

# Comparison of Induced Vapor Phase Fluorescent Responses of Four Polycyclic Ether Antibiotics and Several Lipid Classes on RP-18 and Silica Gel HPTLC Plates

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## Summary

In 1974, *Segura* and *Gotto* reported a TLC method for the analysis of various types of compound containing no native fluorophore in which visualization was achieved by vapor phase fluorescence (VPF) induced by heating the compound, spotted on silica or alumina plates, in the presence of ammonium bicarbonate. Previous reports on the use of this technique have not compared its sensitivity with that of other methods of detection; we have demonstrated that statically spotted polycyclic ether antibiotics and various types of lipid have VPF fluorescence responses in the low nanogram range on silica plates but that when the same compounds are developed on the plates their responses decrease to the  $\mu\text{g}$  range.

In efforts to improve the sensitivity of this technique, VPF responses on silica and RP-18 HPTLC plates have been compared for both classes of compound. Fluorescence response on RP-18 and silica varied greatly depending on the type of compound: the response of the polycyclic ether antibiotics narasin and salinomycin on RP-18 plates was three times greater than on silica. Lipids, on the other hand, could, with the exception of cholesterol, be detected only on silica plates.

## 1 Introduction

Several techniques have been reported for inducing fluorescence responses in compounds which have no native fluorophores on TLC adsorbents [1-6]. In one of these, developed by *Segura* and *Gotto*, fluorescence is induced by heating the substances on silica gel or alumina TLC plates in the presence of ammonium bicarbonate [2] and although the

technique has been successfully applied to lipids, carbohydrates, and sterols, sensitivity levels have not, however, been compared with those of other methods.

We have applied the VPF visualization method of *Segura* and *Gotto* to the sodium salts of the polycyclic ether antibiotics (ionophores): lasalocid, monensin, narasin, and salinomycin. Fluorescence responses for these antibiotics on RP-18 and silica gel HPTLC plates were compared for both statically spotted and solvent-developed samples. Attempts were made to compare the results from this study with those obtained using a vanillin spray visualization technique [7] which has previously been reported as a detection method for the ionophores.

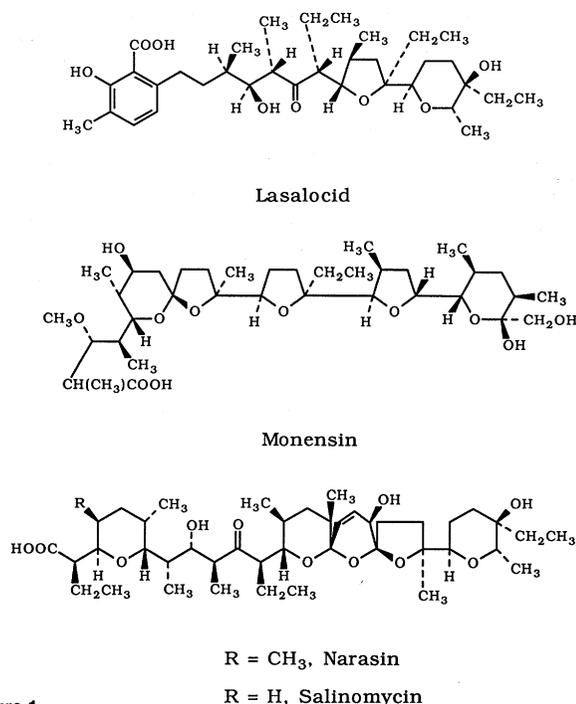
A limited investigation of the VPF fluorescence responses of several classes of lipid has also been performed in order to determine whether lipids co-extracted with the polycyclic ethers would interfere with VPF detection.

## 2 Experimental

### 2.1 Chemicals, TLC Materials and Fluorodensitometry

The four polycyclic ether antibiotics were gifts from their manufacturers: lasalocid sodium (100%) from Hoffman-La-Roche (Nutley, NJ, USA), monensin sodium (96%) and narasin sodium (94%) from Eli Lilly (Indianapolis, IN, USA), and salinomycin sodium (97%) from A.H. Robins (Richmond, VA, USA). The chemical structures of the compounds are shown in **Figure 1**. Lipids were obtained from Sigma (St Louis, MO, USA) and ammonium bicarbonate (99%) from Aldrich (Milwaukee, WI, USA). The solvents used were of HPLC grade.

