

Simple, At-Site Detection of Diethylene Glycol/Ethylene Glycol Contamination of Glycerin and Glycerin-Based Raw Materials by Thin-Layer Chromatography

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This paper describes a rapid, inexpensive thin-layer chromatographic (TLC) method that separates diethylene glycol (DEG) from glycerin and other glycols. Studies with collaborating laboratories of the World Health Organization have shown that about 6% DEG in glycerin and about 2% DEG in acetaminophen (paracetamol) elixirs may be detected by direct visual inspection of the developed TLC sheets. Staining the sheet permits detection of DEG at less than 0.1%. The method costs less than \$1.00 per test and takes 20 min by visual inspection, longer when staining is required. The visual method can be performed without laboratory facilities by personnel having little previous training. Samples testing positive by the visual method can be submitted to a laboratory for confirmation and quantitation of DEG.

In 1937, 105 Americans died of renal failure after ingesting a sulfanilamide elixir containing 72% diethylene glycol (DEG) as a diluent (1). Despite this notorious incident, additional deaths due to DEG poisoning continue to occur (2). The fourth largest outbreak of DEG poisoning associated with pharmaceutical products occurred in Haiti in 1996, 59 years after the U.S. sulfanilamide elixir episode, and was linked to the deaths of more than 80 children (3,4).

Glycols such as glycerin are present in many formulations of medicinal, cosmetic, and food products. As a

result of world trade, glycols contaminated by DEG or ethylene glycol may unwittingly be accepted for use in manufacture of pharmaceuticals. Investigations of DEG-related poisonings in various countries have repeatedly revealed lack of testing for contamination and other quality-control measures at ports of entry or during the pharmaceutical manufacturing process (2-6).

The minimum safe level for humans for ingested DEG is not known, but at a 1997 interagency workshop (6) supported by the U.S. Centers for Disease Control and Prevention (CDC), the U.S. Food and Drug Administration (FDA), and the World Health Organization (WHO), a detection level of 0.1% DEG was considered adequate for screening raw materials and glycerin-based elixirs.

Earlier, we reported the development of a novel TLC system for rapid screening analysis of many drugs (7-9). The purpose of this work was to adapt this method to detect DEG in glycerin in raw materials and in liquid oral pharmaceuticals.

We required that the method (1) distinguish DEG from related components (e.g., glycerin, and ethylene and propylene glycols) often present in glycerin and glycerin-based materials and products; (2) be sufficiently sensitive and reliable to prevent future DEG-related fatalities; (3) allow detection of gross DEG contamination (about 6% DEG in glycerin and about 2% DEG in elixirs) through direct visual inspection of the sheet without spraying and at locations without laboratory facilities, such as at ports of entry; and (4) allow detection of DEG at 0.1% through spraying or staining the sheet. (Throughout, we use percentage of DEG to refer to the percentage in the original sample before dilution.)

Separation of glycols by TLC has been reported previously (10,11), although with developing solvent systems not used here. Because glycols are not visible by UV illumination, researchers (12,13) have used a variety of staining sprays to make the spots visible.

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Experimental

The chemicals and solvents used for the TLC developing solvent (toluene, acetone, and ammonium hydroxide), as well as reference DEG, glycerin, ethylene glycol, propylene glycol, and sorbitol, were all pure grade obtained from commercial sources. Toluene and acetone were chromatographic grade, but reagent grade solvents also are satisfactory.

Samples were prepared or obtained from various sources. Some were representative samples that had been analyzed by the CDC, and the FDA National Forensic Chemistry Center (NFCC) in Cincinnati, OH, by gas chromatography (GC) and GC/mass spectrometry (GC/MS).

The rapid screening TLC apparatus used in this work (Figure 1) and its operation have been previously described (7, 8; U.S. Patent 5,350,510). The apparatus was produced by Granite Engineering (Granite City, IL). Although the TLC method described here is not specific for this apparatus, the apparatus was used because of its ease, reliability, cost, and portability.

