TLC as previously described (6, 7). Lipase (EC 3.1.1.3) from *Rhizopus arrhizus* and phospholipase A_2 (EC 3.1.1.4) from bee venom were obtained from Boehringer Mannheim, Canada.

Lipase and phospholipase A_2 reactions

Total fatty acid content and distribution were determined in each lipid sample, before enzyme action, by GLC of FAME after methylation in 0.2 N HCl in dry methanol using methyl pentadecanoate as internal standard (8). In both reactions, approximately 2–3 μmol of purified plant lipid was dissolved in 0.5 ml chloroform–methanol 2:1 (v/v) containing 2–3 mg Triton X-100, and dried under nitrogen in screw-capped glass tubes (100 × 15 mm, Pyrex, Corning, NY).

Lipase

The lipase reaction method of Fischer, Heinz, and Zeus (2), with minor modifications, was used with the glycolipids (MGDG, DGDG and SQDG) and TAG. Tris-HCl buffer (40 mM, pH 7.2) (0.5 ml) containing 50 mM sodium borate (to reduce positional migration of fatty acids) was added to the dried lipid sample and the mixture was sonicated for 10 min. Twenty μl of lipase (100–120 units) was added to the sonicated mixture and incubated at 22°C for up to 30 min with continuous shaking.

Phospholipase A_2

Phospholipase A_2 reactions were performed on phospholipids according to the procedure of Griffiths, Stobart, and Stymne (9), with minor modifications. Purified plant phospholipids (1–2 μmol) were dissolved in 0.5 ml diethyl ether, 0.5 ml borate buffer (100 mM, pH 8.9) was added and the mixture was sonicated for 15 min. To the mixture was added 40–100 units of phospholipase A_2 and the reaction mixture was then shaken continuously for 2 h at 37°C. The diethyl ether was then evaporated under N_2 until the solution cleared.

Both lipase reactions were stopped by the addition of 0.5 ml 0.1 N acetic acid and all of the lipids were extracted three times with 2.0 ml chloroform–methanol 2:1 (v/v). The combined extracts were dried under N_2, methyl pentadecanoate was added as an internal standard, and the lipid was redissolved in 0.5 ml chloroform–methanol 2:1 (v/v).

Methylation reactions

The following methylation reactions were then carried out separately on two aliquots of the lipid solution that were dried under N_2 before the addition of: *1)* 1.0 ml of 0.1 N NaOH in dry methanol for 30 min at room temperature, and *2)* 1.0 ml of 0.2 N HCl in dry methanol heated in a microwave oven for 2 min (8).

The FAME from each methylation were extracted into hexane after the addition of 0.2 ml water to the reaction mixture.

The hexane extracts of the NaOH reaction were washed once with water to remove any traces of NaOH before drying under N_2.

The extracts were dried and the FAME were redissolved in hexane and analyzed by GLC using a Hewlett-Packard model 5890 gas–liquid chromatograph with a 30 m DB-225 capillary column programmed from 160°C to 210°C at 3°C min^{-1}. The FAME were estimated quantitatively from the pentadecanoate internal standard.

Calculation of data

The enzyme activity can be determined from the amount of fatty acid released by the lipase. This is assumed to be complete (enzyme reaction, $R = 1$) when 50% of the fatty acid is removed from diacylglycerols, and 66.6% (lipase) or 33.3% (phospholipase A_2) from TAG.

For diacylglycerols:

$$R = 2\times(1-(T_{NaOH}/T_{HCl})) \qquad Eq.\ 1)$$

where, T_{NaOH} and T_{HCl} = total FAME determined after methylation by NaOH and HCl, respectively.

For TAG, with lipase, $R = 1.5\times(1-(T_{NaOH}/T_{HCl}))$, and with phospholipase A_2, $R = 3\times(1-(T_{NaOH}/T_{HCl}))$.

Determination of positional distribution

The positional distribution of fatty acids can be determined either from the free fatty acid released by the enzyme, if the enzyme reaction is incomplete ($R < 1$, method A), or from the lysolipid remaining after enzyme treatment, if the reaction is complete ($R > 1$, method B).

