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Rapid and sensitive method for the quantitation of non-polar lipids by high-performance thin-layer chromatography and fluorodensitometry

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ABSTRACT

Non-polar lipids were separated by high-performance thin-layer chromatography on silica gel plates and detected by use of a new reagent that induced fluorescence in the separated components. Developed thin-layer plates are dipped into a solution of sulfuric acid–ethanol–hexane (1:35:64, v/v), heated and the lipid classes are quantified by fluorescence densitometry. This technique allowed detection of certain standard lipids at the 5-ng level, is well suited for the rapid and efficient analysis of large numbers of samples and offers distinct advantages over other *in situ* fluorescence inducing methods. The method was successfully applied to the analysis of the non-polar lipids that occur in enzymatically hydrolyzed beef tallow.

INTRODUCTION

Thin-layer chromatography (TLC) has been used extensively for the analysis of lipids [1] and more recently, high-performance TLC (HPTLC) has been supplanting tradional TLC methodology. While extremely useful for lipid separations, broad applications of thin-layer techniques have been limited due to a lack of reliable quantitation of separated components [2]. This limitation was addressed by the development of quantitative methods such as absorbance densitometry of charred [3,4] or reagent-stained [5,6] thin layers, which did allow quantitation, but at the expense of many of the intrinsic advantages of TLC such as speed, sensitivity, minimal sample and plate pretreatment and reproducibility. In addition, absorbance/reflectance densitometry has been shown to yield non-linear calibration curves which have the potential to introduce large errors into a quantitative method [7].

Thin-layer chromatography combined with fluorodensitometric detection obviates many of the difficulties associated with absorbance TLC quantitation and has been used in a number of variations to analyze a broad range of components [8]. A vapor-phase fluorimetric procedure for the *in situ* derivatization of organic compounds on thin-layer plates was developed by Segura and Gotto [9] and this method has been applied to the analysis of lipids [10]. In other work, Schmitz and Assman [11]

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made use of a reagent mixture that induced fluorescence of lipids after immersion and subsequent heat treatment of developed TLC plates. While the advantages of fluorescence detection, such as superior reproducibility, lower detection limits and linear response of calibration standards are obtained with these techniques, they are somewhat complex and time consuming to perform and not conducive for use in routine analysis or large scale studies.

In this report, we present a method for the analysis of neutral lipids by HPTLC and fluorodensitometry. We developed this method for the analysis of product streams from immobilized lipase reactors designed for the hydrolysis of beef tallow [12]. Tallow is composed primarily of triacylglycerides, minor amounts of cholesterol and partial glycerides and typical lipase reactor products are mixtures of unreacted triacylglycerides, free fatty acids, diacylglycerides and monoacylglycerides. We required a simple and accurate method of analysis that could quantitate all of the above components found in partially hydrolyzed tallow that would be amenable to the large number of samples required for engineering studies of this process system. The HPTLC method described here appears to satisfy these requirements and would appear to be applicable in the quantitation of many other types of analytes. We have also found, in comparative studies, that this method is more sensitive than those previously reported for the induced fluorescence of lipids [8,11]. Because of this sensitivity the method would appear to be especially suitable for use in studies where only minute amounts of lipid are available for analysis.

EXPERIMENTAL[a]

HPTLC plates

Precoated HPTLC plates, 10 × 10 cm, silica gel 60 (Merck, Darmstadt, Germany), without fluorescent indicator, were used for all experiments.

Standards

For method development, commerical lipid-class standards, all with the same fatty acid substitution were used. These were: triolein, oleic acid, 1,2-diolein, 1,3-diolein and monoolein (Nu Chek Prep, Elysian, MN, USA).

Reagents

Hexane and methanol used in this work were HPLC grade (Burdick and Jackson, Muskegon, MI, USA), diethyl ether was analytical-reagent grade (Mallinckrodt, Paris, KT, USA), ethyl alcohol was USP dehydrated 200 proof (Pharmco, Bayonne, NJ, USA) and sulfuric acid was ACS reagent grade (J. T. Baker, Phillipsburg, NJ, USA). The tallow used in this study was edible-grade beef tallow (Ed Miniat, Chicago, IL, USA).

Fluorescence detection

Fluorescence detection of HPTLC-separated components was obtained with a TLC scanner II (Camag, Muttenz, Switzerland) equipped with a mercury lamp.

