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Use of hematoporphyrin as a fluorescent stain for detection of lipids in high-performance thin-layer chromatography

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ABSTRACT

The use of hematoporphyrin as a sensitive and selective fluorescent dye for the detection of various lipids after separation by high-performance thin-layer chromatography is reported. To maximize the fluorescence signal, one of the visible lines of an argon ion laser is used for fluorimetric excitation; to minimize background noise, a novel approach involving the rapid treatment of the chromatogram with a dilute solution of copper(II) nitrate for the efficient quenching of background fluorescence from hematoporphyrin adsorbed on the silica gel substrate is presented. Excellent limits of detection in the low nanogram range are obtained for the detection of cholesterol, cholesteryl esters, sphingomyelin, lecithin and triolein. Some preliminary investigations regarding visual detection limits, selectivity and photostability of hematoporphyrin are also reported.

INTRODUCTION

Thin-layer chromatography (TLC) is an important separation method for the qualitative and quantitative determination of lipids in various biological materials [1]. For the *in situ* visualization or quantification of lipids on thin-layer chromatograms, chromogenic reagents such as molybdophosphoric acid, bromothymol blue and copper(II) sulfate can be used for the production of colored compounds with lipids of interest [2]. The major disadvantage of these reagents is that an acid charring process is often necessary, which is time consuming and, more important, the charring process is difficult to control and could lead to destruction of the lipid samples. To increase the detection sensitivity and to simplify the sample treatment procedure, methods involving fluorogenic reagents can be used [3]. These include treating the chromatogram with fluorescent dyes such as 8-anilino-1-naphthalenesulfonate (ANS), 2′,7′-dichlorofluorescein and rhodamine B. Among these fluorescent dyes, only rhodamine has been shown to be a popular fluorogenic reagent owing to its high fluorescent quantum yield and photostability; however, destructive sample treatment procedures involving preliminary hydrogenation of samples are required before separation and detection can be performed [4]. Recently, a new fluorescent dye, nile red, has been introduced as a general-purpose reagent for the detection of lipids and other hydrophobic compounds after separation by TLC [5]. Among the major advantages of this reagent are that it is intensely fluorescent, the fluorescence is relatively photostable and fades slowly and the dye preferentially dissolves in hydrophobic compounds. A common disadvantage among almost all fluorescent dyes, including nile red, is that significant background fluorescence can be observed from dyes adsorbed directly on the stationary phase, resulting in a noisy background and a decrease in detectability and reproducibility. Quantification of lipids on thin-layer chromatograms using nile red staining requires the use of a dilute bleach solution for the

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reduction of background fluorescence. However, careful selection of the bleach concentration is necessary to prevent oxidation of lipids and/or dye dissolved in the lipid bands.

It has been known for a long time that protoporphyrin possesses affinity for certain lipids [6], and it has been used as a selective fluorogenic reagent for the detection of a number of lipids on paper chromatograms [7]. It has shown that the use of protoporphyrin as a fluorescent stain offers several advantages over rhodamine dyes for the visualization of lipids on paper chromatograms, the most significant of which is the enhancement in detectability due to the relatively high fluorescent quantum yield of protoporphyrin and the appearance of a very low background fluorescence. Further, it has been shown recently that hydrophobic porphyrins, including protoporphyrin and hematoporphyrin, bind strongly to serum lipoprotein and are responsible for the photosensitivity of patients suffering from porphyrias and the accumulation of hematoporphyrin derivatives in tumors accompanying the phototherapy of cancer [8].

The separation of different individual components of lipid subclasses usually involves the use of two-dimensional TLC techniques [9]. However, with the improvement in separation efficiency in high-performance (HP) TLC, the rapid separation of several lipid fractions with good resolution can be achieved in one-dimensional development [10]. Compared with conventional TLC plates, the decrease in particle size in HPTLC requires the application of smaller sample volumes, which places a greater demand on the detection system to achieve the best sensitivity possible. The ease with which the laser beam can be focused to a very small spot while maintaining sufficient power makes it an attractive light source for the fluorimetric excitation of sample spots on HPTLC plates. Excellent detectabilities have already been reported in the use of laser fluorimetric techniques for the determination of biologically important molecules such as aflatoxins after separation by TLC [11]. Further, other laser-based techniques such as photoacoustic spectroscopy, non-linear luminescence and photothermal deflection spectroscopy have also been successfully applied to improve analytical performances in TLC [12].