Preparation of TLC Sheets

Merck plastic-backed silica-coated 20 × 20 cm sheets or equivalent, either with or without fluorescent

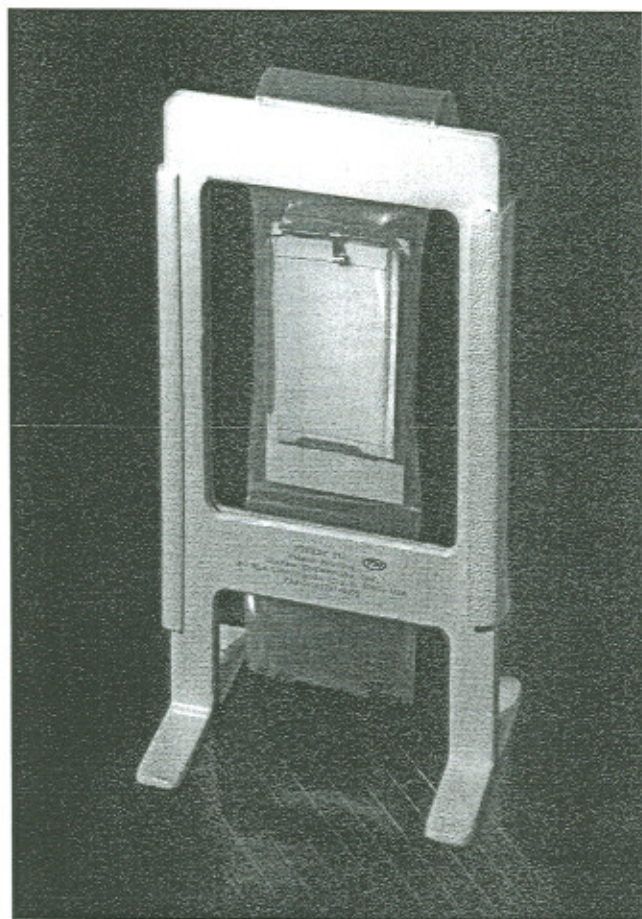


Figure 1. Apparatus for rapid-screening TLC.

indicator, were used: At low DEG concentrations, best results were obtained with fluorescent sheets.

Sheets were cut into 8 equal parts, each 5 × 10 cm. To prevent edge effects, 1 mm of the coating layer was removed from the sides and the bottom of the sheet by bearing down on a No. 2 pencil along a straight edge. A spotting line was drawn 2.5 cm from the bottom of the sheet without cutting into the coating by very lightly applying a pencil tip. Three spotting locations were marked at 1.5, 2.5, and 3.5 cm from the left edge. The development stopping line was marked by placing a straight edge across the sheet 1 cm from its top and removing 1 mm of the silica coating by bearing down on a pencil.

Preparation of Solutions

To reduce viscosity, glycerin and other glycols were diluted 3-fold with methanol before spotting. Typically, 1 mL methanol was added to 0.5 mL glycol in a 1.5 mL plastic centrifuge tube graduated at 0.5, 1.0, and 1.5 mL. Lower concentrations were prepared by diluting portions of the initial solutions with methanol. Solutions of pure glycols were used as reference materials to assign spot positions. The developing solvent was toluene-acetone-5M ammonium hydroxide (5 + 85 + 10), which was prepared daily.

Spotting and TLC Sheet Development

This TLC system accommodated 2 sheets, with 3 samples or reference solutions spotted on each sheet. The sample or reference solutions were spotted at the marked positions with a 3 μ L capillary pipette. A reference solution of DEG was always applied as one of the spots on each sheet. The spots were allowed to dry for ca 10 min.

The spotted sheets were clamped onto aluminum trays in the system. (The developing solvent may be reused for additional analysis during the same day by replacing the volume lost in the saturation pads.) Developing solvent (24 mL) was added to the plastic bag, and the assembled sheets were inserted into the polyethylene bag until the large saturation pad just touches the solution of developing solvent. The assembly in the bag was positioned with a wire rod (fabricated from a coat hanger), which also serves as a tool for removing the assembly from the bag after development. The bag containing the assembly was clamped in the system's metal support, which sealed the top of the bag to maintain a uniform environment.