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[a] Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Plates were scanned in the fluorescence/reflectance mode at a rate of 0.5 cm/min using an excitation wavelength of 366 nm with a 400-nm cutoff filter in place before the photomultiplier detector. The scanning beam was set at a 2-mm thickness and a 6-mm beam width. Sensitivity of the instrument was set manually at 200 arbitrary units (a.u.) and a span of 25 a.u. For automatic scanning of HPTLC lanes the instrument was programmed to scan at 1.0-cm intervals. Fluorescence output was monitored with an SP 4290 integrator (Spectra-Physics, San Jose, CA, USA) operated in the peak-height mode for the integration of detected peaks.

Plate cleaning

HPTLC plates were placed into a 10 × 10 cm TLC development tank containing approximately 400 ml of methanol and immersed for 5 min. Plates were removed, drained of excess solvent and dried in an 80–85°C oven for 15 min and stored until needed in a closed dessicator box that contained no dessicant or other chemicals [13]. Two plates were immersed into the methanol at the same time and the methanol was replenished after cleaning 20–24 plates.

Separation of lipids

Initial studies were conducted with mixtures of commercial lipid standards, dissolved in chloroform and applied to precleaned HPTLC plates with a 1.0-μl syringe ((Hamilton, Reno, NV, USA). Aliquots of 1.0 μl of lipid solutions, in the concentration range of 1–100 ng/component, were spotted to test the reproducibility and sensitivity of the method. Spots were applied at a distance of 1.5 cm from the bottom edge of the plate and a line was etched across the plate at a distance of 9.0 cm from the plate bottom. Spots were placed exactly 1.0 cm apart with a maximum of eight spots per 10 × 10 cm plate in order to conform to the TLC scanner lane interval setting for autoscanning.

The spotted HPTLC plates were placed in a developing tank (15 × 30 cm), that was preequillibrated for 10–15 min with hexane–diethyl ether–formic acid (80:20:2, v/v) and solvent was allowed to migrate to a distance of 3.0 cm from the bottom edge of the plate. Plates then were removed, air-dried (10 min) and returned to the development tank, containing the same solvent mixture, until the solvent migrated to the 9.0-cm line. Developed plates were allowed to air-dry (10 min) before the fluorescence induction treatment.

Induction of fluorescence

Fluorescence of the separated components was effected by immersion of the air-dried, developed plate into a mixture of sulfuric acid–absolute ethanol–hexane (1:35:64, v/v). The plate was quickly dipped into this mixture (1–2 s), removed and reimmersed a second time (1–2 s). After this treatment excess solvent was allowed to evaporate from the plate surface. The treated plates were then heated in a forced-air-type oven at 110°C for exactly 45 min.

Tallow standards

Lipids from partially hydrolyzed tallow were purified by preparative TLC on 20 × 20 cm, 500 μm, silica gel G plates (Analtech, Newark, DE, USA). The solvent system for initial class separation of the lipids was hexane–diethyl ether–acetic acid

(70:30:1, v/v) [14]. Diacylglyceride fractions from this separation were purified using benzene–ethyl acetate–diethyl ether–acetic acid (80:10:10:0.2, v/v) [15]. Lipid-class standards applied to the preparative plates were used to identify the separated components. Purified components were combined to form mixed standards, of known concentrations, that correspondend with the lipid-class composition of partially hydrolyzed tallow obtained from the lipase-reactor treatment of beef tallow.

Other methods

Two methods for the induced fluorescence detection of lipids were compared with the method presented in this report. Vapor-phase induction of fluorescence with ammonium bicarbonate was carried out as described by Segura and Gotto [9] in a modified heating chamber developed by Maxwell [16]. Fluorescence was also induced using a reagent comprised of manganese chloride–methanol–sulfuric acid–water described by Schmitz and Assman [11].

Estimates of the free fatty acid composition of partially hydrolyzed tallow as determined using the new HPTLC method were compared with the results obtained with a pH stat titration method described by Taylor [17].

