

An Improved Method for Efficient Predevelopment Washing of HPTLC Plates

Summary

We have previously developed a methanol-based immersion prewashing technique for silica gel HPTLC plates to remove contaminants that interfered with quantitation of certain classes of antibiotic. The procedure yielded adsorbent surfaces significantly cleaner than those prewashed by ascending development. Subsequent to that report, however, tests revealed that some trace-level residues remained on the plates after prewashing by immersion. In this study an alternative approach to the direct-immersion washing technique was developed to minimize surface contamination further, or to eliminate it. Plates from four suppliers were first washed by ascending development with methanol, dried, and then washed by immersion in the same solvent. Surface residues remaining on the plates were visualized by fluoro- and UV densitometry. The improved, two-step ascending development-immersion prewashing technique yielded adsorbent layers that were essentially free from surface contamination, an essential requirement when residues at the ppb level and below are analyzed by this means.

1 Introduction

The activated surface of a silica gel HPTLC plate can adsorb impurities as part of the manufacturing process, upon standing in sealed packages or storage containers, or as the result of handling operations in the laboratory. To achieve satisfactory, reproducible visualization and quantitation by densitometry it is important that plates be properly cleaned before sample application and development.

There is no universally agreed technique for prewashing HPTLC plates. Reported techniques have utilized a variety

of solvent systems [1-4] involving either an ascending development prewash or full immersion in the selected solvent system [5,6]. In earlier work we evaluated the performance of several single- and mixed-solvent-system methods for predevelopment washing of HPTLC plates [7]. For example, we compared ascending development with immersion prewashing and found that the immersion technique consistently gave superior results when visualizing certain classes of antibiotic on silica gel plates [8]. The results of that survey established that methanol alone was more efficient than other solvents and mixtures, such as ethyl acetate, ethyl acetate-methanol, 1 + 1, isopropanol, and chloroform-methanol, 1 + 1, in removing residues from plates when used as part of the proposed immersion prewashing technique.

In the earlier study we observed that a small but detectable level of residual material, as visualized by fluorescence detection, remained on HPTLC plate surfaces after a methanol immersion prewash. Since then we have determined that the original study had several limitations:

- (i) only fluorescence densitometry was used to detect background contamination after plate washing whereas investigators also routinely use UV densitometry as a means of detection and this might yield artifactually enhanced signals for some plate residues not detectable by fluorescence detection;
- (ii) only data for plates from one supplier whose plates seemingly had the lowest initial background fluorescence response were reported, although plates from several suppliers were tested; and
- (iii) the textual figures were inadvertently transposed during publication of our original report [7], which made the description of the method difficult for the reader to understand and apply.

For these reasons we have re-investigated our reported plate cleaning method. Our main purpose was to determine

whether an improved plate cleaning technique could be developed that would result in adsorbent surfaces that were freer from residues than was achieved in the originally proposed method. In this study plates from four suppliers were used to compare the effects of several cleaning operations on both residue removal and changes in the surface characteristics of the activated layers before and after washing. The outcome of this study was a variant of our original method that resulted in significantly cleaner plate surfaces than could be obtained by use of the one-step methanol immersion method.

2 Experimental

2.1 Chemicals, TLC Materials, and Fluoro- and UV Densitometry

Ammonium bicarbonate (99%) was obtained from Sigma (St Louis, MO, USA). Methanol was HPLC grade from Burdick and Jackson (Muskegon, MI, USA). Plate dipping and ascending development experiments were performed in Camag (Wrightsville Beach, NC, USA) twin-trough tanks. The HPTLC silica gel 60 plates were of four types: HP-GHL 56077 from Analtech (Newark, DE, USA); NANO-SIL-.20 M811012 from Macherey–Nagel (Aston, PA, USA); silica gel 60 from Merck (Gibbstown, NJ, USA; #5633); and HP-K 4807–425 from Whatman. The Analtech silica gel adsorbent layers were 150 μm thick; those from the other three suppliers were 200 μm thick. For the purpose of this study, the plates from the four suppliers were randomly assigned a letter designation from A–D and are referred to by those letters throughout the text. Induced vapor-phase fluorescence (VPF) experiments using the method of Segura and Gotto [9] were performed in a heating device developed and assembled in this laboratory [10]. Background fluorescence was measured by densitometry with a Camag TLC Scanner II and Camag Labdata Station AT, and CATS Software. The excitation wavelength was 366 nm (mercury lamp) and a $\lambda = 400$ nm emission cut-off filter and a 2 mm \times 6 mm slit were used; the scan speed was 4.0 mm s⁻¹. Background absorption from residues were also measured in the UV absorbance mode on the same instrument at $\lambda = 200$ and 254 nm (deuterium lamp) with a 2 mm \times 6 mm slit and a 4.0 mm scan speed.

