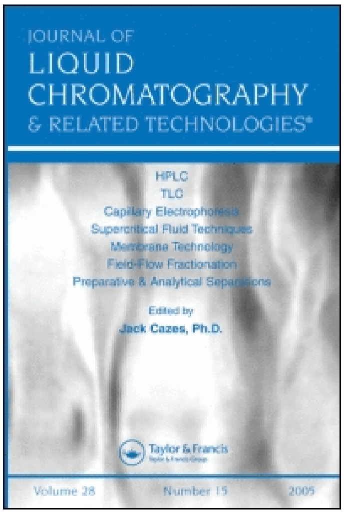

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TWO PHASE, TWO-DIMENSIONAL TLC FOR FINGERPRINTING AND CONFIRMATION PROCEDURES

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Two-dimensional /2D/ chromatography was first described by Kirchner in 1951. A sample applied to one corner of a 20 x 20cm plate approximately 1.5cm from each edge is developed in a mobile phase that resolves the polar constituents. It is then dried, rotated 90 degrees to the first direction and placed in a second mobile phase designed to resolve the more non-polar components. In this manner, a multitude of diverse compounds can be resolved.

The versatility of an open surface to separate a variety of complex samples gained momentum and in

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1962 Stahl's Handbook on TLC referred to this method as the SRS technique and noted its ability to characterize changes in a given matrix after environmental exposure to Gamma rays, X rays, U.V. light, gases or heat. By 1967, Kirchner's Volume XII of Techniques of Organic Chemistry on TLC cited 77 references on two-dimensional TLC.

The technique further diversified to the use of dual media combinations. Strips of magnesium silicate with silicic acid were used on Bergamot oil, carbon and silica gel on ketones, and quite common was the use of parafin, silicone oil or undecane impregnated in the layer after the first development to create a reversed phase partition mode for the second direction. A wide variety of compounds were separated by these techniques from simple antioxidants in motor oil to serum proteins all dealing with complex matrices.

In studying these works and the comments of Stahl, it is apparent that not only can individual compounds be identified, but that the pattern of developed spots in a 2D development can become a fingerprint of the sources and further, that changes within the source can be identified as normal or abnormal, based on that pattern. This technique has been referred to as fingerprinting. Literature references on the identification of oil sources from ocean oil spills are now quite common.

Developing procedures for this method can prove quite harrowing, however, especially given the crude or complex nature of the starting sample. Samples often streak in the second dimension if composition differences are great. Reproducibility is poor under the best of conditions.

Recently it has been demonstrated that reliable procedures can be developed for fingerprinting emploxing a TLC plate which is composed of a three centimeter octadecyl bonded silica gel strip for reversed phase chromatography in one dimension and the remainder of the plate composed of a standard silica gel with high capacity for adsorption chromatography in the second dimension. Whatman refers to this combination as the Multi-K CS5 plate. The uniqueness of the combination resides in the ability to chromatograph without transfer, a complex mixture by both a non-polar mechanism and by the usual adsorption mechanism that deals with the compound's polarity differences.

The sample is applied to the reversed phase strip of the plate at a point 1.5cm from the edges and developed in the reversed phase partition mode, utilizing polar solvents. A <u>major benefit</u> at this point is that when working with crude samples, the usual interfering polar mass migrates to the solvent front, allowing a much cleaner analysis in the adsorption mode. Several operational points must be made at this stage.

1. The entire layer should be prewashed when dealing with these complex mixtures. This is accomplished by sandwiching several plates together with paper wicks at the end so all elutable material will migrate to the paper. Methanol/Acetone /50/50/ is best suited as a solvent for this task. Several plates may be washed simultaneously overnight and then activated at 110°C for one hour. Plates should then be stored in a dessicator so as not to readsorb environmental contaminants.

2. Chambers should be equilibrated for 10 minutes with paper pads for all developments.

3. To obtain an even solvent front in the first direction, scrape sufficient silica gel off the plate so that it is not immersed in the first mobile phase. An alternative to this is to spray the silica gel lightly with ethanol /masking the reversed phase strip/. In this method, the objective is to deactivate the silica gel sufficiently for the solvent front to migrate uniformly /Fig. 1 /.

4. Finally, the reversed phase strip, because of its binding requirements, must contain 0.5 M sodium chloride or equivalent buffer salt when mobile phases are used that contain in excess of 40% water. Ammonium acetate has also been used successfully, which of course, completely volatizes on drying the plate at 110°C for 10 minutes.

