zolium chloride. Apparently there are other factors also affecting germination which act independently of rancidity development, but rancidity seems to play an important role in this process, at least under normal storage conditions and in the early stages of viability deterioration. The loss of linolenic acid through respiration, which has been suggested by Mirov as the cause of loss of viability and longevity of oleaginous seeds, seems to be a rather indirect factor because of the close association of linolenic and other unsaturated acids, losses, and the development of rancidity. Experiments with okra seeds showed a similar correlation between rancidity development and the loss of germinability. Preliminary experiments by coating okra, onion, and pine seeds with an antioxidant (starch phosphate) proved beneficial for the preservation of viability of okra and onion seeds during storage at room temperature.

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The Determination of Glycerine in Polyol Mixtures by Paper Chromatography

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RAPID AND ACCURATE METHODS for the determination of glycerine in polyhydric alcohol mixtures, containing polyols such as ethylene glycol, propylene glycol, erythritol, butanetriols, sorbitol, and mannitol have not been available. Precision distillation has often been used, but this technique is exacting and time-consuming. In the case of glycols and glycerine it is difficult to separate the glycerine from the glycols by distillation. A recent paper by Rosenberger and Shoemaker (7) used an azeotropic distillation for the determination of glycerine in an ethylene glycol water mixture. Dal Nogare (1), employing a partition chromatographic technique, was able to separate C_2 to C_4 glycols on a silicic acid-Celite column. However no work was done with glycerine and higher polyhydric alcohols, which would be removed very slowly from the column. We have devised a paper chromatographic technique by which glycerine can be separated quantitatively from glycols and higher polyhydric alcohols such as sorbitol, erythritol, and butanetriols. The suggested procedure may be adapted to semi-micro scale very easily and does not require the full attention of the analyst as in a column partition chromatographic technique.

In the development work on this procedure, paper chromatography was found to be the most satisfactory method to separate glycerine from other polyhydric alcohols, using Whatman No. 1 filter paper. To determine whether a descending or ascending technique would provide an efficient separation and to determine the approximate concentration of glycerine, a qualitative chromatogram was run on the unknown samples. The developing solvents for ascending and descending chromatograms are sec-butanol-water (saturated), and n-butanol-water (saturated), respectively. The glycerine is located by spraying a parallel chromatogram with an indicating reagent. The corresponding area in the unsprayed sample chromatogram is cut out of the paper and extracted with warm water. Others (2, 3, 8) reporting on the quantitative paper chromatographic separation of sugars have found that sugars are readily extracted from the paper simply by immersing the paper in water. Since this recovery is simple and requires no special devices, the authors adopted this procedure.

After elution the glycerine is determined on an aliquot of the eluate by using the procedure of MacFadyen (4). It is well known that periodate cleavage of vicinal hydroxyls will produce formaldehyde and, in some cases, formic acid (5, 6). The periodate oxidation of glycerine produces two moles of formaldehyde and one mol of formic acid. MacFadyen showed that micro quantities of formaldehyde could be determined by reaction with chromotropic acid. Thus the chromatographic separation of glycerine from the accompanying constituents, followed by the application of the two above-mentioned reactions, should furnish a method for the determination of glycerine in a glycol, polyhydric alcohol mixture.

Experimental

APPARATUS

Paper Chromatographic Chambers. These are ascending and descending types with accompanying accessories made of glass or stainless steel.

Pipettes. These are of 6 microliter capacity and volumetric 1 ml., 2 ml., 5 ml.

Glass Atomizer.

Test Tubes. 50-ml capacity.

Flasks. These are volumetric 25 ml., 50 ml., 250 ml., and 1,000 ml.

Spectrophotometer. This should be capable of measuring percentage of transmittance at 570 millimicrons.

MATERIALS USED

Chromatographic Filter Paper. This is designated as Whatman No. 1, 18¼ x 22½ in. sheets.

Developing Solutions. a) Sec-butanol saturated with water; b) n-butanol saturated with water.