In this paper we report the use of hematoporphyrin as a sensitive and selective fluorescent dye for the detection of certain lipids separated on HPTLC plates. To reduce the background fluorescence, a novel approach involving the rapid and mild treatment of the chromatogram with a dilute copper(II) nitrate solution for the efficient quenching of hematoporphyrin molecules adsorbed on a silica gel substrate is presented. Using the 488-nm line of an argon ion laser for excitation, low nanogram amounts of cholesterol, cholesteryl esters, triolein, sphingomyelin and lecithin are detected fluorimetrically. Similar amounts of cholesterol and cholesteryl esters are also detected visually using a conventional ultraviolet (UV) light source for fluorimetric excitation.

EXPERIMENTAL

Chemicals

Hematoporphyrin and protoporphyrin were obtained from Fluka Biochemicals (Ronkonkoma, NY, USA). All the lipids (cholesterol, choslesteryl oleate, cholesteryl linoleate, cholesteryl linolenate, cholesteryl palmitate, sphingomyelin, lecithin, triolein and testosterone) were of research grade and purchased from Sigma (St. Louis, MO, USA). Chloroform, carbon tetrachloride and methanol were of HPLC grade and other organic solvents were of analytical-reagent grade; all were obtained from Fisher Scientific (Pittsburgh, PA, USA). Copper(II) nitrate, *n*-octanol, caproic acid and acetic acid were purchased from Eastman Kodak (Rochester, NY, USA).

Chromatography

Separations of various lipids were performed on 5-μm silica gel HPTLC plates equipped with a pre-adsorbent area (Whatman, Hillsboro, OR, USA). The plates had dimensions of 10 × 10 cm and a layer thickness of 200 μm. To minimize background fluorescence from impurities on the plates, the plates were first washed with methanol followed by an additional wash with the development solvents. A sample volume of 100 nl was spotted on the plates with a 0.5-μl syringe. Before development, the silica gel plates were activated at 100°C for 30 min. The plates were developed in a nano-developing chamber designed to accept 10 × 10 cm HPTLC plates (Anspec, Ann Arbor, MI, USA) and equipped with

a heavy glass lid to fit the chamber flanged top. The chamber was lined with four filter-papers and then filled with the appropriate solvent system to allow saturation of the chamber atmosphere for about 60 min before introduction of the spotted plate. The chromatographic solvent systems described recently which allow the rapid HPTLC separation of 21 different lipid components within 15 min [10] were adopted in our experiments for the separation of various lipids. For the separation of cholesteryl esters, solvent A (carbon tetrachloride) was used; sphingomyelin and lecithin were separated with solvent B, chloroform–methanol–methyl acetate–water (100:60:16:8, v/v), and cholesterol and triolein with solvent C, chloroform–ethylacetate (94:6, v/v).

Staining

A stock solution of 0.33 m*M* hematoporphyrin was prepared by first dissolving appropriate amounts of hematoporphyrin in a small amount of 2 *M* sodium hydroxide solution and then diluting to an appropriate volume with 0.01 *M* phosphate buffer (pH 7.4). A stock solution of 100 m*M* copper(II) nitrate was prepared in doubly distilled, deionized water (DDW). To stain lipids for either laser-based or visual detection, the plate was dipped for 10 s in a tank of 0.25 m*M* hematoporphyrin solution followed by rinsing with DDW. To reduce background fluorescence by chelation with copper (II) ions, the plate was dipped for another 10 s in a 10 m*M* solution of copper(II) nitrate. The plate was dried by heating at 100°C for about 5 min for the laser-based experiment; however, the plate was inspected wet for the visual experiment.