Spot Migration and Detection

The system allowed 2 min to equilibrate. The bottom of the bag was gently pulled down, forcing the developing solvent onto the TLC sheets to initiate migration. At this point, the bottoms of the TLC sheets

were immersed in the developing solvent to a depth of 1 cm. The developing solvent was allowed to migrate upward until the solvent front reached the scored top line on the sheets (ca 20 min). Assembly containing the sheets was removed from the bag with the positioning rod, and the sheets were allowed to dry in air.

At high concentrations of DEG (6% or more in glycerin; 2% or more in elixirs), the spots could be detected visually by transmitted daylight. The spots appeared dark relative to the sheet because of the difference in refractive index. At lower concentrations of DEG, the sheets were stained to make the spots visible. The Appendix details 2 methods for staining.

Results and Discussion

Three developing solvent mixtures were evaluated: a, *n*-butyl alcohol saturated with 1.5M ammonium hydroxide; b, chloroform-acetone-5M ammonium hydroxide (10 + 80 + 10); and c, toluene-acetone-5M ammonium hydroxide (5 + 85 + 10). The development time for solvent a was 75 min, whereas solvents b and c each required 20 min. Because the WHO has banned use of chloroform in a TLC developing solvent because of its carcinogenicity, the chloroform in solvent b was replaced with a mixture of toluene and acetone (1 + 1) to form solvent c to achieve approximately the same solubility behavior. Solvent c proved to be the most

satisfactory because its migration time was less than that of solvent a and it did not contain toxic chloroform found in b. The development times for solvents b and c were identical.

To detect 0.1% DEG, we tried different staining procedures. Most involved spraying with highly toxic (e.g., benzidine) or costly materials, requiring use of a well-ventilated hood, a face mask for the operator, and a well-equipped laboratory. Essentially, they ruled out performing the test in open, nonlaboratory environments.

Visual observation of spots in bright transmitted white light was the simplest and the most effective detection method when the DEG content was >2% in elixirs or >6% in glycerin. Figure 2 shows the appearance of spots on developed sheets. Circles were drawn around the spots to mark their locations. Sample A was pure glycerin. Sample B contained 4.5% glycerin and 25.5% DEG; DEG was readily detected but not glycerin. Sample C contained 18% glycerin and 18% DEG; both DEG and glycerin were easily seen. Samples D and E were syrups containing no drug. Sample D contained 2.2% glycerin and 14% DEG; sample E contained 67% glycerin and no DEG.

Table 1 lists retardation factors (R_f values) for reference glycols in the 3 solvent mixtures (solvents a, b, c), and illustrates that glycerin, DEG, and other glycols can be detected by matching R_f values of sample spots