Silver Nitrate Solution. Prepare the following two solutions: a) Dissolve 50 g. of silver nitrate in 450 ml. of distilled water. Store in an amber bottle. b) Dilute amonium hydroxide solution; add 175 ml. of concentrated ammonium hydroxide to 350 ml. of distilled water. For spraying the chromatograms, combine equal volumes of a) and b) for use as a spray reagent.

Methanol. This is absolute.

Periodic Acid Reagent (0.03M). Prepare by dissolving 1.71 g. of periodic acid (H_5IO_6) in 50 ml. of 0.25 M sulfuric acid, transfer the solution to a 250-ml. volumetric flask, and dilute to the mark with 0.25 M sulfuric acid.

Chromotropic Acid Reagent. Dissolve 0.500 g. of chromotropic acid (1, 8-dihydroxy-naphthalene-3, 6 disulfonic acid) in 10 ml. of distilled water in a 250-ml. volumetric flask. Dilute to the mark with 15 M sulfuric acid.

Stannous Chloride Reagent. Prepared freshly before use to approximate 0.125 M in 0.3 N hydrochloric acid. Dissolve 8.0 g. of stannous chloride ($SnCl_2 : 2H_2O$) in 175 ml. of 0.3 N HCl. Transfer the solution to a 250-ml. volumetric flask and dilute to the mark with 0.3 N HCl. The stannous chloride solution should be titrated with the periodic acid reagent immediately before use and so adjusted that 10 ml. of stannous chloride reagent will titrate 10.2 ml. of the periodic acid reagent. For the titration 10 ml. of concentrated hydrochloric acid are added to 20 ml. of stannous chloride plus 1 ml. of starch indicator solution (a blue color indicates the end-point).

Sulfuric Acid. This is 8M.

ANALYTICAL PROCEDURE

The sample applied to the paper should contain approximately 0.50 to 1.0 mg. (depending on the glycerine concentration) of total polyol mixture in a volume of 6 microliters.

Expected Glycerine Content	Approx. weight of sample applied to paper	Approx. weight of sample to be weighed for analysis
100 – 95%	0.50 mg.	2.0 g.
95 – 40%	0.70 mg.	3.0 g.
less than 40%	1.00 mg.	4.0 g.

Weigh accurately (± 0.001 g.) into a 30-ml. beaker the optimum weight of sample as indicated by the above table and dissolve the sample in methanol. A small amount of water may be added to facilitate solution. Transfer to a 25-ml. volumetric flask and dilute to the mark with methanol.

Qualitative chromatograms should be run before quantitative work is attempted in order to determine the approximate concentration of glycerine in the sample and to determine which one of the two techniques, descending or ascending, produces the better separation of the components. Where possible, the ascending procedure should be used because of its simplicity. In running the qualitative chromatogram the sample weight applied should be of the same order of magnitude as the subsequent portion taken for analysis.

Descending Chromatograms. If descending chromatograms are to be used, the Whatman No. 1 filter paper sheets are cut into pieces to fit the solvent trough of the chromatographic apparatus. Serrate the lower edge of the paper to insure uniform solvent drip from the paper. At least 24 hrs. before chromatographing the sample, a petri dish, which contains the developing solvent, is placed on the bottom of the jar in order to saturate the enclosed space in the jar with the solvent vapor.

By means of a micro pipette, 6 micro-liters of the sample solution are applied to the paper, approximately 5.5 cm. from the left edge. This application will serve as the reference sample. With the 6-microliter pipette, apply additional portions of the sample to be analyzed to the paper, with spaces of 6 cm. between samples and parallel to the reference sample with respect to the top edge of the paper. Rinse the pipette once with pure methanol, and apply this rinse solution to the paper after the first application has dried. After the spots have dried, place the paper into the solvent trough in the chromatograph jar in such a manner that the sample spots are slightly below the anti-siphon rods. The solvent trough is filled with water-saturated n-butanol developer solution, and, preferably at a constant room temperature, the chromatogram is developed for 16 hrs. When the development is complete, the paper strips are removed and dried under adequate ventilation away from open flames.