2.2 Plate Cleaning Sequences

HPTLC plates were newly purchased in shrink-wrap packages from four suppliers. Initially the new plates were visually inspected for surface imperfections and those plates with imperfections were discarded. Two 10 cm \times 10 cm plates from each supplier were labeled A and A', B and B', C and C', and D and D', respectively. Each plate then was scored and snapped into two 5 cm \times 10 cm sections. The subsets from each plate set were designated as follows: the subset from plate A was labeled A₁ and A₂ and the subset from plate A' was labeled A'₁ and A'₂. Similar designations were applied to the plates from the other three sets. The four plates from each set were scanned by UV densitometry at $\lambda =$

200 and 254 nm to establish base-level background signals and then washed by one of the two pathways outlined in Figure 1 using the plates from set A as an example. The techniques used for ascending development and immersion plate washing are described in detail in Section 2.3.

2.2.1 Original Plate-Washing Scheme (Immersion Wash \rightarrow Ascending Development Wash)

Plates A₁ and A₂ were immersed for 5 min in a developing tank filled to a depth of 15 cm with methanol (Section 2.3.2) after which they were removed from the tank, air-dried and heated in an oven set at 80°C for 15 min. Upon cooling, the plates again were scanned by UV densitometry at $\lambda = 200$ and 254 nm. From this point the two plates were processed separately. Plate A₁ was subjected to induced fluorescence (VPF), in the manner described in Section 2.4, then fluorescence densitometry at $\lambda_{\text{ex}} = 366$ nm (Figure 1) whereas plate A₂ was placed in a tank containing methanol and washed by ascending development (Section 2.3.1). After air and oven drying, plate A₂ was scanned by UV densitometry, subjected to induced fluorescence (VPF) and finally scanned by fluorescence densitometry. The same sequence of washing experiments was performed on plates from the other three suppliers (B₁ and B₂, C₁ and C₂ and D₁ and D₂) simultaneously with those performed on plates A₁ and A₂.

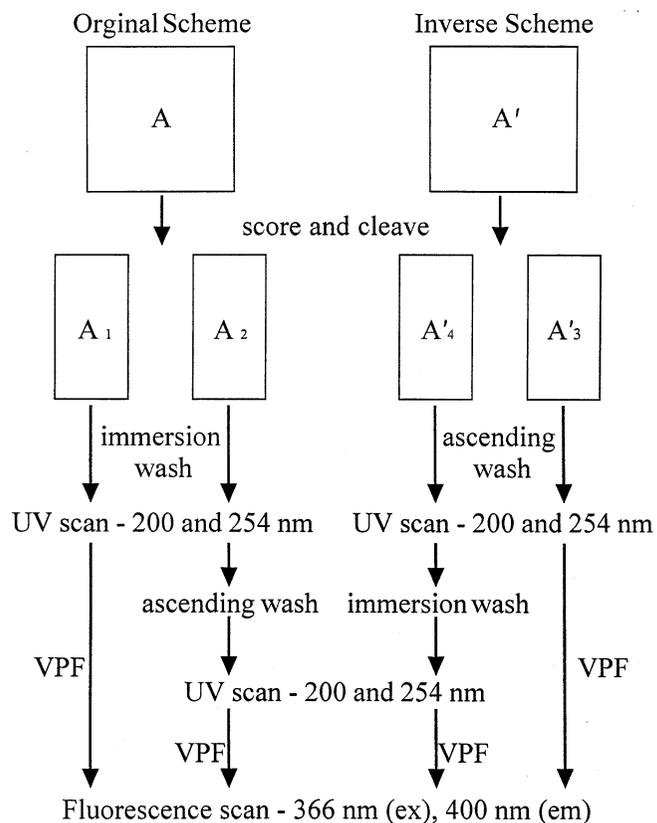


Figure 1

Schematic diagram of two methanol-based plate-washing schemes performed on two 10 cm \times 10 cm (A and A') silica gel HPTLC plates from the same supplier.