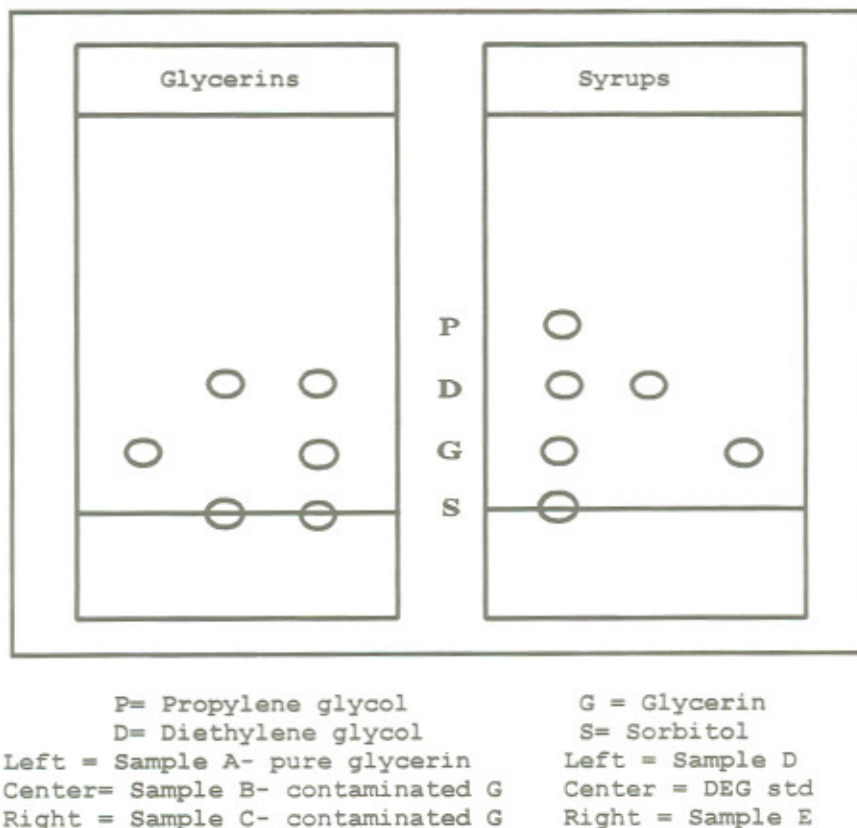


Figure 2. TLC separation of diols.

Table 1. Retardation factor values (R_f) for glycols

Glycols	Solvent	Solvents
	A	B and C
Glycerin	31	23
Diethylene glycol	52	33
Ethylene glycol	57	34
Propylene glycol	65	56
Sucrose	0	0
Sorbitol	0	0

with those of authentic references. No study of the possible variation of R_f with temperature or humidity was made.

The method also can be used to estimate the concentration of DEG contamination in glycerin or syrup samples, by spotting solutions with different concentrations of DEG and comparing spot intensities. In this procedure, the intensity of a sample spot (and hence the amount or concentration of DEG) can be estimated visually to be between certain limits, bracketed by 2 reference DEG spots of different concentrations. Detection with only transmitted light worked well with the samples studied, because the concentration of DEG in the glycerin ranged from 18 to 25%.

Several stains that enhance the detection of glycols (10) have been reported, but most are toxic and costly, require operation in a hood to protect the analyst from the harmful sprays, and lack sensitivity. Many recommended stains are applicable only for high concentrations of glycol. Use of these stains was judged impractical after several trials. As recently as 1996, the

Pharmacopeia of India (14) described a staining procedure using benzidine to detect DEG at the 0.1% level. This method worked well in our hands but is not recommended for use in open places or without suitable laboratory facilities. Two procedures were found feasible for use in open, nonlaboratory places, namely, iodine vapor/starch spray and oxidation of the glycols with potassium permanganate. Details are described in the Appendix. Figure 3 shows detection of DEG in glycerin at concentrations ranging from 0.1 to 0.75% as measured by densitometry at 400 nm after sheets were stained with iodine vapor and sprayed with starch. The densitometer was used to obtain performance data and to show that spot intensities were a function of concentration. The densitometer response for the 0.1% concentration was low and erratic, and that point is not shown; however, the spot was visible to the eye. Responses were linear, with a correlation coefficient of >0.99 , based on average values obtained in duplicate runs by 3 analysts. Responses from solutions of higher DEG concentrations were also linear. Intensities from different sheets could not be compared because of differences in staining and fading; standards and samples must be run on the same sheet. An estimate of the concentration can be determined visually without instrumentation by spotting solutions of bracketing concentrations of DEG along with sample.

Children's acetaminophen elixir obtained from a local pharmacy was spiked with DEG. Samples of the laboratory-contaminated product were diluted to various concentrations down to 0.1% DEG. The developed spots were oxidized with potassium permanganate. An actual separation at the level of 0.25% DEG is shown