Chromatography was performed on 10 × 10 cm silica gel 60 and RP-18 HPTLC plates from E. Merck (Cherry Hill, NJ, USA). Samples were applied to the plates as 5 mm wide



**Figure 1**  
**Chemical structures of the polycyclic ether antibiotics (ionophores).**

bands by means of a Camag automatic TLC Sampler III (Wrightsville, NC, USA) and plates were developed in either Camag twin-trough chambers or a Camag horizontal developing chamber. Vapor phase derivatization was performed in a chamber designed and assembled in this laboratory [8]. Induced fluorescence was quantified using a Camag TLC Scanner II operated with CATS 3 software. The excitation wavelength was 366 nm (mercury lamp) and the emission wavelength was  $> 400$  nm (cut-off filter). The scanning slit was  $3 \times 0.1$  mm and plates were scanned at 5 mm/s. For each series of compounds the sensitivity and span were held constant for both types of plate.

## 2.2 TLC Solvent Development

Silica gel and RP-18 plates for both static and solvent-developed experiments were pre-cleaned before sample application using a methanol dipping technique developed in this laboratory [9].

### 2.2.1 Lipids

All lipids were chromatographed by ascending development to a distance of 7 cm. Silica gel plates were developed with cyclohexane – diethyl ether – acetic acid (9 + 1 + 0.1, v/v). RP-18 plates were developed with chloroform.

### 2.2.2 Ionophores

On silica gel plates the four ionophores were chromatographed by ascending development with ethyl acetate – water (100 + 3, v/v) to a distance of 6 cm. RP-18 plates were twice developed horizontally, to a distance of 5 cm, with methanol – 5% aqueous acetic acid (9 + 1, v/v), after phase conditioning for 8 min with the same solvent system.

## 2.3 Visualization Procedures

### 2.3.1 VPF

After development, the HPTLC plates were placed beneath a stream of nitrogen gas to remove residual traces of solvent and then placed in the removable rack of the VPF heating chamber [8]. Sodium bicarbonate (3 g) was placed in the bottom of the chamber and the chamber assembled and sealed. Plates spotted with the polycyclic ether antibiotics (ionophores) were heated at 130 °C for 4 h in a mechanical forced air oven; those spotted with lipids to 150 °C for the same time. At the end of the heating period the VPF chamber was removed from the oven and set aside to cool. The plates were then removed from the chamber and scanned.

### 2.3.2 Vanillin Reagent

Plates were sprayed with a solution of vanillin (3% m/v) in 2% (v/v) conc. sulfuric acid in methanol and heated for 10 min at 60 °C according to a reported procedure [7].