2.2.2 Inverse Plate-Washing Scheme (Ascending Development Wash → Immersion Wash)

The washing sequences performed on plates A'_1 and A'_2 were the inverse of those described for A_1 and A_2 , in that both plates were washed first by ascending development (Section 2.3.1) and scanned by UV densitometry. Plate A'_1 then was subjected to induced fluorescence (VPF) followed by fluorescence densitometry at 366 nm whereas Plate A'_2 was immersion washed, dried, scanned by UV, treated by VPF and finally scanned by fluorescence densitometry at $\lambda_{\text{ex}} = 366$ nm. (Figure 1). The same sequence of washing experiments was performed simultaneously on the plates from the other three suppliers.

2.3 Ascending Development and Immersion Plate-Washing Procedures

2.3.1 Ascending Development Wash (One-Dimensional)

Methanol (*ca* 10 mL) was placed in each trough of a Camag twin-trough development tank. The HPTLC plates were placed on one side of the trough and developed to within 5 mm of the upper edge. They were then removed from the tank, air-dried for 5 min, oven dried for 15 min at 80°C, cooled, and stored in a desiccator until needed.

2.3.2 Immersion Plate Washing

A Camag development tank was filled to a depth of 15 cm with methanol. The HPTLC plates were immersed in the tank for 5 min, removed and air-dried for an additional 5 min. The plates then were placed in an oven 80°C for 15 min, cooled, and stored in a desiccator until needed.

2.4 Vapor Phase Fluorescence (VPF) Visualization of HPTLC Plate Surfaces

Enhanced induction of background residue signals on the HPTLC plates was performed by the technique of *Segura* and *Gotto* [9]. An in-house designed and assembled glass heating chamber [10] was charged with ammonium bicarbonate (2 g). The HPTLC plates were placed on a stainless steel rack inserted into the glass heating chamber. The chamber then was sealed with a gasket, glass cover, and clamp, and placed for 90 min in a forced air oven at 130°C. After heating, the chamber was cooled, unsealed, and the plates were removed and scanned in the fluorescence mode with the Camag TLC Scanner II.

2.5 Two-Step Plate-Cleaning Method for Surface Residue Removal

1. Place HPTLC plate in a twin-trough tank containing 10 mL methanol in each trough.
2. Develop plate until solvent reaches the upper edge.
3. Remove plate from tank and air-dry for 5 min.

4. Immerse plate for 5 min in a second tank containing 500 mL (15 cm) methanol.

5. Remove plate from tank, air-dry for 5 min, and oven-dry for 15 min at 80°C.

6. Cool plate in a desiccator before use.

3 Results and Discussion

In our earlier investigation of plate-cleaning techniques, we compared the efficacy of cleaning HPTLC plates by ascending development and immersion washing [7]. In those comparisons plates were first prewashed in methanol using either ascending development or immersion dipping. Each plate then was examined by fluorescence densitometry after pretreatment by vapor phase fluorescence (VPF) visualization (Section 2.4). (VPF is a technique developed by *Segura* and *Gotto* [9] that induces or enhances fluorescence in non-to weakly fluorescent compounds on silica gel TLC plates). The results of that study are illustrated by the densitograms shown in **Figure 2**. Figure 2A is a densitogram of a plate from that study after an ascending development wash. Note that the surface residue on the plate was carried with the solvent front during development and subsequently occupies a large portion (70–90 mm) of the upper surface of the plate.

In contrast with that result, the chromatogram of a plate after immersion dipping was, visually, relatively free from interfering background fluorescence response Figure 2B. However, when the plate washed by immersion (Figure 2B) was further subjected to an ascending development wash (Figure 2C), diffuse residual material migrated to the upper portion (75–85 mm) of the plate and could then be visualized, demonstrating that immersion washing had not entirely removed all of the surface residue.

