

A New Reagent for the Analysis of Lipids by High Performance Thin Layer Chromatography and Fluorodensitometry

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Summary

A method has been developed for the high performance thin layer chromatographic (HPTLC) separation of lipids and the *in situ* induction of fluorescence of the separated compounds by immersion of the developed plate into a mixture of sulfuric acid, absolute ethanol, and hexane and heating at 110 °C. During excitation with light at $\lambda = 366$ nm the sample components exhibit fluorescence which can be measured with a TLC scanner.

The method has been applied to the analysis of lipids from partially hydrolyzed edible beef tallow and fungal mycelia. The advantages and utility of the method for routine lipid analysis are discussed in the context of the two types of sample analyzed.

1 Introduction

A high performance thin layer chromatographic (HPTLC) method for the separation and fluorodensitometric detection of neutral lipids has been previously reported [1] and further details regarding the development of the method have also been presented [2]. In brief, a reagent comprising sulfuric acid – absolute ethanol – hexane (1 + 35 + 64, v/v) could be used to induce fluorescence in lipids separated by HPTLC. The fluorescent response of standard lipids was found to be linear across a concentration range in some cases as low as 5 ng, to 100 ng.

In addition to the potential high sensitivity, the method also enabled rapid, economical, and efficient treatment of the large numbers of samples required for many of the studies supported by this laboratory. In comparative studies, this new reagent, when tested with standard lipid mixtures, yielded better quality fluorodensitograms and offered sensitivity superior to that of other common reagents used for fluorescence induction.

In this report we present some preliminary results of the application of this technique to the analysis of lipids in samples from two different sources. The advantages and limitations of the method will be discussed within the context of the applications.

2 Material and Methods

2.1 Lipid Samples

Edible beef tallows were partially hydrolyzed in an immobilized lipase reactor system [3] and samples were periodically withdrawn for analysis. The liquefied, partially hydrolyzed tallow (PHT) was allowed to equilibrate to room temperature, after which the now solidified fat could be accurately weighed and dissolved in chloroform to give an approximate concentration of 2 mg/ml.

Mycelia of the fungus *Saprolegnia parasitica* were collected, washed and lyophilized [4]. The dried mycelia were extracted with supercritical carbon dioxide [5] and the extracts purified as described by *Folch* [6]. The lipid phase of the *Folch* extract was dried under a stream of nitrogen, accurately weighed, and the residue dissolved in chloroform to yield a final concentration of approximately 2 mg/ml.

2.2 Lipid Separation

Chromatography was performed on 10 × 10 cm silica gel 60 HPTLC plates (Merck) which had been cleaned by immersion in methanol for 5 min, dried in an oven at 80-85 °C for 15 min, and stored in a closed box until used [7]. Sample aliquots (1 μ l), containing approximately 2 μ g of lipid, were applied to the plates, at a distance of 1.5 cm from the bottom edge, by means of a 1 μ l syringe (Hamilton). Plates were developed in an unsaturated type N chamber [8] (Camag) which had previously been equilibrated with the mobile phase for at least 10 min.

For PHT samples, plates were developed twice with hexane – diethyl ether – formic acid (80 + 20 + 2, v/v). In the first development, solvent was allowed to migrate to a mark 3.0 cm from the bottom edge of the plate; plates were allowed to dry and then developed until the solvent reached a mark 9.0 cm from the bottom of the plate.

Plates spotted with fungal lipid extracts were developed once, with hexane – diethyl ether – formic acid (60 + 30 + 2, v/v), to a distance of 9.0 cm from the bottom of the plate.