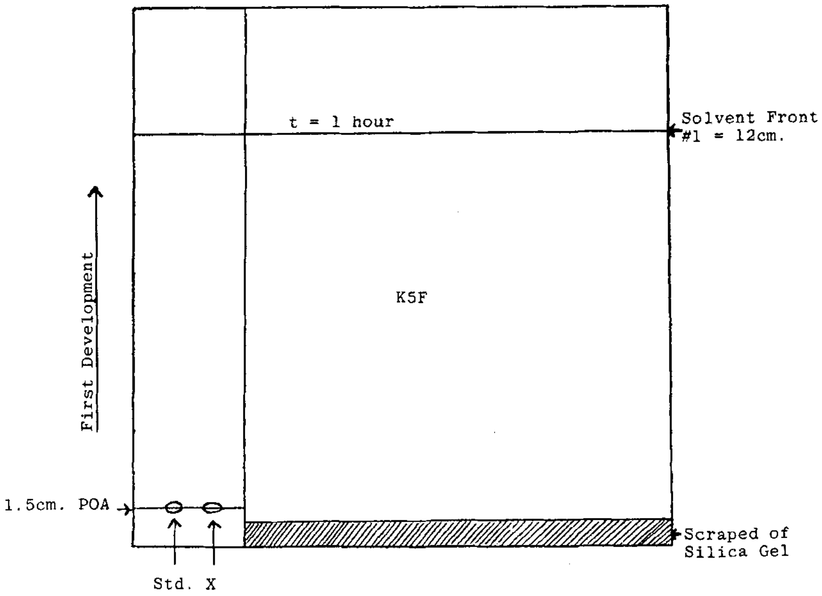

FIGURE 1

Separation in the second direction, i.e., the adsorption mode, on the CS5 plate, generally utilizes solvents of lower polarity to further resolve the components of the mixture. Typical K5F silica gel separations are obtained. A key feature at this point is the ability of these types of mobile phases to admix standards into the unknown while in the reversed phase strip to confirm identity /Fig. 2 /.

A known reference standard is, in effect, co-chromatographed in the second dimension. Admixing is commonly used in the adsorption mode by placing a known standard over the unknown mixture and co-chroma-

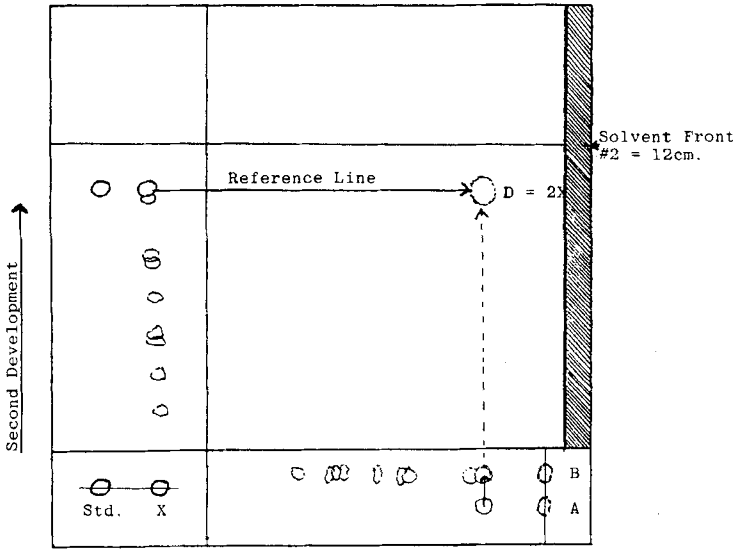

FIGURE 2
A moves to B and migrates as D with an increase in intensity = A + B

tographing. If the known and the unknown do not indicate separation after developing in the two directions, confirmation of the unknown's identity is assumed. Combining these two widely differing separation mechanisms, reversed phase partition chromatography in the first dimension, with adsorption chromatography in the second dimension, increases the reliability of this technique.

An example of this methodology could be the confirmed presence of morphine in a suspected urine specimen. A urine sample was extracted in the usual manner and 10 μl of extract was applied to the reversed phase strip with a known quantity of standard analyte /Morphine/ applied next to it. It was developed up the reversed phase strip in Methanol /0.5 M Sodium Chloride solution /65/35/. After drying at 65°C for 20 minutes, the plate was again spotted with extract and standard at 90 degrees to the first direction and developed in the second mobile phase of Ethyl Acetate/Methanol/Water/28% Ammonium Hydroxide /85/13.5/1.0/0.5/ to 12 cm. After detection with methanolic Iodoplatinate reagent, the original suspected spot showed a greater intensity, indicating co-chromatography and confirmed identity.

Another example in a more difficult area for toxicology would be methadone confirmation.