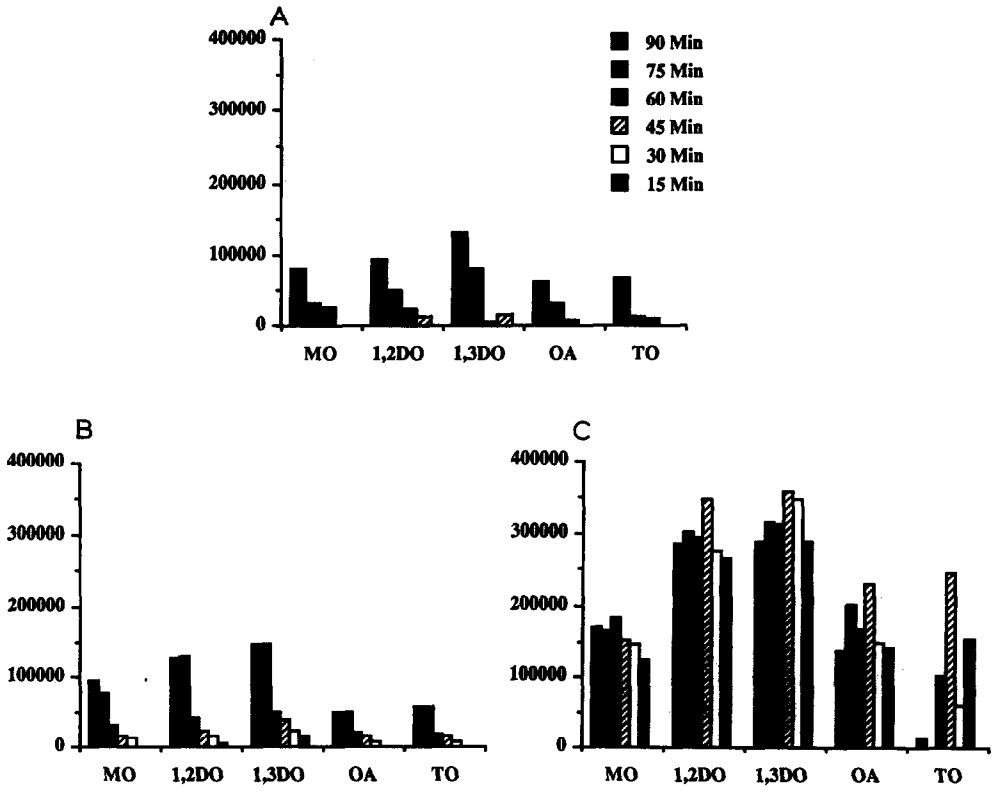

Fig. 1. Effect of temperature and time on the fluorescent response of lipid standards. Triolein (TO) 0.56 μg, oleic acid (AO) 8.97 μg, 1,3-diolein (1,3DO) 7.45 μg, 1,2-diolein (1,2DO) 6.66 μg, monoolein (MO) 6.18 μg. A = 90, B = 100, and C = 110°C.

RESULTS

Optimization of lipid detection

The solvent system of hexane–diethyl ether–formic acid (80:20:2, v/v) was arrived at after examining the more widely used hexane–diethyl ether–acetic acid solvent systems for lipid separation [1]. The solvent mixture that contained acetic acid provided a complete separation of the major lipid classes (monoacylglycerides, diacylglycerides, sterols, free fatty acids, triacylglycerides and sterol esters) but resulted in plates containing a line at the approximate R_F of the free fatty acid region after fluorescence induction. When these plates were scanned in the fluorescence mode this line interfered with the readings obtained for the free fatty acids in the test mixtures. By substituting formic acid, which is more volatile, for acetic acid, this interference was eliminated and the resolution of the major lipid classes remained unaltered.

Developed HPTLC plates, containing lipid standards and treated with the sulfuric acid–ethanol–hexane reagent, were heated at three temperatures, 90, 100 and 110°C, over a time range of 15–90 min (Fig. 1). With the exception of monoolein, optimal fluorescence was detected for all other lipids after heating at 110°C and 45 min (Fig. 1C). Monoolein appeared to give an optimum response after heating at 110°C and 60 min; however, the 45-min response was sufficient for quantitative purposes. This observation plus the fact that the response of the other test compounds decreased significantly when heated beyond 45 min led us to select the 45-min time interval and 110°C oven temperature as the optimum conditions for fluorescence induction with the new reagent. At oven temperatures greater than 110°C the fluorescent response decreased dramatically and charring of the spots became evident.

Comparisons between the use of gravity ovens and forced-air-type ovens for the heating stage, revealed that reproducible results and optimum fluorescence can only be obtained in the uniform temperature environment of the forced-air oven.