2.3 Lipid Detection

The developed, air-dried plates, were immersed into a mixture of sulfuric acid – absolute ethanol – hexane (1 + 35 + 64, v/v). Plates were held in the reagent for 1-2 s, removed, and re-immersed for an additional 1-2 s. Excess reagent was drained and plates were allowed to dry in air. The dry plates were heated at 110 °C for 45 min in a forced air type oven in order to induce fluorescence in the separated components. Fluorescence detection of the separated lipids was accomplished with a TLC Scanner II (Camag) equipped with a mercury lamp. Scanning was performed in the fluorescence – reflectance mode at a rate of 0.5 cm/min with an excitation wavelength of 366 nm and a 400 nm cut-off filter installed in front of the photomultiplier. Fluorescence output was measured with an SP 4290 Integrator (Spectra Physics) operated in the peak height measurement mode.

2.4 Calibration

Lipid standards (Nu Chek Prep Inc.) consisting of mixtures of monoolein, 1,2-diolein, 1,3-diolein, cholesterol, oleic acid, and triolein were spotted at varying concentrations and separated and detected as above. Standards of PHT and fungal lipids were prepared by preparative TLC on 500 μ m Silica Gel G plates (Analtech) [9,10]. The purified lipids were combined to produce mixed standards of known concentration which were then analyzed as outlined above.

3 Results and Discussion

Comparative studies of various fluorescence induction techniques were performed in order to evaluate the described method in relation to other commonly used techniques. A standard lipid mixture was separated by HPTLC, and fluorescence induced both by the vapor phase method [11] in which ammonium bicarbonate vapor is generated in a sealed vessel containing the developed HPTLC plate [12], and also by treating the developed plates with a reagent containing manganese chloride – sulfuric acid – methanol – water [13].

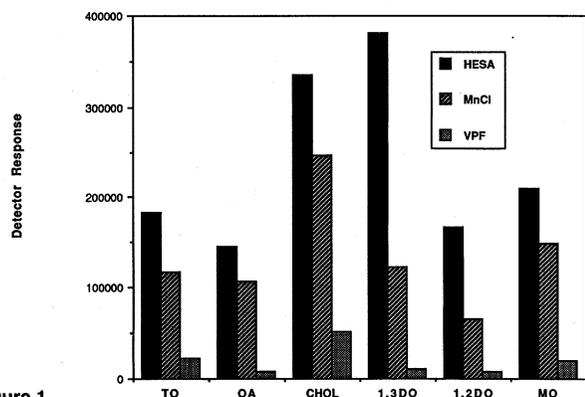


Figure 1

Fluorescent response of standard lipid components (9.5 μ g) subjected to fluorescence induction by various methods: HESA, sulfuric acid – ethanol – hexane; MnCl, manganese chloride – sulfuric acid – methanol – water; VPF, vapor phase fluorescence; TO = triolein; OA = oleic acid; CHOL = cholesterol; 1,3DO = 1,3-diolein; 1,2DO = 1,2-diolein; MO = monoolein.

Figure 1 illustrates the fluorescent responses obtained from each of the different induction techniques. The sulfuric acid – ethanol – hexane reagent generated the highest level of fluorescence; the manganese chloride-based reagent was next best. The vapor phase technique produced a response which was barely detectable at the sample level used in this comparison (9.5 μ g/component) and, in fact, below the 9.5 μ g/component level the vapor phase technique failed to generate a response measurable by the scanner. The response produced by the manganese chloride-based reagent also tended to deteriorate at lower sample levels, but not as drastically as that of the vapor phase method.

Observation of plates treated by the vapor phase and manganese chloride methods revealed surface distortion of the silica layer and discoloration of the plate surfaces; this could explain the baseline instability and diminished sensitivity encountered with these techniques. The severe treatment required for the vapor phase fluorescence induction (10 h, 150 °C), and the presence of water, which is generally recognized to have a deleterious effect on silica gel layers, in the manganese chloride-based reagent, could explain the distortion observed.

Plates were dipped very quickly into the sulfuric acid – ethanol – hexane reagent, and the short contact time appeared to minimize the spot spreading which can occur when the dipping reagent is capable of dissolving the sample components. Since the dipping reagent contains no water, major distortions of the surface of the plate are avoided and the silica gel layers appear to be less prone to flaking and discoloration after the heating stage. The result was fluorodensitograms with a minimum of baseline irregularities and separated peaks which appeared to be better resolved than those obtained by the other methods, even though the HPTLC separations were performed identically.