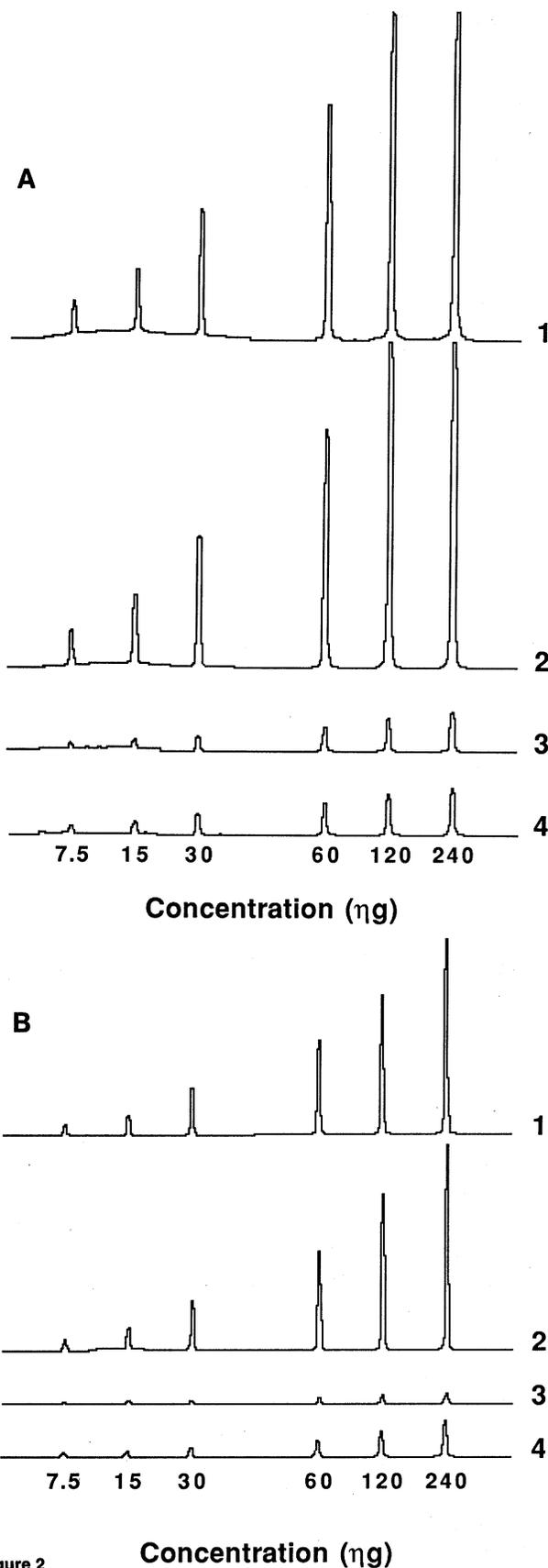
## 3 Results and Discussion

The ionophores are administered to poultry and cattle in medicated feeds for the control of coccidiosis in the former and to increase the feed utilization efficiency of the latter [10]; they are so named because of their ability to carry ions across lipid barriers, including artificial and biological membranes [11].

Tests are required to determine the levels of the compounds both in feed, and in tissue after slaughter. Two TLC detection techniques are currently used to visualize these compounds, vanillin spray reagent and bioautography [7,12]. The former exploits the reaction which occurs on heating the ionophores in the presence of vanillin; colored fluorescent spots are produced which presumably are specific for each ionophore. The second visualization method, bioautography, is used to reveal zones of inhibited microbial growth on areas of the TLC plate where the ionophores are present. In this work, only the technique employing vanillin was compared with the VPF technique: bioautography requires a clean room environment and a lengthy incubation period, and our goal was to find a simple, rapid method for visualization of the ionophores.

Studies comparing the VPF technique with use of the vanillin reagent were performed on silica plates. Solutions (350 ng/ $\mu$ l) of lasalocid, monensin, narasin, and salinomycin were prepared and 1  $\mu$ l volumes spotted on two silica plates. After development, one plate was visualized by the VPF technique and the other with the vanillin reagent. Spots of the four ionophores could be observed on the VPF plates under long wavelength UV light, whereas none was detectable on the vanillin plate.

Tests were also conducted in which the ionophores were applied to the silica plates at the 1  $\mu$ g level and again visualized by both techniques. At this level, all of the ionophores could be quantified by densitometry after VPF derivatization. This was not so following use of the vanillin reagent: although the

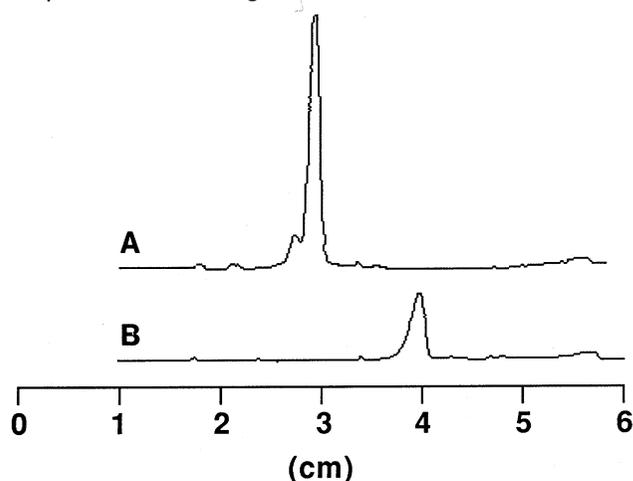


**Figure 2**  
 Fluorescent intensity of statically spotted ionophores after VPF derivatization on (A) RP-18 and (B) silica gel HPTLC plates: 1, salinomycin; 2, narasin; 3, monensin; 4, lasalocid.

four ionophores could be observed on the plate, the spots quickly faded and could not, therefore, be quantified by densitometry.