In this study, we have re-investigated the above HPTLC plate-cleaning technique with the aim of developing a cleaning procedure that will completely eliminate surface residue. To perform this investigation we devised the washing sequences outline in Figure 1, which shows the pathway of our original scheme (plate A) and the newly devised inverse of that scheme (A'). Each washing scheme then follows two pathways. In the original scheme [7] a single HPTLC plate, represented in Figure 1 as A, was cleaved into two sections A_1 and A_2 . Both sections were subjected to an immersion wash. Plate A_1 was then scanned by UV densitometry, subjected to VPF, and finally scanned by fluorescence densitometry. (It was not possible to perform all the steps in this sequence on a single plate, because the VPF treatment irreversibly alters the surface characteristics of the plate.) The UV and fluorescence densitograms for plate A_1 after immersion washing are shown in **Figure 3**. The plates from suppliers B_1 , C_1 , and D_1 after immersion washing also are presented in the same figure and column as A_1 . Before immersion washing, densitograms of the plates (A–D) were acquired from the plates as they were received from the four suppliers. The densitograms (not shown) of

cence densitograms for plate A₁' after ascending development are shown in **Figure 4A**. The plates from suppliers B₁'–D₁', which were washed simultaneously with plate A₁', also are shown in the same figure.

The densitograms shown in **Figure 4** dramatically illustrate the disadvantage of using a single ascending development wash to clean HPTLC plates – irrespective of the wavelength scanned a significant, large peak is obtained from compressed surface residues in a wide area of the upper portion of each plate. Compressing the surface residue at the top of an HPTLC plate does, however, offer an important advantage when ascending development is used as part of the proposed two-step cleaning sequence. This was demonstrated by subjecting the plates washed by ascending development to a subsequent immersion washing step (Figure 1, plate A₂'). The densitograms recorded for plates A₂'–D₂' from this two-step sequence are illustrated in Figure 4B. Observe in this figure that the plates are, with one exception, almost free from surface residue. The efficiency of this two-step method is especially apparent for the plates scanned at $\lambda = 366$ nm; the densitograms depicted have flat baseline responses with no detectable surface contamination. Additionally, the surface characteristics of the silica gel layers do not seem to be adversely affected by the additional washing step in the sequence, as evidenced by the lack of enhanced background signal noise at all three wavelengths scanned.

The results of these studies illustrate the unique properties of methanol as a solvent for prewashing HPTLC plates. When this solvent is used in a one-step immersion prewash process (Figure 3), the resulting silica gel surfaces might be sufficiently free from residues for most applications. However, the two step methanol prewash method described in Section 2.5 improves upon the single-step process first by concentrating the residue at the top of the plate, then by removing this material from that location more efficiently than when it is distributed over the entire plate surface. The use of this two-step HPTLC plate cleaning method should be considered in critical applications when optimum sensitivity is required [5], and when the presence of any surface residue might interfere with analyte detection and quantitation.

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

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- [4] R.D. Coker, K. Jewers, K.I. Tomlins, and G. Blunden, *Chromatographia* **25** (1988) 875–880.
- [5] H. Celoms, *J. Planar Chromatogr.* **6** (1993) 337–340.
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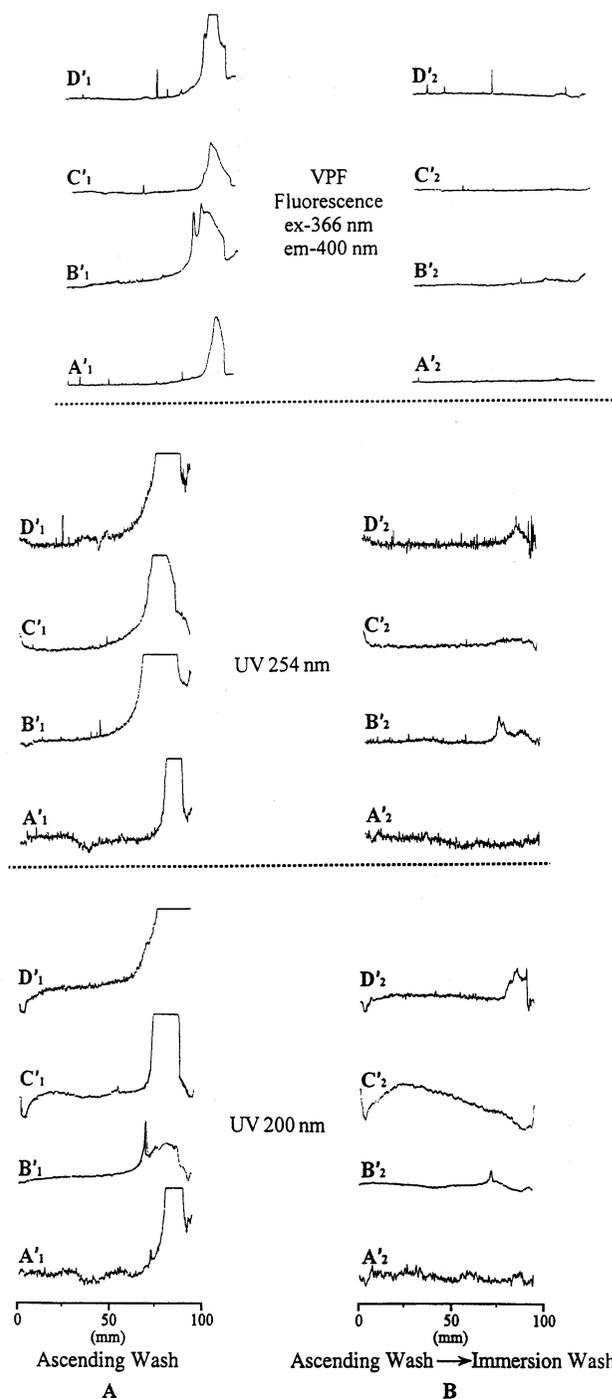