Method A

If $R < 1$, the quantity of a fatty acid released is used to determine the amount of that fatty acid at the *sn*-1 position by correcting for the enzyme activity.

For diacylglycerols de-esterified by lipase:

$$F_1^x = (F_{HCl}^x - F_{NaOH}^x)/R \qquad Eq.\ 2)$$

where, F_1^x = quantity of the fatty acid, *x*, at the *sn*-1 posi-

tion, F^x_{HCl} and F^x_{NaOH} = the quantity of that fatty acid determined from each methylation reaction, respectively.

The quantity of the fatty acid on the *sn*-2 position (F^x_2) is determined by subtraction of F^x_1 from the total of that fatty acid (F^x_{HCl}):

$$F^x_2 = F^x_{HCl} - F^x_1 \qquad Eq.\ 3)$$

The quantities of all fatty acids calculated in this way can then be used to determine the % distribution (M^x_1 and M^x_2) of fatty acids on the *sn*-1 and *sn*-2 positions.

For diacylglycerols using phospholipase A_2 the *sn* positions are reversed in equations 2 and 3.

As lipase is not specific for the *sn*-1 or *sn*-3 positions in TAG, the % distributions for the *sn*-1 and *sn*-3 positions ($M^x_{1/3}$) are averages of the two. Otherwise the calculations are similar for both enzymes and both types of substrates using the appropriate value for *R*.

Method B

If $R > 1$, the method of determining the composition of each *sn* position is to use the % distribution in the fatty acid in the total fatty acid (HCl methylation) (M^x_{HCl}) and the distribution of the fatty acid in the lysolipid (NaOH methylation) (M^x_{NaOH}).

For diacylglycerols de-esterified by lipase:

$$M^x_1 = (2 \times M^x_{HCl}) - M^x_{NaOH} \qquad Eq.\ 4)$$

and $M^x_2 = M^x_{NaOH}$

For diacylglycerols using phospholipase A_2 the *sn* positions are reversed in equation 4.

For TAG and lipase:

$$M^x_{1/3} = (3 \times M^x_{HCl}) - (2 \times M^x_{NaOH}) \qquad Eq.\ 4a)$$

where $M^x_{1/3}$ = the average % distribution of the fatty acid, *x*, on the *sn*-1 and *sn*-3 positions.

Method B is more accurate because it depends less on the accuracy of quantitative determinations using the two reagents and more on the % distribution of fatty acids in each reaction. This method can only be used if the enzyme reaction is complete.

Computer program

As the calculations are time-consuming a computer program was developed to automatically calculate enzyme activity and determine % distribution at each *sn* position. The program accepts the total and the individual fatty acid contents of the HCl and NaOH methylation reactions and gives the % enzyme activity, the % distribution of fatty acids using each methylation reaction, and the % distribution of fatty acids on each *sn* position, calculated using both methods.

Correction or enhancement of data

The program is capable of enhancing the results by correcting for contaminating or nonspecific lipase activity in reactions that are not complete. The program accepts manually entered correction factors (as a % of total enzyme activity) and adjusts the fatty acid composition determination on each carbon, respectively.

For example, with diacylglycerols using lipase:

$$F^x_{2e} = (F^x_2 \times (100 + e)/100) - (F^x_1 \times e/100) \qquad Eq.\ 5)$$

$$F^x_{1e} = F_{HCl} - F^x_{2e} \qquad Eq.\ 6)$$

where, F^x_{2e} and F^x_{1e} = the corrected or enhanced fatty acid level on the *sn*-2 and *sn*-1 positions, respectively, and e = the correction or enhancement factor as a percentage of total enzyme activity.