Sensitivity and linearity

Chloroform solutions of the commercial lipid standards in the concentration range of 1–100 ng/μl were applied to HPTLC plates, developed and fluorescence was

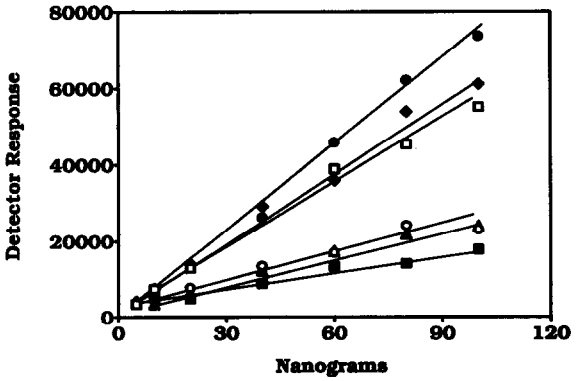

Fig. 2. Effect of concentration on the fluorescent response of lipid standards. See Table I for abbreviation code. ● = TO; ◆ = CHOL; □ = 1,3DO; ○ = 1,2DO; ▲ = OA; and ■ = MO.

TABLE I

SENSITIVITY AND LINEARITY OF STANDARD LIPIDS

The lower limit of detectability and correlation coefficients for each regression line fit shown in Fig. 2 for standard lipids. TO = Triolein; CHOL = cholesterol; 1,3DO = 1,3-diolein; OA = oleic acid; 1,2DO = 1,2-diolein; MO = monoolein.

Lipid standard	Lower limit of detection (ng)	Correlation coefficient
TO	20	0.988
CHOL	5	0.991
1,3DO	5	0.992
OA	10	0.981
1,2DO	10	0.975
MO	10	0.969

induced using the optimum conditions described above. A plot of the fluorescent response *versus* concentration (Fig. 2) revealed that the detected fluorescence differed for each class of standard lipid in this concentration range, the highest quantum yield produced by triolein and the least by monoolein. It is clear from these results that for each lipid class tested, the fluorescent response is distinct and dependent upon the structural properties of each lipid class. Table I lists the lower detectable limit, which is the minimum amount that could be accurately quantitated, for each lipid standard and the correlation coefficient for each regression line generated in the plot shown in Fig. 2. A linear relationship between fluorescence and concentration was found in the range starting at the lower limit of detection to 100 ng for all of the lipid standards analyzed using this method of fluorescence induction. The coefficients of variation for the fluorescent response of triplicate samples of the standard lipids were 2.8% for triolein, 2.3% for cholesterol, 3.4% for 1,3-diolein, 1.6% for oleic acid, 3.7% for 1,2-diolein and 2.6% for monoolein. Comparisons of values obtained for known amounts of standard lipid from standard curves resulted in an accuracy that was within 5% of the known amount spotted when calibration standards and test samples were run on separate HPTLC plates. Duplicate plates, each spotted with four samples of mixed lipid standards at the 40-ng level, showed a plate-to-plate variation in the fluorescent response of $\leqslant$ 2.9%. These results, however, were obtained when plates were cleaned together and treated with the same batch of dipping reagent. When changes are made, such as replenishment of cleaning solvent or dipping reagent, a significant difference in similarly spotted plates was noted. Due to these observations it is recommended that for maximum accuracy standards should be included on the same plate as the samples.

Effect of fatty acid composition

The effect of fatty acid unsaturation on the fluorescent response obtained after reagent treatment and heat is illustrated in Fig. 3A. The response of stearic acid (18:0) was very low but the mono-unsaturated oleic acid (18:1), at the same concentration, had a quantum yield over eighteen times greater than the saturated fatty acid. The