The reference strip is cut from the chromatogram and is sprayed evenly with silver nitrate reagent by means of a glass atomizer in a hood. The sprayed strip is heated in an oven at 105°C. for 5 to 10 min. The polyol zones appear as black spots on a light background. The reference strip is placed parallel to the unsprayed sheet, and the area on the unsprayed sheet which corresponds in size and location to the glycerine zone on the reference strip is carefully cut out. With a pair of forceps the portion of paper which contains the glycerine is placed into a 50-ml. beaker.

The glycerine is eluted from the paper in the beaker with 3 or 4 portions (5-10 ml.) of warm distilled water. After each addition of the distilled water the polyol solution is filtered through a filter paper, previously wetted with distilled water, into a volumetric flask of such capacity that a 2 ml. aliquot will contain 0.015 to 0.040 mg. of glycerine for analysis. Dilute to the mark with distilled water.

Ascending Chromatograms. For the ascending chromatograms the sample weights are the same. The Whatman No. 1 filter paper sheets are stapled with a one-inch fold along the lower edge and the two long sides to increase the rigidity of the subsequently formed clylinder. A 6-microliter sample is applied approximately 5.5 cm. from the long edge and 4.5 cm. from the lower or short edge of the paper. This is the reference sample. The samples for analysis are applied at intervals of 6 cm.; the first is about 8-10 cm. from the reference sample. After the spots are dry, the

sheet of paper is rolled into a cylinder and the edges are stapled together. The cylinder is placed vertically into the jar, the edge rests in the solvent dish which contains water-saturated sec-butanol developer solution at the bottom of the jar, 1-2 cm. deep. The chromatogram is allowed to develop for 16 hrs.

The technique for indicating and recovering the glycerine is identical to that described under the descending technique.

Colorimetric Procedure. A 2-ml. aliquot, measured with a volumetric pipette, is taken from the glycerine solution resulting from the elution of the paper and is transferred to a 50-ml. test-tube. With a volumetric pipette transfer 1.0 ml. of periodic acid into the glycerine sample in the test-tube, swirl gently to mix the contents, and allow to stand at room temperature for 15 min. After the oxidation period transfer 1.0 ml. of stannous chloride reagent into the test-tube and swirl gently. The chromotropic acid reagent, 5 ml. measured with a volumetric pipette, is added to the sample in the test-tube. The test-tube is swirled gently during the additions to prevent any localized concentration effects.

The test-tube is then placed into a boiling water bath for 30 min. to develop the red-violet color of the formaldehyde-chromotropic acid complex. At the end of the heating period the test tube and sample are removed from the boiling water bath and cooled to room temperature. The sample is transferred into a 50-ml. volumetric flask, rinsing the test-tube with 2- to 10-ml. portions of 8 M sulfuric acid and diluting to the mark with the same reagent.

A reagent blank is prepared by pipetting a second 2-ml. aliquot of the eluted solution into a 50-ml. test-tube. Pipette into the test-tube 1.0 ml. of stannous chloride reagent and 1.0 ml. of periodic acid reagent.

The foregoing reagents are added in the order given to prevent the oxidation of the glycerine present. The 5-ml. portion of chromatographic acid reagent is added to the contents of the test-tube, and the blank is treated in the same fashion as the sample.

A portion of the reagent blank is poured into the reference cell, and the sample is transferred to the matched sample cell. The percentage of transmittance (or optical density) is measured at 570 millimicrons with the spectrophotometer. The weight of glycerine, in milligrams, present in the sample is determined from a standard curve in which the milligrams of glycerine are plodded *versus* the percentage of transmittance (or optical density).

$$\% \text{ Glycerine} = \frac{\text{mg. of glycerine from std. curve} \times 100}{\text{wt. of sample in mg.} \times 100} \times A$$

A = Dilution factor if final volume differs from 50 ml.

Results and Discussion

The average glycerine recovery attained by the recommended procedures is shown in Table 1 to be 101.0%. The glycerine content varies from 100% to 15.6%, and, as shown in the table, the average deviation from the mean is 0.3% with an estimated precision of 0.7%. The sample sizes taken for analysis range from 2.6 to 4.6 g., depending on the expected glycerine content.