Detection

Fluorimetric detection of the hematoporphyrin-stained lipids was accomplished with the apparatus used in previous work [13]. Briefly, radiation from a Coherent Innova 90-4 argon ion laser was first passed through a 488-nm line filter before it was focused with a 90-mm focal length lens and directed at an angle of almost 45° relative to the plate surface. The excitation and emission wavelengths were found to be optimum at 488 and 630 nm, respectively. Typical output power of the laser was 80 mW. To obtain a chromatogram, the thin-layer plate was scanned in front of the focused laser beam at *ca.* 30 mm/min using a precision d.c. motor-driven translational stage (Newport Research, Fountain Valley, CA, USA). The fluorescence signal was collected normal to the plate with an *f*/1, 25-mm focal length lens. The signal was then focused by an 80-mm focal length lens onto the entrance slit of an *f*/4 monochromator (PTR Optics, Waltham, MA, USA). The monochromator was fitted with 600-μm slits and the band pass was *ca.* 3 nm. Further fluorescence isolation was provided by a Corion 10-nm band-pass filter centered at 620 nm. The signal was measured with an R928 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA) operated at 800 V. The photocurrents were fed to a picoammeter (Oriel, Stratford, CT, USA) and the output signal was filtered through a 1.0-s time constant before recording on a strip-chart recorder. To reveal lipids separated on HPTLC plates, the plates were excited with a UV hand lamp (UVP, San Gabriel, CA, USA) which was held *ca.* 5 cm from the surface of the plate. This lamp was operated at a wavelength of 366 nm ("long-wave" mode) and produced a typical peak 366-nm intensity of *ca.* 200 μW/cm^2 at a 15-cm distance. The visual detection limits were established by observing the pink fluorescence from the sample spots and recording the lowest lipid concentrations at which the fluorescence could no longer be seen in a dark room.

RESULTS AND DISCUSSION

In general, the fluorescence of porphyrins is most efficiently excited at the major absorption band at about 400 nm (the so-called "Soret" band) using a conventional light source; however, fluorimetric excitation using a laser light source at one of the minor absorption bands, which centers around 500 nm in the blue-green region of the visible spectrum and happens to fall conveniently within the most intense emission region of an argon ion laser, can be of great advantage for improved sensitivity and selectively as the high intensity of the laser will more than compensate for the sacrifice in the magnitude of the molar absorptivity and only a small number of organic molecules present in biological samples absorb efficiently in the visible region and yield appreciable fluorescence signals. These advantages have already been demonstrated in our earlier work on the determination of porphyrin profiles in human urine using HPTLC for separation and visible laser

fluorimetry for excitation and detection [13]. Another interesting spectroscopic property of porphyrins is that chelation of iron and other transition metal ions with porphyrins usually leads to severe quenching of the fluorescence [14]. For example, heme (iron protoporphyrin) has a fluorescence yield that is probably smaller than 10^{-10}. To the best of our knowledge, analytical exploitation of this particular property has not been reported.

In our experiments, a significant reduction in the background fluorescence on HPTLC plates is accomplished by the rapid chelation of Cu(II) ions with hematoporphyrin bound to the silica gel substrate. It should be noted that for most metal-free porphyrins, Cu(II) is the most rapidly incorporated ion in aqueous solution followed by Zn(II), Co(II), Fe(II) and Ni(II) [14]. Fig. 1a shows a chromatogram of 25 ng of cholesterol eluted on an HPTLC plate. Using hematoporphyrin as the fluorescent stain and the 488-nm line of an argon ion laser for fluorimetric excitation, it is clear that the intensity of the fluorescence signal from hematoporphyrin bound to the lipid band is higher than that of hematoporphyrin adsorbed directly on the stationary phase, partly owing to more efficient quenching of the stain by the silica gel. Fig. 1b shows the efects of treating the chromatogram as shown in Fig. 1a with a dilute solution of copper(II) nitrate. It is obvious that background fluctuations are significantly reduced owing to efficient chelation of Cu(II) ions and subsequent quenching of the hematoporphyrin molecules that are adsorbed on the silica gel. However, the intensity of the fluorescence signal arising from hematoporphyrin bound to the lipid also seems to decrease, but certainly to a much lesser extent. It should be noted that in order to record the chromatogram without changing the sensitivity on the recorder and the picoammeter, a suppression current provided by the internal circuitry of the picoammeter was used to attenuate the larger d.c. background obtained from plates that have not been treated with copper(II) nitrate solution. The results presented in Fig. 1a and b suggest that the rate of copper chelation with hematoporphyrin is sensitive to the microenvironment. When compared with the relatively polar silica gel substrate, the hydrophobic environment of the lipid band appears to provide a medium in which the rate of copper chelation with hematoporphyrin is slower, resulting in enhancement in signal-to-noise ratio (S/N). By comparing the average peak heights and peak-to-peak noise for five sets of chromatograms similar to that shown in Fig. 1a and b for the detection of cholesterol, an average improvement in S/N by a factor of *ca.* 5 can be estimated. This improvement factor remains relatively constant over a period of 30 min while keeping the chromatograms in a dark room. However, it is likely that the rate of copper chelation with hematoporphyrin dissolved in other lipids would be slightly different, resulting in varying degrees of S/N improvement.