The fluorescence generated by use of the sulfuric acid – ethanol – hexane reagent with the standard lipid mixture was compared with that obtained from the PHT lipid fractions purified by preparative TLC. The responses obtained from identical concentrations of the two lipid mixtures are shown in Figure 2. The standard lipid mixture, which consisted of oleic acid and oleate-substituted acylglycerides, generated significantly higher fluorescent responses than the corresponding lipid class fraction purified from tallow hydrolyzate. This data confirmed our earlier observations [1,2] that the fluorescence obtained by this procedure is dependent upon both the structure and fatty acid composition of the lipid sample, and that the standard lipid mixture is not suitable for calibration and quantitation of actual lipid samples.

The oleic acid-containing lipid mixture was, however, useful as a qualitative indicator of the composition of the PHT samples, as is illustrated in Figure 3. The fluorodensitogram in Figure 3A was obtained after separation of the standard lipid mixture; that in Figure 3B depicts a sample of PHT. From this we were able to ascertain that cholesterol was not present in the PHT samples; this enabled us to prepare stan-

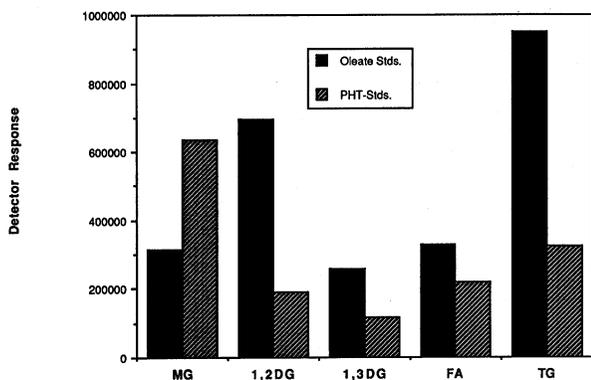


Figure 2

Comparison of the fluorescent response produced by use of the sulfuric acid – ethanol – hexane reagent with a standard mixture (oleate standards) and purified lipid fractions obtained from partially hydrolyzed beef tallow (PHT): mixtures were spotted at $0.4 \mu\text{g}/\text{component}$; MG = monoacylglycerides; 1,2DG = 1,2-diacylglycerides; 1,3DG = 1,3-diacylglycerides; FA = free fatty acids; TG = triacylglycerides.

standard mixtures for calibration purposes which matched the actual composition of the sample.

Calibration standards were prepared from fractions obtained from PHT or fungal lipid extracts which had been purified by TLC. For PHT samples, a mixed standard was prepared containing the lipid fractions apparent from Figure 3B in the concentration range $0.1\text{--}0.8 \mu\text{g}/\text{component}$. Calibration curves were prepared for each lipid class and plots of detector response against concentration showed a linear relationship