The known mixtures were chromatographed, using the ascending technique on the 100% glycerine solution, mixtures 3 and 4, and the descending technique on the mixtures 1, 2, 5, and 6. In Figures 1 and 2

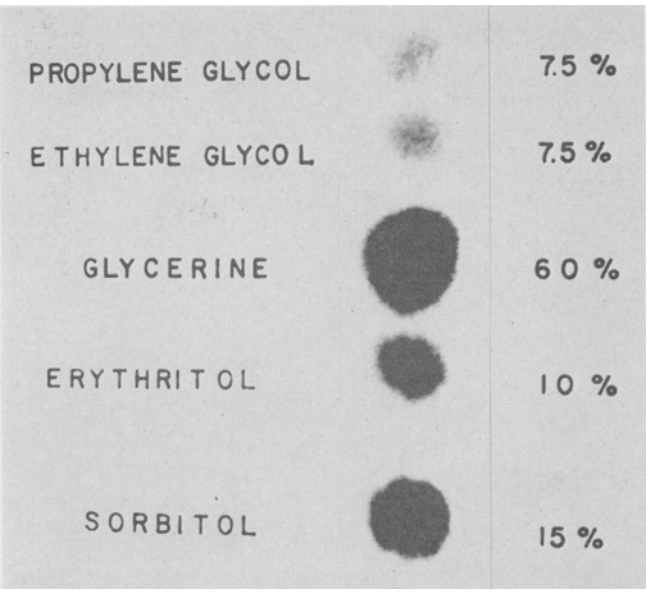

FIG. 1. Ascending chromatograms of polyhydric alcohol mixture.

TABLE I
Analysis of Known Mixtures for Glycerine

Mixture	Components	Composition known	% Glycerine found	% Recovery	Av. deviation from mean	% Relative precision
Std. glycerine	Glycerine	100	100.4, 99.6 98.7 av. 99.6	99.6	0.6	0.6
1	Glycerine Erythritol 1, 2, 4 butanetriol	93.5 3.9 2.6	93.7, 93.7 93.7, 93.7 av. 93.7	100.2	0.0	0.0
2	Glycerine Erythritol 1, 2, 4 butanetriol	80.9 12.7 6.4	83.2, 82.4 82.3, 82.4 av. 82.6	102.1	0.3	0.4
3	Glycerine Erythritol Sorbitol	63.8 20.5 15.7	65.6, 64.7 66.0 av. 65.4	102.5	0.5	0.8
4	Glycerine Erythritol Sorbitol	43.9 22.9 33.2	42.1, 43.7 43.7, 44.3 av. 43.5	99.1	0.7	1.6
5	Glycerine Erythritol Sorbitol	30.8 24.3 44.9	31.6, 31.5 av. 31.6	102.5	0.1	0.3
6	Glycerine Erythritol Sorbitol 1, 2, 4 butanetriol	15.6 24.3 50.1 10.0	16.0, 15.6 16.0, 15.6 av. 15.8	101.3	0.2	1.3