The most intriguing application of this technique is in the area of fingerprinting. An example is lubricating oils taken from three different sources. The crude samples were extracted by shaking with 0.5% Acetic Acid in Methanol in equal volumes for 5 minutes and centrifuging to obtain a clear supernatant. Ten microliters of this supernatant were applied to the reversed phase and developed in 18 minutes to the 12 cm level in Acetonitrile/Water/Acetic Acid /90/10/1/, dried for 20 minutes at 65°C and then developed in the second direction to 12 cm /22 minutes/ in Hexanes/Chloroform /80/20/. After drying the plate, it was reacted by dipping in a 5% solution of Phosphomolybdic Acid and heated for 5 minutes at 120°C. Characteristic and reproducible spot patterns

/fingerprints/ were obtained which may be used in screening to denote changes in composition and/or to identify additives and therefore, possible sources.

Of particular note is the ability to develop mirror images by spotting the reversed phase strip at either end and developing toward the center line in the first mobile phase. Comparisons are made more easy /Fig. 3 /.

Secondly, known standards may be spotted at the outside edges in the second mobile phase development to establish identity. It is then possible to go back to scheme one to establish confirmation.

Coefficients of Variation for these oil patterns ran from a low of 4% for one spot to a high of 12%. It appears that some compounds are more variable and under some unknown influence contributing to this variation more than others.

A more recent example of the use of this technique is the identification and quantitation of several targeted sulfonamides in the muscle and liver tissue of turkeys and chickens. There are thirteen known sulfonamides currently in use. Of these, five are suspected carcinogens and levels have been set by the United States Department of Agriculture for their presence in meat products. They are often used in combination and therefore assays had to be developed to quantitate each of the targeted sulfonamides.