The lack of sensitivity of the vanillin reagent prompted a detailed investigation of the VPF technique with the polycyclic ethers. The technique was first evaluated by determination of the induced fluorescent responses of statically spotted ionophores on both RP-18 and silica gel plates. The resulting densitograms are shown in **Figures 2A** and **2B**. Several differences were noted in the responses of the four compounds on the two plate surfaces. On RP-18 the fluorescence responses of lasalocid and monensin were in the same range, whereas on silica that of monensin was significantly lower: the absolute response of monensin was found to be 80 % greater on the RP-18 surface than on silica.

Because of their similar molecular structures (Figure 1), narasin and salinomycin gave similar fluorescent responses on both types of plate, although the absolute responses of both compounds were 68 % greater on RP-18.



**Figure 3**  
 Comparison of induced VPF response of salinomycin after solvent development on (A) RP-18 and (B) silica gel HPTLC plates: sample size, 0.5  $\mu$ g.

We next conducted experiments to compare the VPF responses of the ionophores after solvent development. The four ionophores were each spotted at two concentrations on both types of plate which, after development, were subjected to VPF; the fluorescent responses obtained were quantified by densitometry. The densitograms obtained from salinomycin on both types of plate are reproduced in **Figure 3**; the peaks represent the responses obtained from 0.5  $\mu$ g of sample.

The fluorescence response for salinomycin on the RP-18 plate was 3 times greater than that on silica. The histogram presented in **Figure 4** depicts the responses obtained, after solvent development, from two concentrations of each of the four ionophores on both silica and RP-18. Because of the low responses observed for lasalocid and monensin (Figure 2), they were applied to the plates at twice the concentration used for narasin and salinomycin. Comparison of the histogram bars for all four ionophores shows that the fluor-

escence response was, in general, 2-3 times greater on RP-18 than on silica.

The fluorophores on RP-18 plates do not fade on storage (> 1 month) and can be visually observed under illumination with long wavelength UV. The induced fluorophores from narasin and salinomycin are visible at levels down to *ca* 0.15  $\mu\text{g}$  whereas clear observation of the fluorescence induced from monensin and lasalocid is only possible if levels exceed 0.5  $\mu\text{g}$ . Compared with visualization with vanillin [7] for which levels > 1  $\mu\text{g}$  are required and the spots exist for a short time only, this technique is, however, a significant improvement.

Efforts to enhance further the responses of the ionophores on these plates after VPF derivatization by use of other reported techniques gave inconsistent results and could not be used reliably. Reports have indicated that dipping plates in paraffin [13] or Triton X-100 enhances fluorescence yields for many types of compound [14]. These reagents were tried with the ionophores on silica plates, but with mixed results. *Segura* and *Navarro* described the use of metal salts as fluor-

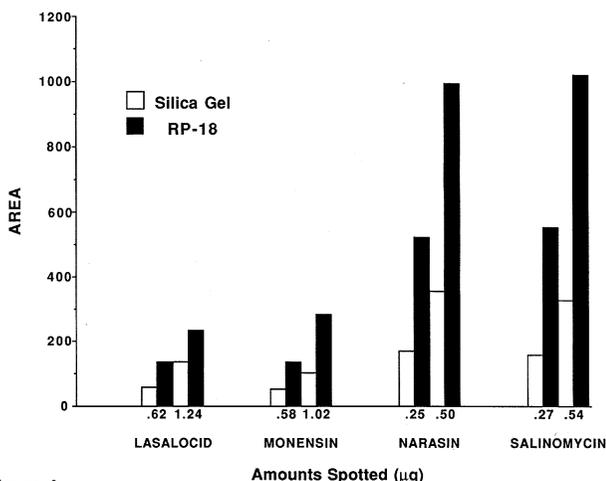


Figure 4

Histogram of area response from the four ionophores, spotted at two levels, after solvent development on silica gel and RP-18 plates.

escence-inducing reagents [15]. We conducted experiments in which their best reagent, zirconyl sulfate, was used with the ionophores statically applied on silica gel plates. No increase response was observed for the ionophores (the comparison limited to silica gel plates).

A unified mechanism for the process by which compounds can be induced to fluoresce on silica gel and reversed phase plates has yet to be proposed. *Segura* and *Gotto* suggested that a chromophore of the type  $\text{RN}=\text{CH}-\text{CH}=\text{CH}-\text{NH}-\text{R}$  is produced by a mechanism similar to that of the production of malonaldehyde and *Schiff* bases by X-ray irradiation of various compounds [2].