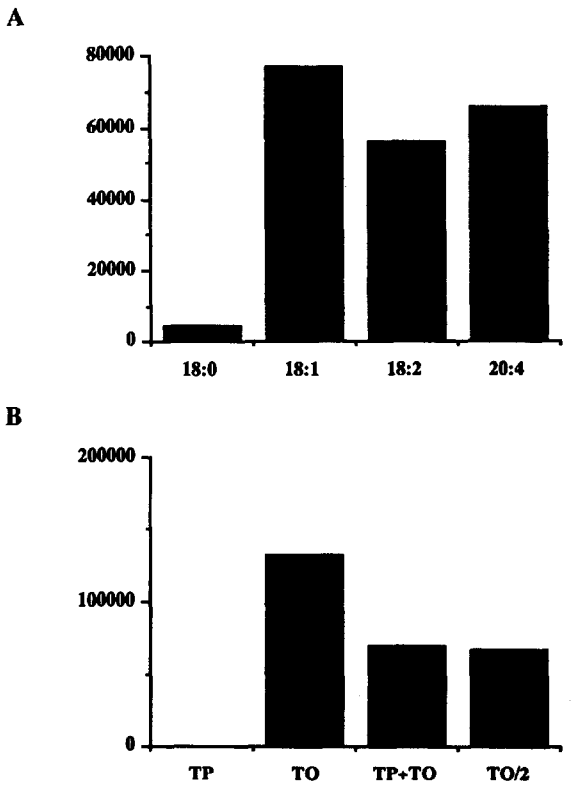

Fig. 3. Effects of unsaturation and lipid class on fluorescent response. (A) 1.0 μg of stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4). (B) 0.58 μg of tripalmitin (TP), triolein (TO), tripalmitin + triolein; 1:1, w/w mixture (TP + TO), triolein response divided by two (TO/2).

di-unsaturated linoleic acid (18:2) produced fluorescence that was 27% less than oleic acid and the polyunsaturated arachidonic acid (20:4) yielded fluorescence at a response that was 15% less than that of oleic acid. These results indicate that saturated fatty acids will probably produce the least fluorescence but that the fluorescent response is not directly proportional to the degree of unsaturation of the fatty acid.

The effect of fatty acid composition on triacylglyceride response is illustrated in Fig. 3B. Tripalmitin (16:0) failed to produce measurable fluorescence, while triolein (18:1), at the same concentration (0.58 μg), produced a significant response. A 1:1 (w/w), mixture of triolein and tripalmitin (TP + TO) produced a response slightly better than half (51.4%) of the triolein response (TO/2, theoretical 1/2 of the triolein response) and indicates that the fluorescence induced is attenuated, almost directly, in the (TP + TO) mixture, by the amount of nonfluorescent tripalmitin. This suggests that the fluorescent response obtained by this procedure for the tested triacylglycerides is dependent upon the fatty acid composition of the triacylglyceride.

Comparisons with two other induced fluorescence detection methods

Duplicate HPTLC plates containing standard mixtures were prepared and de-

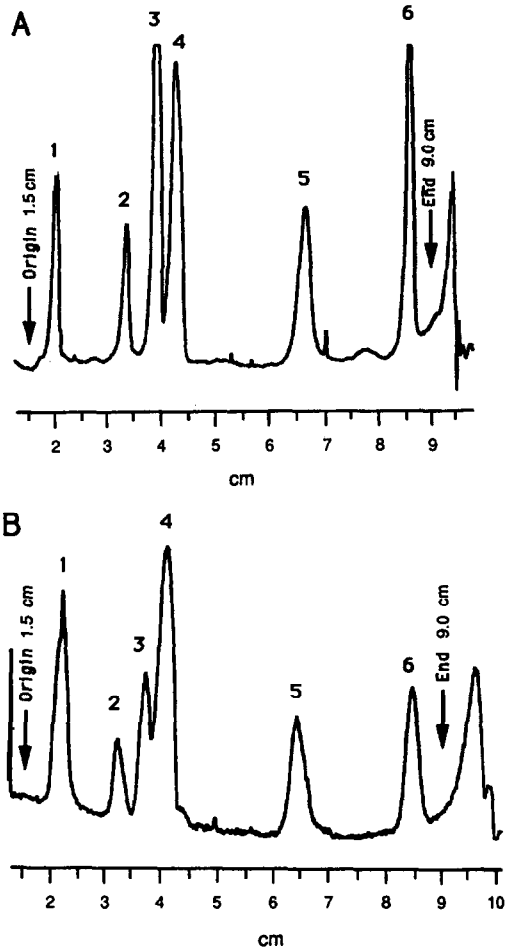

Fig. 4. A concentration of 0.4 μg of each standard was applied and plates were developed with hexane–diethyl ether–formic acid (see text). Peaks: 1 = monoolein; 2 = 1,2-diolein; 3 = 1,3-diolein; 4 = cholesterol; 5 = oleic acid; 6 = triolein. Fluorescence was induced by (A) the sulfuric acid–ethanol–hexane reagent or (B) the manganese chloride–methanol–sulfuric acid–water reagent.