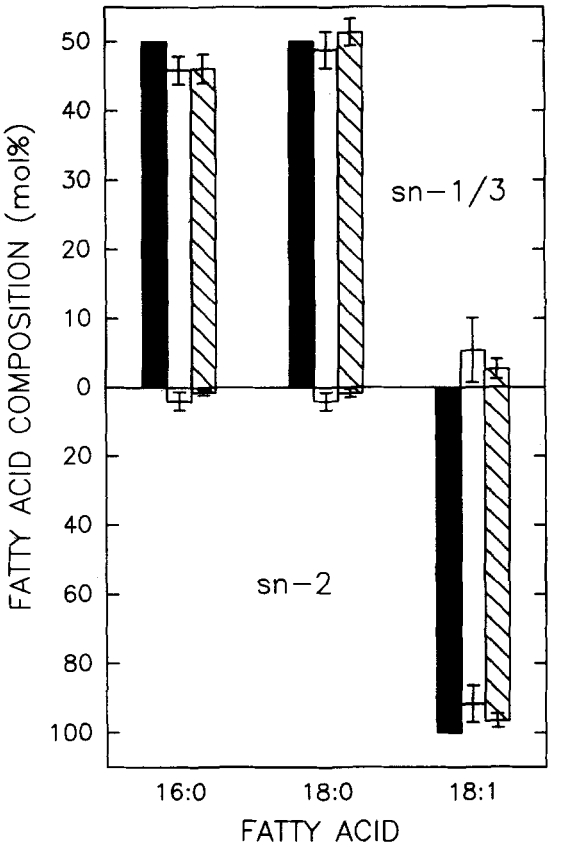

Fig. 1. Comparative positional analysis data from standard TAG(POS) determined using lipase and the standard TLC separation method (open bars) or our new method (shaded bars). In the standard technique *sn*-1 position was determined from the free fatty acid fraction of the TLC plate and the *sn*-2 position from the lysolipid; in our technique the positional analysis was determined using method B. The theoretical distribution is represented by solid bars. The number of analyses used in the standard technique was 4: in the new method, 17. The standard deviations are shown as vertical error bars.

These equations partially correct for contaminating phospholipase A_2 activity but it is necessary to repeat the calculation several times using the newly calculated values of F^x_{2e} and F^x_{1e} and adjusting the correction factor, which was determined empirically from theoretical data sets, each time.

$$e = e_o^2/50 \qquad Eq.\ 7)$$

where e_o is the original value of e. This successive use of correction factors to correct the distribution results in better than 99.9% accuracy after 3–4 cycles.

For TAG with lipase: $F^x_{2e} = (F^x_2 \times (100+e)/100) - (F^x_1 \times e/200)$, and with phospholipase A_2: $F^x_{2e} = (F^x_2 \times (100+e)/100 - (F^x_1 \times e/50)$.

The correction of data is valuable when the reaction is not complete and there is significant fatty acid removed from the 'wrong' position due to contamination from other lipases or nonspecificity of the lipase.

RESULTS

To test the procedure we used standard TAG (POS) and plant MGDG, DGDG, SQDG, PC, PE, and PG extracted from leaves of *Brassica napus*. We used the standard techniques of Fischer et al. (2) with lipase and Griffiths et al. (9) with phospholipase A_2, separating the products of lipase action by TLC for comparison with our new procedure.

Analysis of standard TAG

Figure 1 contains the results of determination of positional distribution on a standard TAG (POS) using lipase. The positional distribution using the standard technique of TLC and the new method are compared with the theoretical distribution. The data show that the new method is closer to the theoretical positional analysis. In the new method the low levels of these fatty acids may be due to unreacted lipid, fatty acid migration, or to the fact that the standard TAG is not 100% pure in terms of positional distribution. Higher levels of 16:0 and 18:0 at the *sn*-1/3 position in the standard method may also be due to streaking or contamination of the sample on the TLC plate.