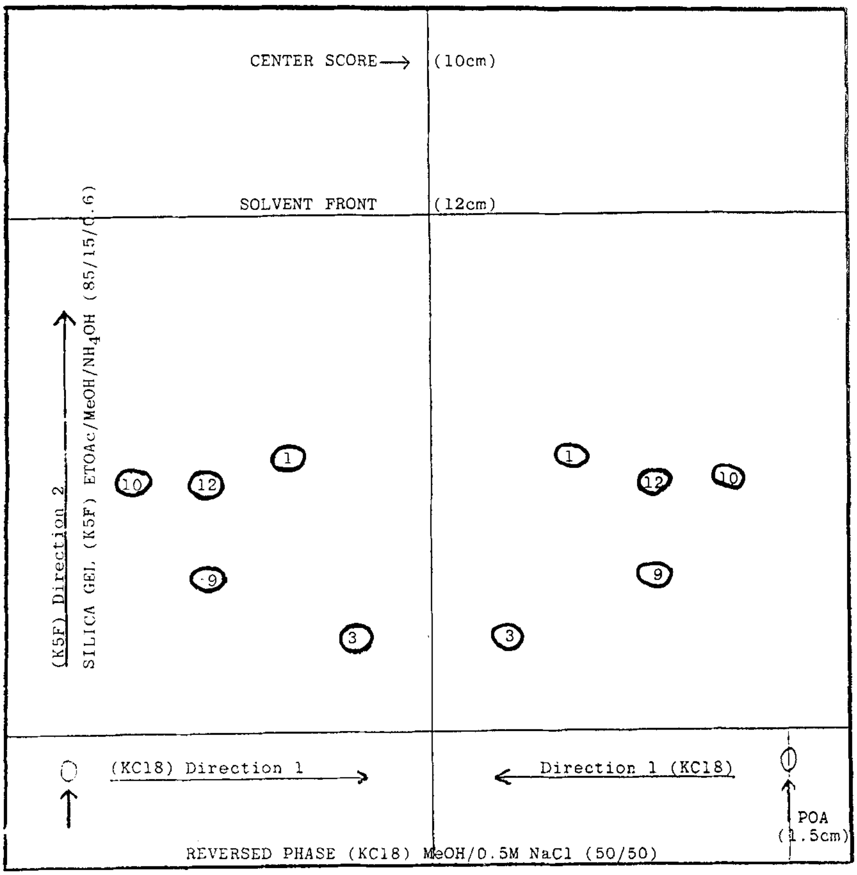

FIGURE 3
SEPARATION OF FIVE TARGET SULFONAMIDS

1. Sulfamethazine
3. Sulfathiazole
9. Sulfaquinoxaline
10. Sulfabromomethazine
12. Sulfadimethoxine

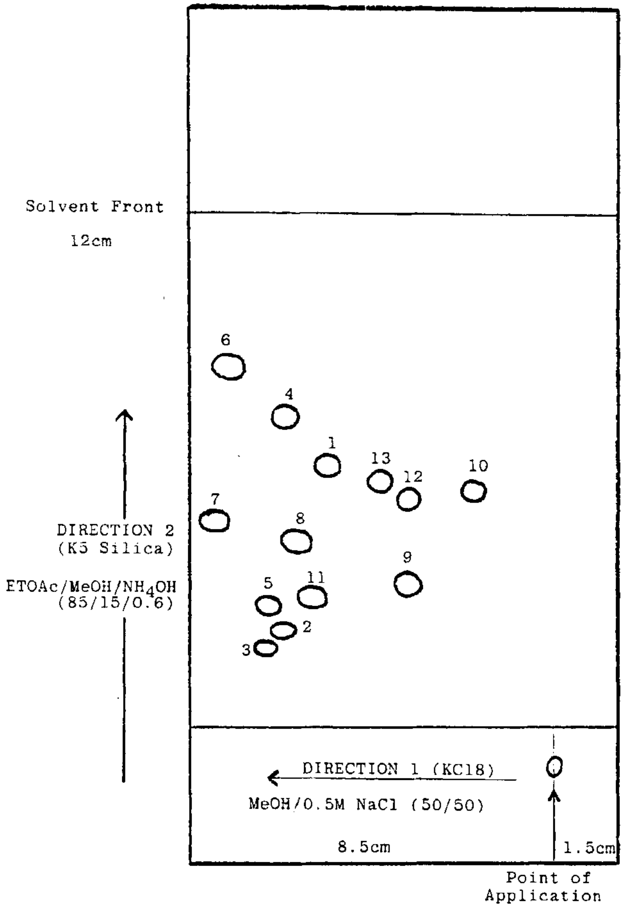

FIGURE 4.

1. Sulfamethazine
2. Sulfisoxazole
3. Sulfathiazole
4. Sulfapyridine
5. Sulfadiazine
6. Sulfanilamide
7. Sulfaguanidine
8. Sulfamerazine.
9. Sulfaquinoxaline
10. Sulfabromomethazine
11. Sulfachlorpyridazine
12. Sulfadimethoxine
13. Sulfaethoxypyridazine

Standards of the thirteen sulfonamides separated from the matrix indicated a very clean and clear picture. A transparency can be prepared therefore, that when overlayed on a developed and visualized chromatogram, unknowns may be quickly identified. Both liver and muscle tissue responded identically /Fig. 4/.

An internal standard curve is recommended for quantitating. This can be accomplished by spotting two

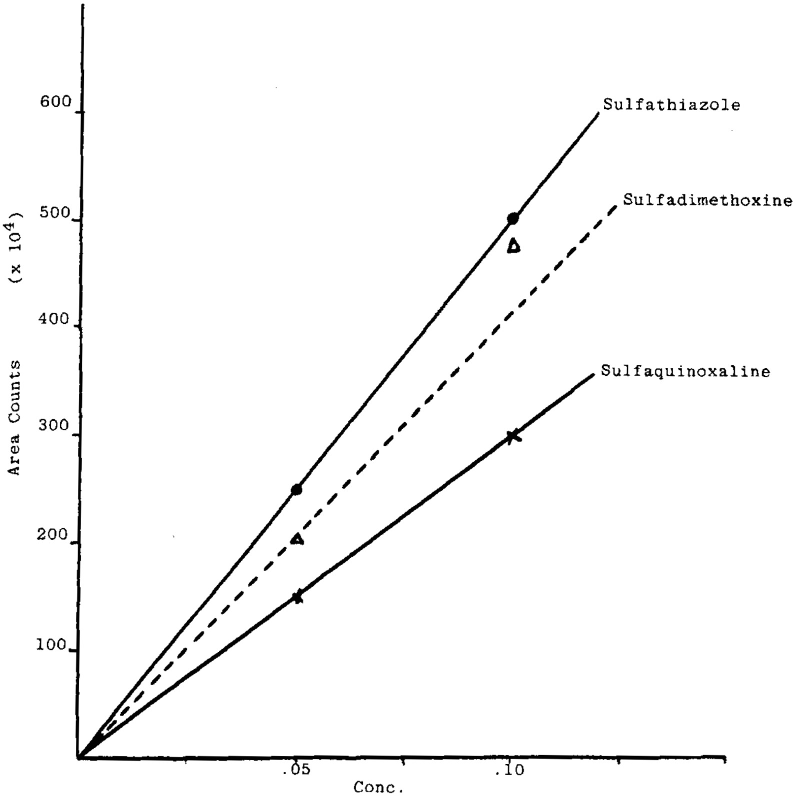

FIGURE 5

dilutions of chosen standards at the outside edges in the second development. From a scan of these two samples, a deviation from the standard curve can be established and a correction computed /Fig. 5/.

For the sulfonamide assay, standard deviations of 0.005 to 0.29 were computed, giving a coefficient of variation of 1-8%.

The two dimensional, two phase separation technique can be a reliable method for fingerprinting complex mixtures in crude extracts and perhaps,more importantly, as an identification method for solutes by comparison to the migration of authentic reference compounds in this two stage chromatography method.