Figure 4

UV and fluorodensitograms of silica gel HPTLC plate surfaces after (A) initial ascending development washing and (B) after subsequent immersion washing.

- [7] R.J. Maxwell, S.W. Yeisley, and J. Unruh, *J. Liquid Chromatogr.* **13** (1990) 2001–2011.
- [8] R.J. Maxwell and J. Unruh, *J. Planar Chromatogr.* **5** (1992) 35–40.
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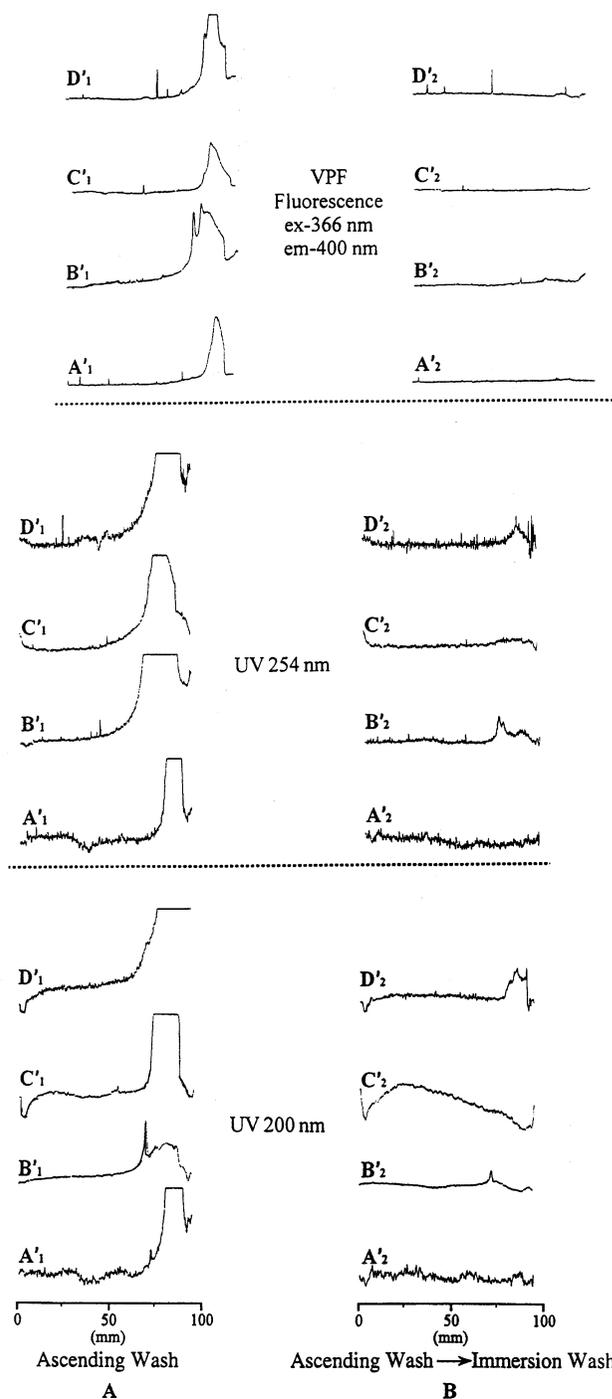


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