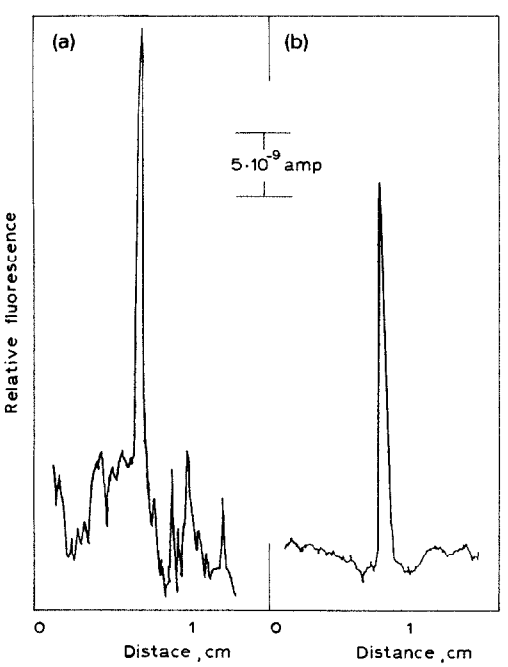

Fig. 1. HPTLC of 25 ng of cholesterol (a) without treatment and (b) with treatment in copper(II) nitrate solution. amp = Ampere.

The fluorescence of hematoporphyrin is pH and concentration dependent; the influence of these factors on the detection of lipids separated on HPTLC plates was investigated. Buffered hematoporphyrin solutions with pH in the range 2–11 were used for the study and the results indicated that the fluorescence of hematoporphyrin dissolved in lipid bands increased non-linearly with increasing pH and reached a plateau between pH 7 and 11. The concentrations of hematoporphyrin and copper(II) nitrate which gave the best S/N in our experiments

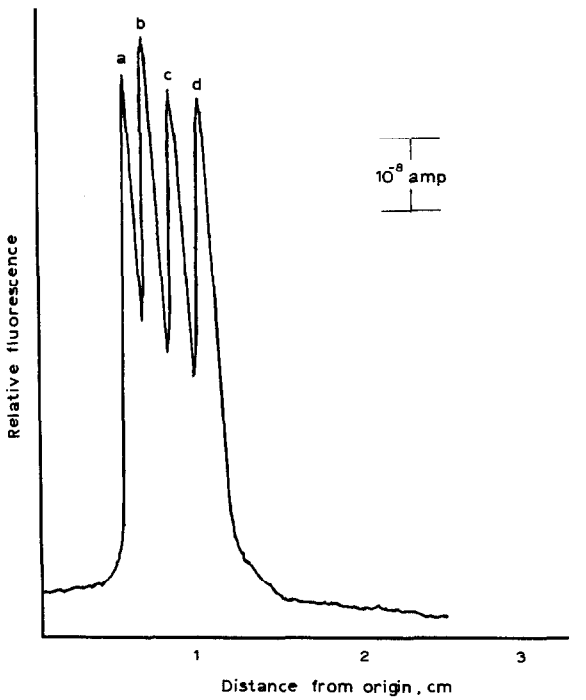

Fig. 2. HPTLC of 50 ng each of (a) cholesteryl palmitate, (b) cholesteryl oleate, (c) cholesteryl linoleate and (d) cholesteryl linolenate.

were found to be 0.25 and 10 m*M*, respectively. Also, a dipping time of 10 s appeared to offer the optimum S/N for the treatment of the plates with either hematoporphyrin or copper(II) nitrate solution.