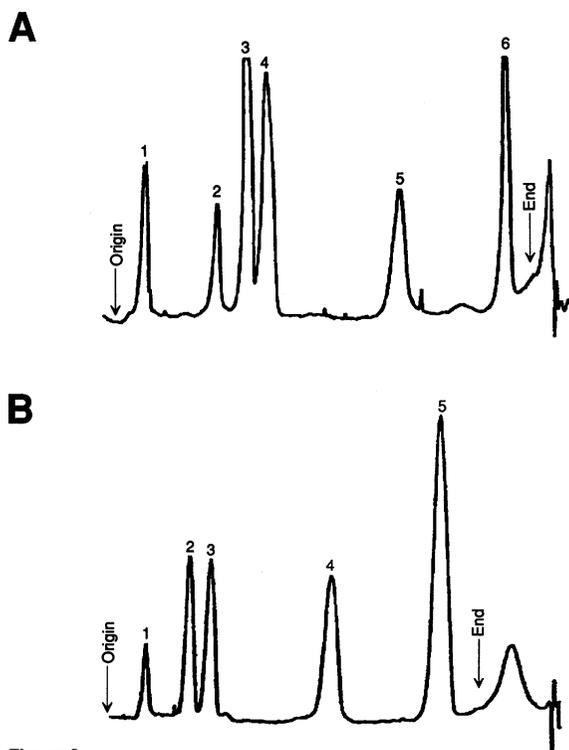


Figure 3

Fluorodensitograms of a $0.4 \mu\text{g}/\text{component}$ lipid standard (A) and $2.1 \mu\text{g}$ of a sample of PHT (B): in A, 1 = monoolein, 2 = 1,2-diolein, 3 = 1,3-diolein, 4 = cholesterol, 5 = oleic acid, and 6 = triolein; in B, 1 = monoacylglycerides, 2 = 1,2-diacylglycerides, 3 = 1,3-diacylglycerides, 4 = free fatty acids, and 5 = triacylglycerides.

over this concentration range: this range was found to be adequate for the quantitation of the lipid classes in typical PHT when PHT samples of approximately $2 \mu\text{g}/\text{spot}$ were applied to HPTLC plates. Regression analysis of the calibration plots yielded correlation coefficients of 0.994 for the monoacylglycerides, 0.967 for the diacylglycerides, 0.959 for the 1,3 diacylglycerides, 0.998 for the free fatty acids, and 0.973 for the triacylglyceride standards.

In order to verify the utility of the HPTLC method, free fatty acids were simultaneously determined by a titration method [14] and the values obtained compared with those measured by HPTLC. Fatty acid weight percent values were derived from the HPTLC determination of all the lipid fractions present in the PHT sample and good correlation was obtained between these values and the results from the alternative method (Figure 4). In preliminary studies of lipase reactor systems we have used this method to record baseline product profiles and monitor any changes in the lipid profiles of the effluent from the reactor. The method enable rapid sample turnover and the processing of the large numbers of samples required for studies involving the optimization of the hydrolytic process.

In preliminary work on the extraction of lipids from the mycelia of *S. parasitica*, our initial concern was whether the polar lipids were removed, and if so at what levels. For quantitation of the fungal polar lipid fraction, a calibration standard was prepared from polar lipids obtained from the fungal source and purified by TLC. Serial dilutions were prepared from the polar lipid fraction, and HPTLC, fluorescence induction, and fluorescence scanning performed as described above. Analysis of these dilutions indicated a minimum detectable level of $0.03 \mu\text{g}$. A linear relationship was obtained between detector response and concentration of polar lipids for quantities between 0.03 and $0.25 \mu\text{g}$ and a correlation coefficient of 0.983 was obtained for the linear fit.

Within this calibration range 1.5% polar lipids could be detected following application of a spot containing $2 \mu\text{g}$ total

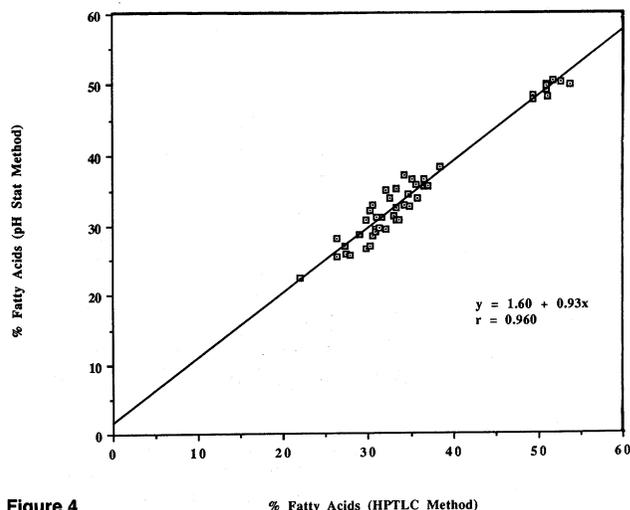


Figure 4

Correlation between values determined by a titration method and the HPTLC – fluorodensitometric method for free fatty acids in PHT samples.