Analysis of plant MGDG, DGDG, and SQDG using lipase

Figure 2 contains data derived from lipase reactions on MGDG, DGDG, and SQDG from leaves of *Brassica napus*, a 16:3 plant. It has been previously shown that in 16:3 plants, MGDG and DGDG contain predominantly 18:3 esterified to the *sn*-1 position and both 16:3 and 18:3 es-

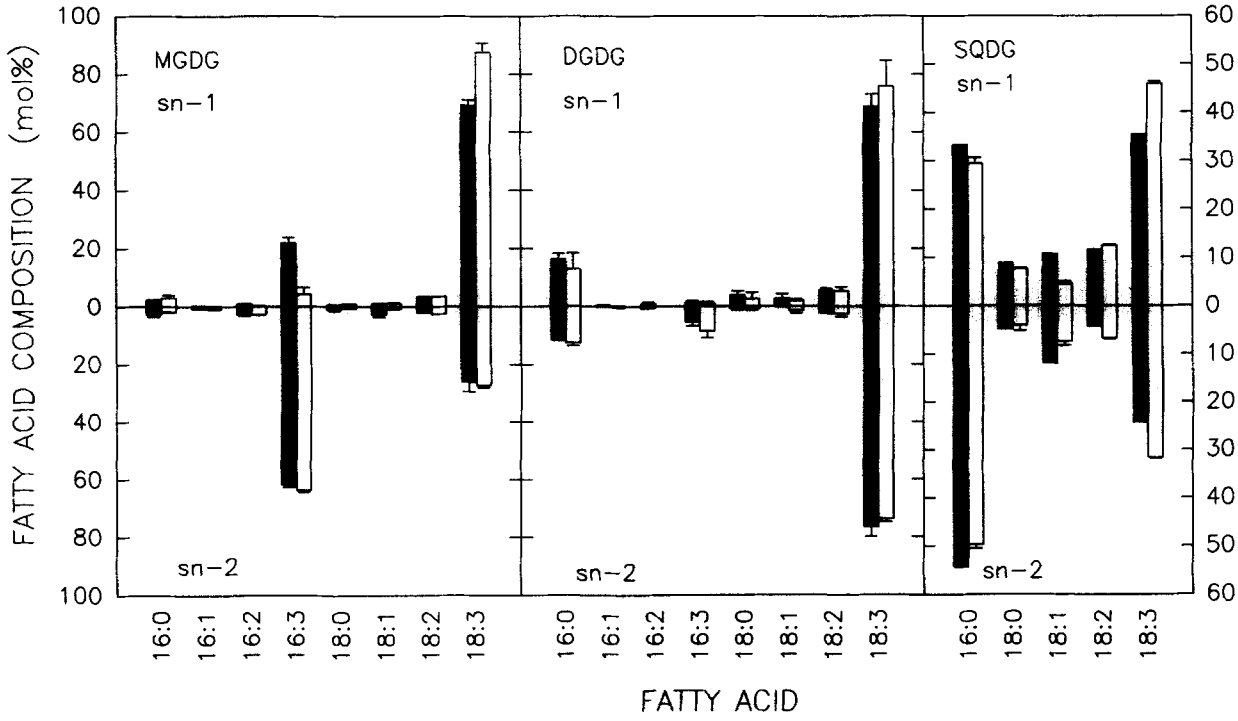

Fig. 2. Comparative positional distribution analysis of MGDG, DGDG, and SQDG from *Brassica napus* using lipase and the standard TLC separation method (solid bars) or our new method (open bars). In the standard technique the *sn*-1 position was determined from the free fatty acid fraction of the TLC plate and the *sn*-2 position from the lysolipid; in the new method the positional analysis was determined using method B. The number of analyses for *a)* the standard TLC method were MGDG, 2; DGDG, 3; and SQDG, 1, and *b)* the new method were MGDG, 4; DGDG, 2; SQDG, 3. The standard deviations are shown as vertical error bars.

terified to the *sn*-2 position (10, 11); SQDG and DGDG contain 16:0 and C18 fatty acids at both *sn*-1 and -2 positions in 16:3 plants (12). Data obtained by the standard technique confirm this distribution and are similar to values obtained using the new method.

In the standard method, the quantities of free fatty acid (FFA) and lysolipid recovered from the TLC plate were lower than those obtained using the new technique (data not shown). This loss is probably due to streaking on the TLC plate, oxidation, and/or incomplete recovery. These problems are magnified when using small quantities of lipid. Using the TLC separation the levels of unsaturated fatty acid in both the FFA and lysolipid fractions were often lower than in our technique. We believe this may be due to oxidation of unsaturated fatty acid during TLC separation. The MGDG free fatty acid fraction (*sn*-1 position) contains unusually high levels of 16:3 probably from the *sn*-2 position as a result of contaminating lipase A_2 activity. This emphasizes the greater accuracy in determinations using the fatty acid remaining as lysolipid in lipase reactions that are complete.