Figs. 2, 3 and 4 show the HPTLC of cholesteryl esters, phospholipids and neutral lipids, respectively. The corresponding limits of detection (LOD) determined for each of these lipids are presented in Table I. These LOD values are about one to two orders of magnitude better than those reported for TLC [1,5,9] in the determination of lipids using densitometry or other fluorescence techniques. For example, using nile red as the fluorescent stain and a conventional UV light source for fluorimetric excitation, the minimum amount of cholesterol and sphingomyelin detectable is 100–200 ng; for cholesteryl oleate and trioleoylglycerol, the minimum amount detectable is about 50 ng [15]. The improvements in LOD in our method are made possible by the highly fluorescent nature of hematoporphyrin, the enhancement in fluorescence signal derived from the increase in photon flux using a laser source and the reduction in noise from effective quenching of background fluorescence through the rapid chelation of hematoporphyrin with copper(II) ion. It should be noted that hematoporphyrin is the least expensive metal-free porphyrin and contains a mixture of dicarboxylic acid porphyrins. We have compared the S/N for the detection of cholesterol separated on HPTLC plates using hematoporphyrin and protoporphyrin as the fluorescent stain and found that the detection limit obtained by hematoporphyrin staining is better by a factor of 2–3. Calibration graphs drawn for the various lipids exhibited linearity from the LOD up to the amount indicated in Table I, and the corresponding linear regression contents are also tabulated. At higher concentrations, non-linearity may arise from insufficient staining of the larger lipid mass, variation in quenching efficiency of the hematoporphyrin bound to the larger lipid bands and/or a mismatch between the dimension of the focused laser beam and the sample spot size and shape.

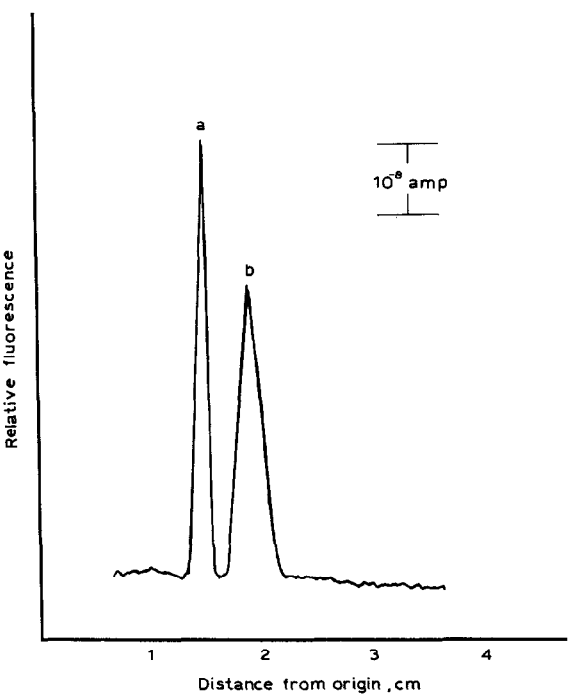

Fig. 3. HPTLC of 50 ng each of (a) sphingomyelin and (b) lecithin.

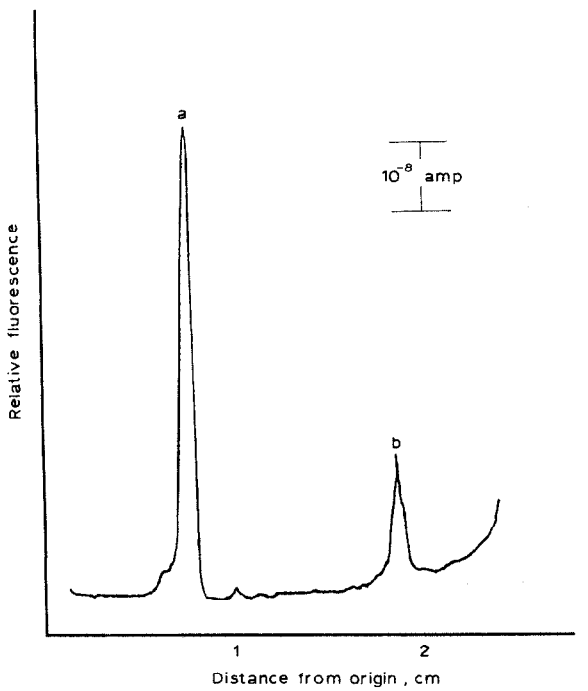

Fig. 4. HPTLC of 50 ng each of (a) cholesterol and (b) triolein.

A major limitation of most *in situ* TLC methods for the quantification of lipids is the varying effect of saturated *versus* unsaturated fatty acid moieties on the level of staining produced. Fig. 2 shows that the relative fluorescence intensity of cholesteryl esters with varying fatty acid composition is similar, suggesting that the influence of saturation of fatty acid moieties of lipids on the observed hematoporphyrin fluorescence is minimum. Fig. 2 also shows that there are some overlaps between the individual cholesteryl ester bands because the laser beam was focused to a diameter of *ca.* 2 mm on the plate surface to maximize the overlap between the laser spot and the various sample spots that are developed on the plate. To improve the resolution, sensitivity and linearity, two-dimensional scanning of the sample spots with a tightly focused laser beam can be performed either along a mutually perpendicular direction or in "meander-scanning" mode by interfacing the scanning table to a computer [16].