*Shanfield et al.* proposed that the conditions used for VPF must be producing free radicals which yield the same type of compound from all starting substances [3,4]. They found that compounds on silica plates subjected to an electrical discharge under vacuum produced the same levels of fluorescence whether the atmosphere above the plates com-

prised nitrogen or ammonium bicarbonate vapors, and that the silica gel appears to be essential for the formation of fluorescent derivatives. Their study did not, however, conclude with an explanation of their results other than the speculation that the process must consist of "energy-transfer" from activated gases to organic compounds [3,4]. Although the mechanism of this process has not been elucidated, other researchers have proposed explanations for the differences in the fluorescence response of compounds on the silica and RP-18.

*Poole* and *Khatib* suggested that the extent of fluorescence quenching often depends on the sorbent medium and is frequently more severe for silica gel than for bonded-phase sorbents [16]. They suggested that adsorption on to silica gel provides additional non-radiative pathways for dissipation of excitation energy and that the extent of this dissipation is at least partly relieved when the silica gel is covered with bonded organic groups.

Although the results of our VPF experiments on the two sorbents with the four ionophores support this concept, it does not account for the differences in quantum yields observed for narasin and salinomycin, compared with those for lasalocid and monensin, when all four compounds are solvent-developed on RP-18 and silica plates (Figure 4). Each of these four compounds have similar functionalities attached to the representative tetrahydrofuran and tetrahydropyran backbones of the structures of the ionophores: each has one carboxy, one keto, and three hydroxy groups linked to the rings (Figure 1).

The only obvious functional feature which distinguishes narasin and salinomycin from lasalocid and monensin is the unsaturated pyran moiety in the former compounds (Figure 1). *Segura* and *Gotto* observed greater fluorescence for unsaturated triglycerides and fatty acids than for the corresponding saturated homologs [2] and the same effect may account, at least in part, for the results obtained in this study.

Our final objective in this investigation was the development of methodology for quantitation of traces of residues of the ionophores in feed or animal tissue. As ionophore-containing extracts may also contain traces of lipid contaminants which could, if they elicit fluorescent responses with the VPF technique, possibly interfere with ionophore detection, a limited investigation of the VPF derivatization method was also made with several classes of lipid.

In initial experiments, comparisons of lipid responses on RP-18 and silica gel plates were made under static conditions. Five representative lipid standards were serially diluted, spotted on both types of plate, and derivatized under VPF conditions without solvent development (Figure 5). The surprising aspect of the results of this study was the low fluorescent responses displayed by these compounds after VPF derivatization. At levels between 0.16 and 0.42  $\mu\text{g}$  the lipids showed poor, non-linear fluorescence: dipalmitoyl L- $\alpha$ -phosphatidylcholine, for example, a saturated phospholipid, gave no response to this reagent (Figure 5).