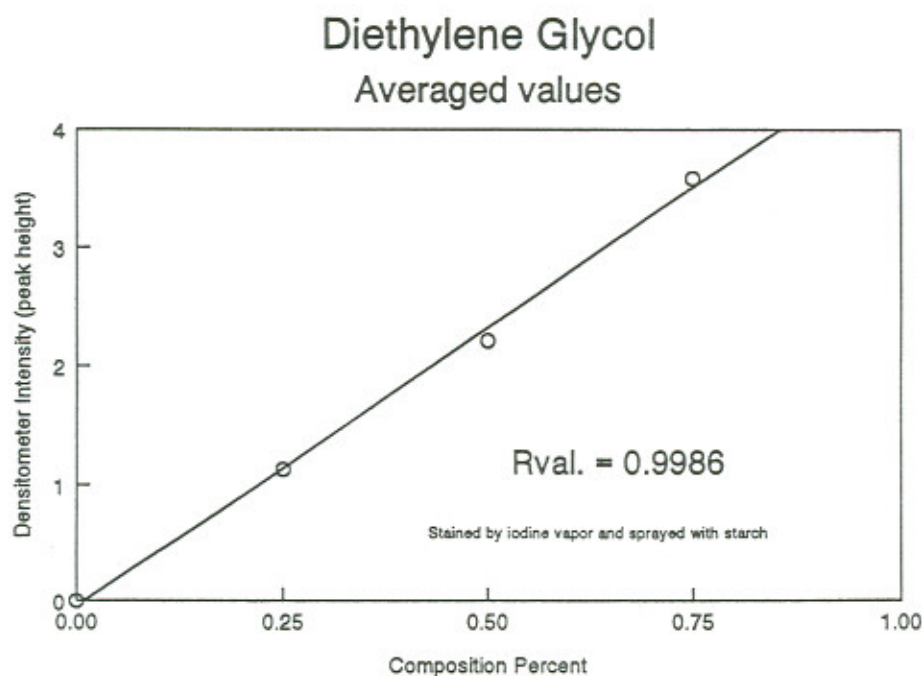


Figure 3. Detection of low DEG concentrations.

in Figure 4 as yellow spots on a purple background, demonstrating the ability to separate sugars (sucrose and sorbitol), glycols, and other components in a final dosage form. (Color photo of Figure 4 can be supplied upon request.) The listed major components of the children's acetaminophen elixir were citric acid, glycerin, propylene glycol, sucrose/sorbitol, and acetaminophen; the minor components did not interfere with separation of glycols.

Because spot size is proportional to concentration, at lower concentrations the spots can be very small and difficult to detect. The detection limit can be lowered just by increasing the volume. If the volume of material spotted is increased from 3 to 15 μL , the detection limit for DEG can be lowered from 0.1 to 0.05%.

Detection levels for contamination of glycerin were different from those for contamination of elixirs or syrups. Because of its high viscosity, glycerin required at least a 3-fold dilution for the contaminant to separate, which meant that the detection limit must be higher for pure glycerin than for samples of elixirs or syrups. With care, DEG could be detected in glycerin down to a level of 0.025% with iodine vapor/starch spray and down to 0.01% in elixirs or syrups with permanganate.

Staining by oxidation with permanganate was faster, easier to perform, and more sensitive, and it eliminated the need for spray equipment and a large glass developing tank, but the spots faded faster. Selection of the

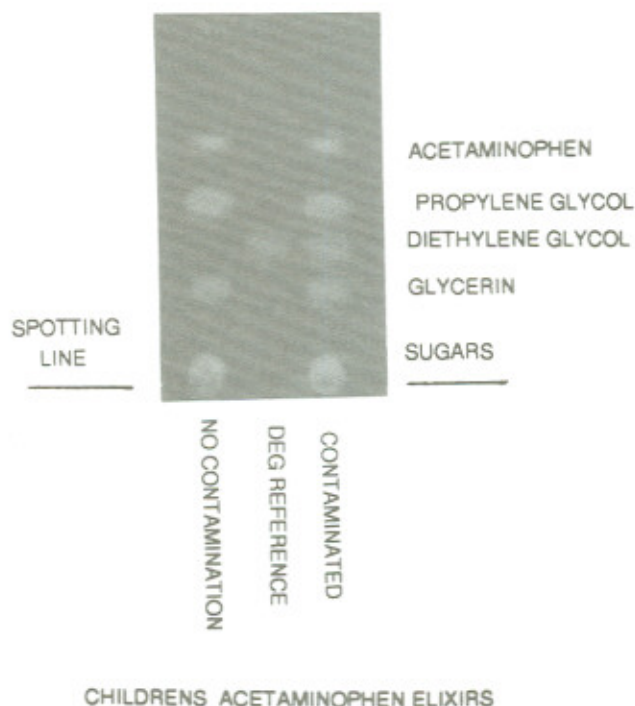


Figure 4. TLC separation of contaminated and noncontaminated elixirs.

2 staining methods was based on low toxicity, low cost, availability of reagents, and ease of operation. Permanent records of the developed TLC sheets can be made by photocopy.