veloped as described previously and fluorescence induction was accomplished with the sulfuric acid–ethanol–hexane (1:35:64, v/v) reagent and the manganese chloride–methanol–sulfuric acid–water reagent described by Schmitz and Assman [11]. For all of the compounds tested at the three different heating intervals, the sulfuric acid–ethanol–hexane reagent resulted in higher fluorescent responses, as illustrated in the histograms, than the manganese chloride–methanol–sulfuric acid–water reagent. In addition, to better overall sensitivity, the new reagent-produced fluorodensitograms with less baseline noise and peak resolution was superior (Fig. 4A) to that obtained with the manganese chloride-based reagent (Fig. 4B).

We next compared the new method with the vapor-phase fluorescence-induction technique of Segura and Gotto [9], wherein fluorescence is induced by heating the

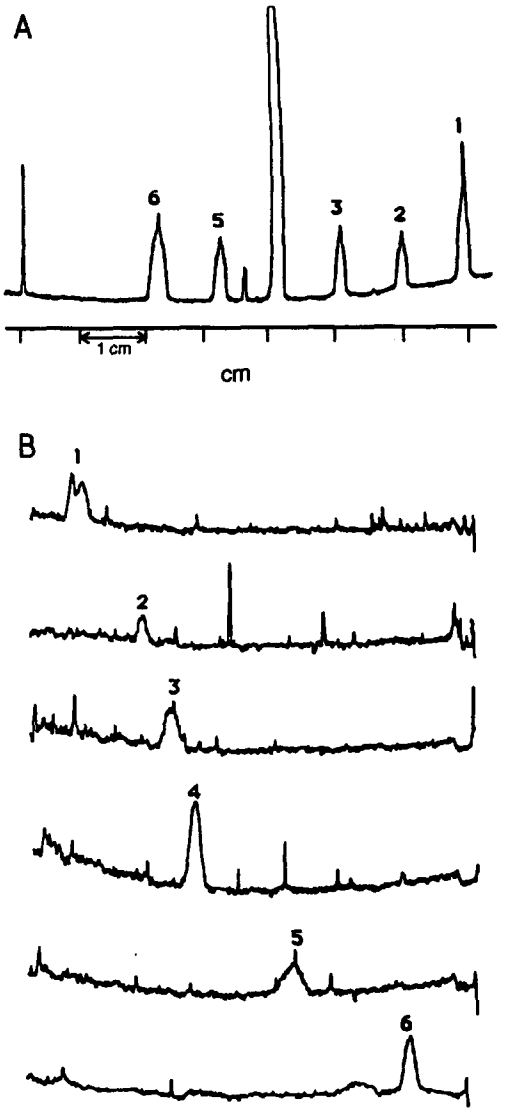

Fig. 5. A concentration of 0.125 μg of each standard applied to HPTLC plates. (A) Static spots and (B) plates developed as described in text. Detection by vapor-phase fluorescence induction. Peaks: 1 = monoolein, 2 = 1,2-diolein, 3 = 1,3-diolein, 4 = cholesterol, 5 = oleic acid and 6 = triolein.

HPTLC plate in a chamber containing an atmosphere of ammonium bicarbonate. With the vapor-phase method, standard lipids were not detectable at levels less than 1.25 μg per component on normally developed plates. When static spots of lipid standards (1.25 μg per component) were subjected to the vapor-phase treatment, they could be detected (Fig. 5A) but the response was found to be greatly deminished if the lipids spotted on HPTLC plates were allowed to migrate by the normal solvent development procedure (Fig. 5B). For detection of lipids on developed HPTLC plates, the

vapor-phase method, as tested here, lacked sensitivity and produced variable responses at the lipid concentrations used in this study.