We have done some preliminary investigations on the photostability of hematoporphyrin as a fluorescent stain. Under the influence of ambient room light, the intensity of the fluorescence signal obtained from a chromatogram as shown in Fig. 1a remains relatively constant for at least 1 h. The intensity decrease by about *ca.* 5 and 10% in periods of 6 and 12 h, respectively. If the chromatogram is kept in the dark, the hematoporphyrin appears to be stable for at least 24 h. Under the influence of laser radiation (a laser power of 80 mW and laser spot diameter of *ca.* 2 mm), there is less than a 3% decrease in the peak height for the detection of 25 ng

TABLE I

DETECTION LIMITS AND LINEARITY AT GIVEN R_F VALUES

Lipid	R_F	Detection limit[a] (ng)	Linearity	
			Upper limit (ng)	Linear regression constant[b]
Cholesteryl palmitate	0.07	7.0	300	0.993
Cholesteryl oleate	0.09	3.3	300	0.993
Cholesteryl linoleate	0.12	7.7	300	0.993
Cholesteryl linolenate	0.14	5.2	300	0.993
Cholesterol	0.30	6.8	600	0.995
Sphingomyelin	0.45	6.8	300	1.000
Lecithin	0.53	10.4	300	1.000
Triolein	0.75	13.8	300	0.997

[a] Detection limits based on a signal-to-noise ratio of 3 according to peak heights.
[b] Linear regression constants determined from detection limits up to the amounts listed for the upper limits.

of cholesterol after five consecutive scans over the same spot while scanning the plate at *ca.* 30 mm/min.

Some preliminary studies have also been conducted in evaluating the visual LOD and the selectivity of hematoporphyrin for the detection of lipids. After dipping an HPTLC plate into a solution of hematoporphyrin for 10 s and subsequently observing the wet plate under UV radiation, cholesterol and cholestryl esters appears as brilliant pink spots on a background with similar color but dimmer intensity. A much better contrast was obtained after further treatment of the plate by dipping it in dilute copper(II)intrate solution for another 10 s, which gave a pink spot for the sample over a dark background. However, no significant improvements in our ability to detect visually the smallest concentrations of lipids was achieved, as hematoporphyrin bound to the lipids was also quenched but to a lesser extent. The visual LOD for cholesterol and cholesteryl esters using either procedure [with or without treatment with copper(II) nitrate] is *ca.* 10 ng, which is slightly better than those obtained by some of the more popular fluorogenic agents such as rhodamine B, ANS and dichlorofluorescein [3]. A number of substances have already been tested with protoporphyrin to determine its selectivity, and certain structural characteristics appear to be necessary for staining by protoporphyrin [7]. Most notably, a carboxyl group in aliphatic compounds of at least eight carbons is necessary. Among the polynuclear compounds, steroids without an isooctane side-chain are negative. In our experiments, testosterone, caproic acid and *n*-octanol were tested with hematoporphyrin to determine its selectivity. Negligible fluorescence signals were observed for these compounds spotted onto HPTLC plates. It appears that hematoporphyrin, having a similar structure to protoporphyrin, would have a similar selectivity to protoporphyrin for the detection of lipids. However, the structural requirements for staining with hematoporphyrin need further investigation.

It should be realized that the excellent LOD obtained for the detection of various lipids on HPTLC plates requires only 80 mW of continuous-wave radiation for fluorimetric excitation, which could be easily provided by a low-cost, air-cooled argon ion laser. Moreover, it is possible that small and inexpensive semiconductor lasers, which provide radiation in the yellow-red region of the visible spectrum, will be available in the near future [17]. Since hematoporphyrin possesses minor absorption bands in the blue-green and yellow-red regions, fluorimetric excitation of one of the minor absorption bands in the longer wavelength region may allow for the application of visible semiconductor laser fluorimetry for the detection of lipids. It should also be noted that the use of a laser is not a requirement, especially for hematoporphyrin-stained lipids separated on conventional TLC plates. As suggested by the excellent LOD obtained for the visual detection of cholesterol and cholesteryl esters, fluorimetric excitation with a conventional light source at the Soret band should provide LODs that are not significantly higher than those reported in Table I for the detection and quantification of certain lipids.

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