A common practice for increasing the sensitivity of TLC is to multiply spots (15) by applying the spot, allowing it to dry, and spotting additional material on top of the dried material. By spotting 3 pipette loadings on a single position, the detection level was effectively increased, making the developed spot more visible.

This method separates either DEG or ethylene glycol from other glycols when present individually. If both are present in approximately equal amounts, the developed spots have the contour of a distorted figure 8. Because both DEG and ethylene glycol are toxic, it does not matter that the pair cannot be distinguished as long as they can be separated from other nontoxic glycols.

Testing with even the simple visual method of detection presented here would have been adequate to detect the contaminated materials that caused the large numbers of deaths among children and adults in the past 60 years.

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Appendix

Staining Procedures for Detecting Low Levels of DEG on Developed TLC Sheets

Method 1. Iodine/Starch Stain

(1) Place an amount (ca 25-50 g) of iodine crystals at the bottom of a glass TLC developing tank that can be sealed with a lid or cap. The tank must be deep enough that the sheet can hang vertically in the iodine vapor. Suspend developed sheet in the tank, cover tank with the lid, and allow the sheet to be exposed to the vapor for 5 min.

(2) Remove sheet and allow it to stand in open air for 1 h to remove iodine absorbed by silica coating on the sheet. If iodine is not removed at this point, detec-

tion of low concentrations of DEG will be masked by the background.

(3) Prepare 1% starch solution by adding 1 g starch to 10 mL water, making a slurry, adding 90 mL water, and boiling until solution becomes clear. (The starch solution will appear turbid when cooled, but this will not harm the solution.) Spray the sheet with 1% starch solution and observe spots. The method of detecting the spots will depend upon the concentration of DEG. Once DEG is detected, it will not be necessary to proceed to another step. Three levels of detection are possible:

(a) *Level 1.*—For DEG levels of 2 to 6% or higher. Observe sheets visually by transmitted light immediately after they are dried. Spots appear dark because of the difference in refractive index. No further staining is necessary. If no spots are detected, proceed to Level 2.

(b) *Level 2.*—For DEG levels of 0.5% or higher. Immerse the sheet in iodine vapor. Iodine-stained spots will be visible. If no spots are visible, proceed to level 3.

(c) *Level 3.*—For DEG levels from 0.1 to 0.5%. Spray sheet with a 1% starch solution. If no spots are observed, concentration of DEG is <0.25%.

Method 2: Oxidation of Glycols by Potassium Permanganate

The method was developed by Robert Prestridge and Kirsten Sharp of Australian Therapeutic Goods Administration Laboratories, Woden, Australia.

Develop sheets and observe in bright transmitted light. If concentration is >6% in glycerin or >2% in elixirs, spots are visible. If no spots are visible, proceed with the oxidation of glycols by potassium permanganate.

(1) Allow developed sheets to air dry for ca 10 min. The presence of a small amount of ammonium hydroxide remaining in sheets speeds up oxidation.

(2) Prepare staining solution immediately before use by adding 75-80 mg solid potassium permanganate to 12 mL used or fresh developing solvent. The permanganate solution is robust enough to allow variation in the amount of permanganate used; it does not have to be weighed exactly. Shake vigorously in a closed container or stir rapidly in an open container until potassium permanganate dissolves. (Use of the developing solvent to dissolve potassium permanganate prevents spots from dissolving or being distorted. *Caution:* Do not use water to make the permanganate solution!) Pour the potassium permanganate staining solution into a shallow vessel such as a watch glass or Petri dish. Attach a clip to the developed sheet, and immerse the

sheet far enough into the potassium permanganate to cover the surface. Allow the sheet to remain covered with the liquid for 2 to 3 s. The staining solution may be used to stain a number of sheets before it must be replaced.

(3) Remove stained sheet and allow it to dry. Oxidized spots of glycols appear yellow on a purple background. They begin to form after short time, and form

at different rates; DEG is the slowest. The first spot appears in ca 5 min, and formation is complete in 30 min. The difference in formation rate may be used to distinguish DEG and ethylene glycol if present individually.

(4) Properly dispose of all used or unused staining solution after treating samples, because it will not keep. Keep volumes to a minimum.