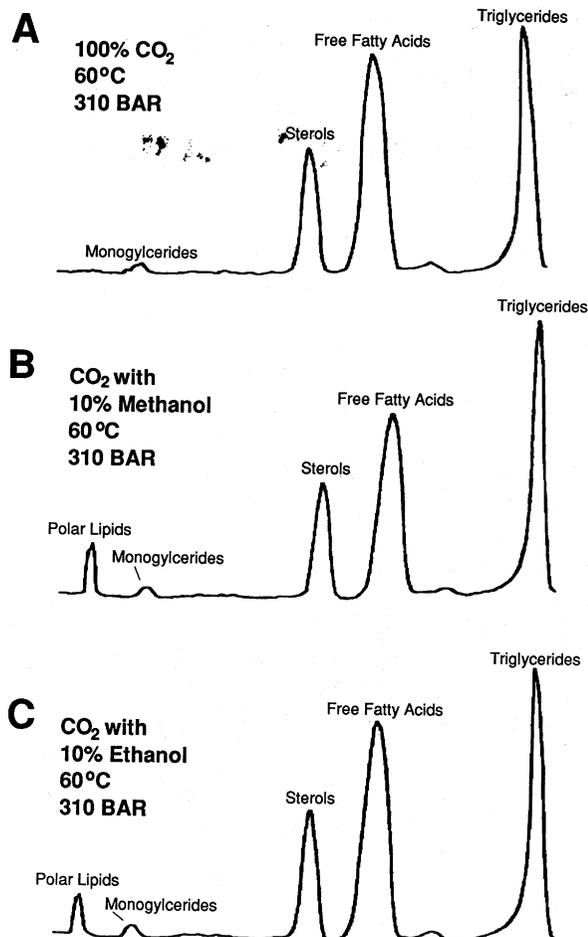


Figure 5
Fluorodensitograms obtained from 2.5 μ g samples of fungal lipids extracted with supercritical carbon dioxide (A), supercritical carbon dioxide plus 10% methanol (B), and supercritical carbon dioxide plus 10% ethanol (C).

fungal lipids. This level of detectability proved adequate for the evaluation of the composition of lipids obtained with a supercritical carbon dioxide extractor.

The results depicted in **Figure 5** show the effects of using both supercritical carbon dioxide and supercritical carbon dioxide containing the cosolvents methanol and ethanol for extraction of fungal lipids. Extraction at identical temperature and pressure, yielded measurable quantities of polar lipids only when methanol or ethanol was used as cosolvent and it can be concluded, from this preliminary data, that extraction with supercritical carbon dioxide alone is ineffective for the extraction of all the lipids contained in the fungal mycelia under study in this work.

Using this information the emphasis of the investigation can now be directed toward the optimization of extraction conditions using mixtures of supercritical carbon dioxide with polar modifiers. With appropriate calibration and standardization the HPTLC method should be applicable to the quantitative determination of all the major lipids in the fungal mycelia; this work is currently in progress. We also plan to apply this method of detection to the HPTLC separation and determination of other polar lipids.

4 Conclusion

This report demonstrates the successful application of HPTLC separation and fluorescence detection for the quantitation of lipids in typical samples analyzed routinely in this laboratory. The method employs a simple pretreatment of the HPTLC plate, straightforward chromatographic procedures, and common reagents for fluorescence induction which enable rapid and efficient processing of large numbers of samples.

Even though the fluorescent response obtained with this reagent is dependent upon the sample's composition and the structures of its components, the high overall sensitivity obtained should facilitate detection at fairly low levels, provided suitable calibration standards can be prepared. In addition, since the mechanism of fluorescence induction using this reagent is, as yet, undefined, the same reagent may also prove to be applicable to the *in situ* detection of any number of compounds separable by HPTLC.

Notice

Reference to brand or company name does not constitute endorsement by the US Department of Agriculture in preference to others of a similar nature not mentioned.

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