Analysis of PC, PE, and PG phospholipase A_2

Figure 3 contains data obtained using the standard technique of Griffiths et al. (9) using phospholipase A_2 on PC, PE, and PG from *B. napus*. As found with lipase and the glycolipids, the results from the two methods are consistent with some apparent loss of unsaturated fatty acids after TLC, particularly in PG.

Figures 1–3 contain results obtained from lipase and phospholipase reactions that were complete. In these cases the positional composition was determined from the lysolipid remaining after treatment. Nonspecificity or contamination of the lipase enzyme is not important as only fatty acid remaining after the reaction is determined in the NaOH methylation.

DISCUSSION

To confirm that NaOH in dry methanol does not methylate free fatty acids, a mixture of free fatty acids was reacted with 0.1 N NaOH in methanol as described in Materials and Methods. No detectable FAME corresponding to the standards used were detected indicating that less than 0.1% of the fatty acid was methylated (data not shown). Our data from a previous publication indicate that 0.2 N HCl in dry methanol heated in a microwave oven completely methylates all fatty acids within 2 min (8).

The calculation of the data in our method is made in two different ways. If the enzyme activity is incomplete then the calculations are made from the amount of free fatty acid released by the enzyme. This fatty acid is

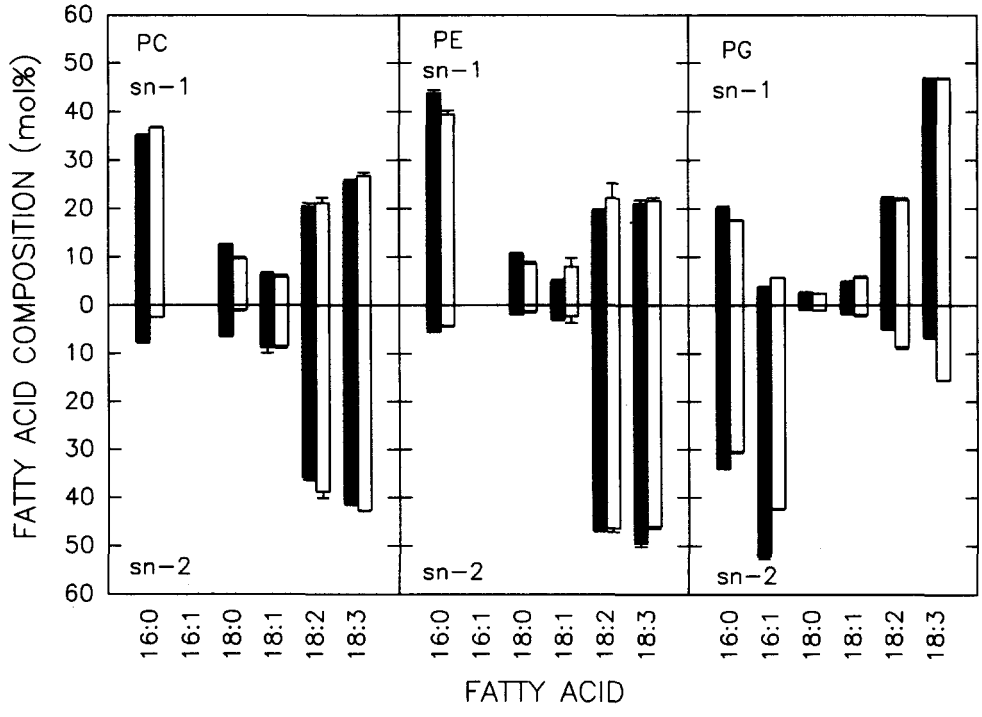

Fig. 3. Comparative positional distribution analysis of PC, PE, and PG from *Brassica napus* using phospholipase A_2. The standard technique is represented by solid bars; *sn*-1 was determined from the lysolipid fraction on the TLC plate and the *sn*-2 position from the free fatty acid fraction; in the new method the positional analysis was determined using method B. The number of analyses for both methods and all lipids was 3. The standard deviations are shown as vertical error bars.

released by the primary lipase activity and also by the alternative lipase activity (either contamination or nonspecificity). In both the standard and the new techniques it is not possible to differentiate between the two enzyme activities. However, using the correction or enhancement routines it is possible to calculate or compensate for this activity with the use of known standards.