Fluorescence stability

We examined the stability of the fluorescence induced with the sulfuric acid–ethanol–hexane reagent over time during the course of this work. For most of the tested lipids (monoolein, 1,2-diolein, 1,3-diolein, oleic acid and cholesterol) fluorescence decreased by an average of 49.8% after 24 h and was further reduced by 56.5% of the original response after three days. Triolein, however, lost only 12.0 and 36.7% of the original fluorescence after one and three days, respectively. At twelve days after fluorescence induction an average of only 5% of the original fluorescence could be detected; however, fluorescent spots could still be detected using a hand-held 254 nm light source. Because of the inconsistent degradation of the fluorescence over time, scans for quantitative determinations should be performed as soon as possible after heat treatment.

Reagent stability

The stability of the sulfuric acid–ethanol–hexane reagent was examined over time and after repeated use of the reagent for the induction of fluorescence in standard lipids on HPTLC plates. When the same 250-ml batch of reagent was used to treat a small number of plates (1 plate per day, 8 plates total) over a one-week period, the resulting fluorescence obtained was approximately the same for all of the test plates. When large numbers of plates (10 per day) were treated similarly, the reagent, based on the fluorescent response obtained, became exhausted after three days of use (30 plates total). Since sulfuric acid is the active ingredient in this reagent mixture (ethanol and hexane did not induce fluorescence), it may become diminished by reaction with components on the HPTLC plates or absorption into the silica gel sorbent layer to a level where it is no longer effective.

Analysis of tallow lipids

Calibration standards for the analysis of tallow samples were obtained by preparative TLC fractionation of partially hydrolyzed beef tallow obtained from a lipase reactor process [12]. The separated fractions of monoacylglycerides, 1,2-diacylglycerides, 1,3-diacylglycerides, free fatty acids and triacylglycerides were dissolved in chloroform and combined to form mixed standards of known concentration. The tallow-derived standards, in the concentration range of 0.1–0.8 μg/component, were subjected to HPTLC and fluorescence induction, as outlined previously. Plots of detector response *versus* concentration of each lipid class resulted in a linear relationship over the tested concentration range. Regression analysis of the calibration data yielded correlation coefficients $r = 0.994$, monoacylglycerides; $r = 0.967$, 1,2-diacylglycerides; $r = 0.959$, 1,3-diacylglycerides; $r = 0.998$, free fatty acids; and $r = 0.973$, triacylglycerides.

Samples of lipase-hydrolyzed beef tallow of unknown composition were dissolved in chloroform to give solutions of approximately 2.0 μg/μl concentrations and 1.0-μl aliquots were applied to HPTLC plates. Plates were developed and fluorescence was induced as described earlier. A fluorodensitogram of a typical sample of partially hydrolyzed tallow is presented in Fig. 6. The response of purified tallow lipid stan-

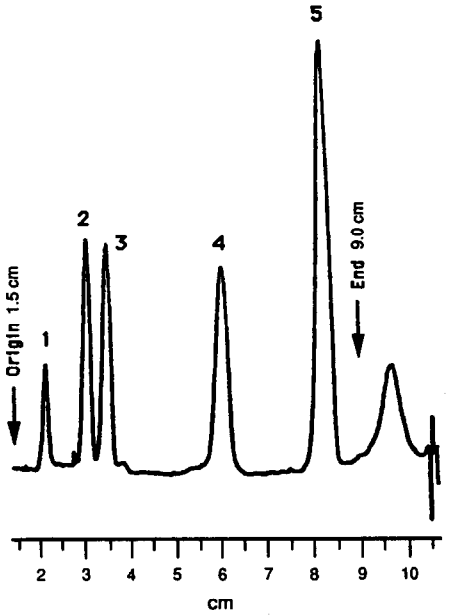

Fig. 6. Fluorodensitogram of 2.10 μg of partially hydrolyzed tallow. Plate was developed and lipids were detected as noted in text. Peaks: 1 = monoacylglycerides, 2 = 1,2-diacylglycerides, 3 = 1,3-diacylglycerides, 4 = free fatty acids, 5 = triacylglycerides.

dards was used to calculate the amount of each lipid class in the unknown samples and the weight percent of each lipid class was derived from this data. The amounts of free fatty acids present in 46 unknown partial tallow hydrolysates were calculated from the weight percent free fatty acids as determined by HPTLC. These data were compared with the free fatty acid amount determined separately, on each of the 46 samples, by a pH stat titration method [17]. Fatty acid values determined by the pH stat method *versus* the HPTLC procedure showed a good correlation between the separate determinations ($r = 0.960$).