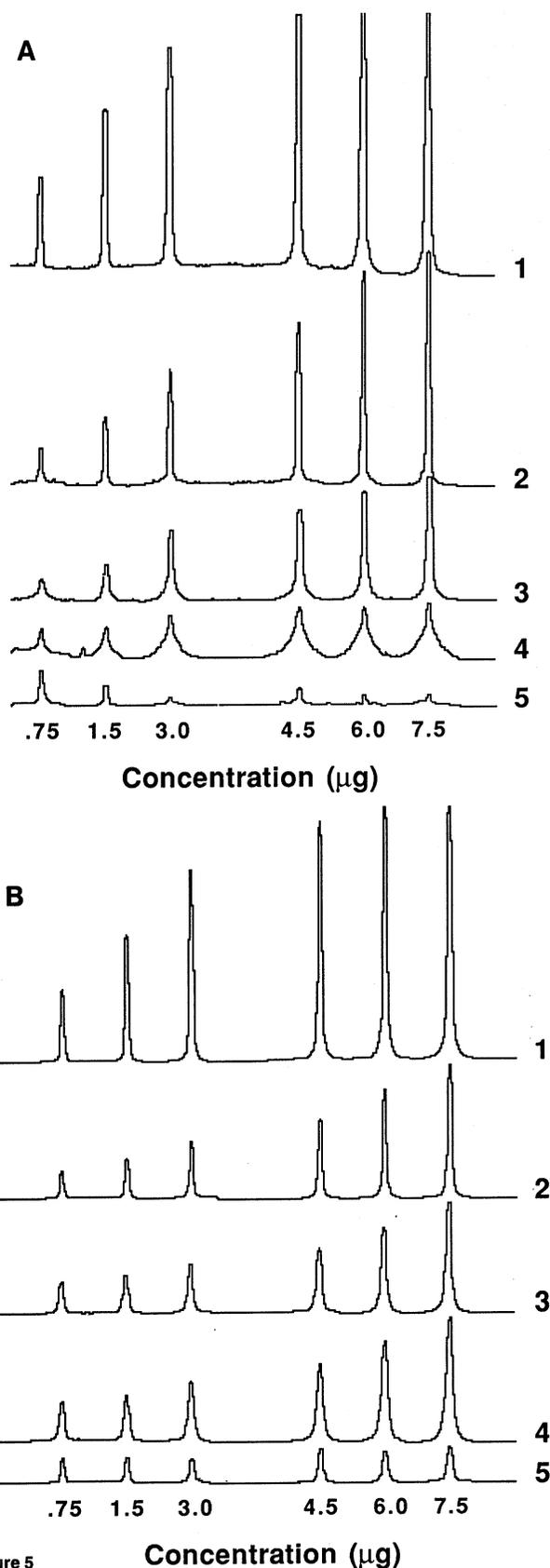


Figure 5

Fluorescent intensity of statically spotted lipids after VPF derivatization on (A) RP-18 and (B) silica gel HPTLC plates: 1, L- $\alpha$ -phosphatidylcholine- $\beta$ -linoleoyl- $\gamma$ -palmitoyl; 2, dipalmitoyl L- $\alpha$ -phosphatidylethanolamine; 3, triolein; 4, oleic acid; 5, L- $\alpha$ -dipalmitoyl phosphatidylcholine.

Fluorescent responses were, in general, about the same for both RP-18 and silica gel plates. The fluorescent responses of the same five lipids, plus cholesterol (which was not tested in static experiments) were measured after VPF derivatization following solvent development. The six compounds were spotted separately on RP-18 and silica gel plates at the 6  $\mu\text{g}$  level and after solvent development (Section 2.2) and VPF treatment, the plates were examined under UV light for evidence of the formation of fluorescent derivatives.

On the RP-18 plates, faint traces of the cholesterol derivative were visible but none of the other lipid classes tested could be detected. Under UV light, all the lipid types could be observed on the developed silica gel plates but because the spots were faint no attempt was made to quantify the results by densitometry. In essence, whereas lipids spotted statically on silica gel HPTLC plates can be detected after VPF derivatization, their fluorescence responses after solvent development are too faint to be useful for visualization.

Although other workers have reported the use of the VPF method for lipid analysis [17], on the evidence of our results it cannot be recommended for general use. In this laboratory, we have developed a method for the visualization of lipids by induced fluorescence [18,19] as an alternative to the VPF technique of Segura and Gotto [2]. In contrast with the high levels ( $> 6 \mu\text{g}/\text{spot}$ ) needed for the VPF technique, 5-30 ng quantities of individual glycerides and phospholipids can be detected after solvent development and, moreover, only relatively short heating periods (45 min) are required to induce the fluorescence [18,19] (the VPF technique requires a minimum of 4 h).

Our results with the VPF visualization technique suggest that it may be used as a qualitative method for the detection of the polycyclic ether antibiotics on RP-18 plates, since the fluorescent responses of these compounds are significantly higher than those generated by the vanillin spray technique currently used [7]: bioautography still is required for quantitative detection of these compounds at regulatory levels. The lack of fluorescence response from lipids on RP-18 plates may, in addition, be an advantage to the analysis of the antibiotics, since crude isolates containing traces of lipid contaminants would not interfere with detection of the target analytes.

In all the earlier reports of the use of the Segura and Gotto technique, VPF derivatizations were performed after separation on silica gel or alumina plates [2]. We found the intensity of the fluorescence induced for both antibiotics and lipids on silica gel plates too low to be considered useful as a visualization technique. In the light of these results, it is apparent that useful applications of this technique may only be possible in conjunction with reversed phase HPTLC plates.

#### Acknowledgments

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