If the major enzyme reaction is complete then the determination often appears to exceed 100% as the alternative lipase activity removes some fatty acid from the second *sn* position. In this case it is necessary to determine the positional distribution from the fatty acid remaining as lysolipid. This method is potentially more accurate because when the primary activity is complete the composition of the lysolipid remains the same regardless of the level of alternative lipase activity. In this case the correction or enhancement program is not used.

Although the comparative data of the two methods in Figs. 1-3 are similar, our method takes significantly less time to perform, can be performed using very small quantities of lipid, and gives an immediate determination of the level of activity of the enzyme. We believe our data are inherently more reliable as the technique requires fewer operations and uses only techniques that are practiced routinely in lipid laboratories. This permits many more determinations using less enzyme preparation.

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REFERENCES

1. Noda, M., and N. Fujiwara. 1967. Positional distribution of fatty acids in galactolipids of *Artemisia princeps* leaves. *Biochim. Biophys. Acta.* **137:** 199-201.
2. Fischer, W., E. Heinz, and M. Zeus. 1973. The suitability of lipase from *Rhizopus arrhizus delemar* for the analysis of fatty acid distribution in dihexosyl diglycerides, phospholipids and plant sulfolipids. *Hoppe-Seyler's Z. Physiol. Chem.* **354:** 1115-1123.
3. De Haas, G. H., F. J. M. Daemen, and L. L. M. Van Deenen. 1962. The site of action of phosphatide acyl-hydrolase (phospholipase A) on mixed-acid phosphatides containing a poly-unsaturated fatty acid. *Biochim. Biophys. Acta.* **65:** 260-270.
4. Mattson, F. H., and R. A. Volpenhein. 1961. The use of pancreatic lipase for determining the distribution of fatty acids in partial and complete glycerides. *J. Lipid Res.* **2:** 58-62.
5. Liu, K-S. 1994. Preparation of metyl esters for gas-chromatographic analysis of lipids in biological materials. *J. Am. Oil Chem. Soc.* **71:** 1179-1187.
6. Williams, J. P., and P. A. Merrilees. 1970. The removal of water and nonlipid contaminants from lipid extracts. *Lipids.* **5:** 367-370.
7. Khan, M., and J. P. Williams. 1977. An improved TLC method for the separation of major phospholipids and glycolipids from plant extracts and phosphatidylglycerol and bis-(monoacylglyceryl)phosphate from animal lipid extracts. *J. Chromatogr.* **140:** 179-185.
8. Khan, M., and J. P. Williams. 1993. Microwave-mediated methanolysis of lipids and activation of thin-layer chromatographic plates. *Lipids.* **28:** 953-955.
9. Griffiths, G., A. K. Stobart, and S. Stymne. 1985. The acylation of *sn*-glycerol 3-phosphate and the metabolism of phosphatidate in microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius L.*) seed. *Biochem. J.* **230:** 379-388.
10. Safford, R., and B. W. Nichols. 1970. Positional distribution of fatty acids in monogalactosyl diglyceride fractions from leaves and algae. *Biochim. Biophys. Acta.* **210:** 57-64.
11. Auling, G., E. Heinz, and A. P. Tulloch. 1971. Combination and positional distribution of fatty acids in plant galactolipids. *Hoppe-Seyler's Z. Physiol. Chem.* **352:** 905-912.
12. Bishop, D. G., S. A. Sparace, and J. B. Mudd. 1985. Biosynthesis of sulfoquinovosyldiacylglycerol in higher plants: the origin of the diacylglycerol moiety. *Arch. Biochem. Biophys.* **240:** 851-858.