DISCUSSION

The analysis of non-polar lipid mixtures by HPTLC and fluorodensitometry after fluorescence induction with the proposed reagent, is a simple and sensitive method for the quantitation of these compounds. This reagent (sulfuric acid–ethanol–hexane, 1:35:64, v/v) was initially developed for use with a class of antibiotics known as polycyclic ethers [18] and various mixtures of the three reagents were tested before arriving at the final combination. This previous work revealed that a volume of 35% ethanol was required to allow solubility of the sulfuric acid and ethanol in hexane. The use of hexane as a carrier solvent for the sulfuric acid offered the advantage of rapid and uniform drying of the plate surface following the immersion step. In addition, hexane lowers the polarity of the dipping reagent which prevents the elution of separated components into the dipping reagent and reduces spot spreading.

We applied this reagent to the analysis of lipids when the need to analyze large numbers of partially hydrolyzed tallow samples arose. At this time, we noted in the literature that Schmitz and Assman [11] reported the use of a reagent for the induced fluorescence of lipids and although the two reagents appear similar, our comparative studies revealed many major differences between them. This water-containing reagent (manganese chloride–sulfuric acid–ethanol–water) dries slowly and blemishes the plate surface. This resulted in poor quality and dereased sensitivity in fluorodensitograms due to background noise caused by surface distortions. In addition, the protracted time of plate immersion recommended with this reagent, appeared to cause spreading of separated spots that resulted in an apparent loss of resolution as observed in the fluorodensitogram.

Vapor-phase induction of fluorescence with ammonium bicarbonate, as originally described by Segura and Gotto [9], proved to be ineffective, as tested here, on solvent-developed HPTLC plates. At the 1.25 μg/component levels used in this study, suitable fluorescence appeared to be induced only when lipids were statically spotted. Migration of sample components into the adsorbent layer during plate irrigation may cause a net reduction in the amount of excitation energy available to the separated components [19]. This could result in a reduction of overall fluorescence relative to the static-spot case, where sample components are situated closer to the surface of the adsorbent layer. The vapor-phase method also requires a long period of heating at high temperature (10 h, 150°C), as well as the use of a gas-tight apparatus to maintain the ammonium bicarbonate atmosphere, thus limiting the sample number and turnaround time for analysis. Both of the methods discussed above relied upon an excitation wavelength of 366 nm to generate a fluorescent response; we utilized the same excitation wavelength for this work in order to directly compare the various methods. Since satisfactory results were obtained with lipid standards and samples, we made no effort to further optimize this parameter and caution that this wavelength may not be the optimum excitation wavelength for maximum fluorescence.

The proposed method for the analysis of non-polar lipids is relatively simple and sensitive, allowing the detection of certian lipid standards at the 5-ng level and the routine determination of 100-ng levels of naturally occurring tallow lipids. The sensitivity of this method compares well with the reported lower detectable limits obtained by the use of such fluorescent indicators as 6-*p*-toludino-2-naphthalene [20], Rhodamine G and 2,7-dichlorofluoresceine [21]; however, no direct comparative studies were conducted at this time.

In this work our interest was primarily in the development of a simple and rapid method to monitor lipase reactor products (partially hydrolyzed tallow) that would allow efficient processing of large numbers of samples. The present method satisfied this purpose as evidenced by the correlation between independent determinations of free fatty acids in samples of the various reactor products. Since sample amounts were not limited in this work, we did not attempt, at this time, to determine the lowest level of lipase reactor products that could be accurately quantitated. The method does, however, appear to be suitable for applications in which high sensitivity is required as evidenced by the comparative studies and literature comparisons, outlined above. The data does indicate, however, that the composition and structure of an analyte will have a significant influence on the fluorescent response obtained with this method. Because of this, sensitivity, calibration and accuracy determinations should be per-

formed in order to evaluate the effectiveness of this method for use with a particular analyte.

CONCLUSIONS

The HPTLC method presented here for the analysis of non-polar lipids relies upon a relatively simple plate-cleaning step and a new reagent for the induction of fluorescence on developed HPTLC plates. The procedure is rapid and sensitive and is well suited for use in large-scale studies or in routine analysis where large numbers of samples are involved. Since the mechanism of the *in situ* fluorescence induction used here appears to be relatively non-specific, the method should be applicable for the detection of a broad range of analytes that are separable by normal-phase TLC.

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