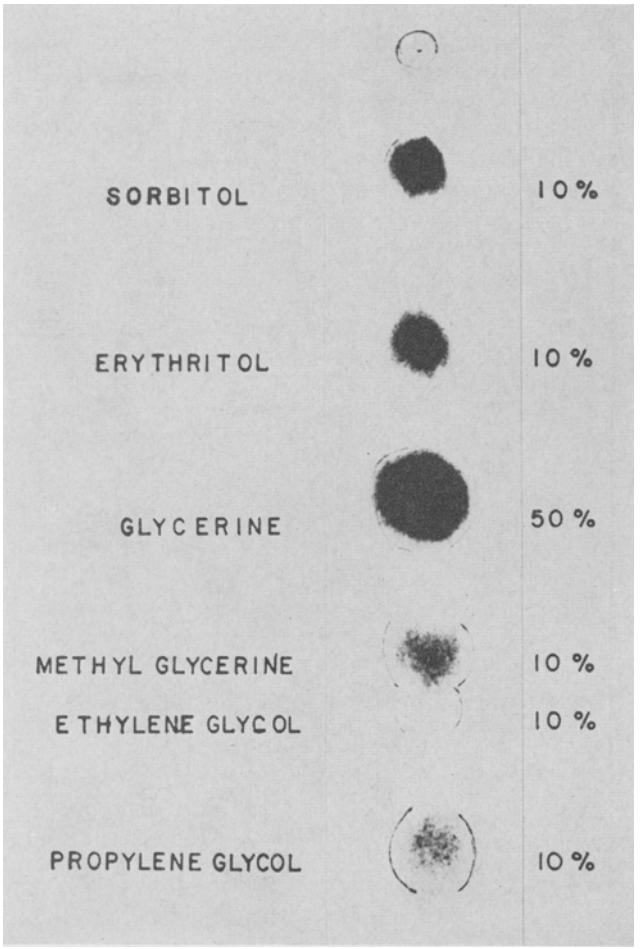

FIG. 2. Descending chromatograms of polyhydric alcohol mixture.

are presented examples of typical ascending and descending chromatograms respectively, with the relative concentrations expressed as percentage by weight. Wherever feasible, the ascending technique is preferred because, first, simplicity marks the equipment and, second, only one reference strip is required for a parallel series of five or six samples on the paper. However it has been found that a better separation can be obtained by using descending chromatograms on mixtures which contain polyhydric alcohols not easily separated from glycerine by the ascending chromatogram. For example, 1, 2, 4-butanetriol, which does not separate completely from glycerine on an ascending chromatogram, is resolved very readily by using the descending technique. Tables II and III present the measured R_f and R_g values of the polyols, using the ascending and descending techniques as proposed in this paper

Redistilled glycerine C.P. was used throughout this investigation. Analysis of the unchromatographed sample indicated 100.1% glycerine by periodate consumption and by the colorimetric procedure. The same solution was also subjected to the recommended procedure and the average percentage of glycerine found on analyzing three samples was 99.6 (Table I). Obviously this indicates that the elution technique as well as the recommended procedure may be considered practically quantitative.

TABLE II

R_G—Values of Polyols (Descending)
based on Glycerine: 0.50
solvent: 1-butanol, saturated with water

Glycerine	0.50
Sorbitol	0.12
Erythritol	0.32
1, 2, 4-Butantriol	0.65
1, 2, 3-Butantriol	0.67
Ethylene Glycol	0.74
1, 2-Propylene Glycol	0.88

TABLE III

R_f—Values of Polyols (Ascending)
solvent 2-butanol, saturated with water

Sorbitol	0.21	Ethylene glycol	0.65
Xylitol	0.28	1, 2, 5-Pentantriol	0.67
Erythritol	0.40	1, 3-Propylene glycol	0.73
Glycerine	0.53	1, 2-Propylene glycol	0.74
1, 2, 4-Butantriol	0.62	1, 2, 6-Hexantriol	0.74
1, 2, 3-Butantriol (Methyl glycerine)	0.63		

Brief experiments were conducted to show that a paper blank, which would correct for any periodate oxidation sensitive impurity in the paper, was not required. Portions of the paper, equivalent in size and location to the glycerine zone, were cut from the paper sheet which had been subjected to the chromatographic conditions. The paper blanks were eluted, and the resulting solutions were treated according to the recommended procedures. The analysis performed on the "blanks" showed no significant difference between the reagent blank reference and the paper blanks, *i. e.*, 99 +% transmission reading.

The standard curve, which is referred to in the recommended procedure, was prepared by analyzing standard distilled water solutions of c. p. glycerine by the recommended procedure and by omitting the chromatographing portion of the method. The standards contained from 0.20 to 1.20 mg. of glycerine per 50 ml., and a 2-ml. portion of each standard was taken for analysis. The percentage of transmittance values *versus* the milligrams of glycerine per 50-ml. concentrations were plotted on semi-log paper. It was possible to connect the resulting points with a straight line which indicates that, up to a glycerine concentration of about 1.20 mg./50 ml., Beers Law is followed.

Summary

Paper chromatography has been found to be a very satisfactory method for the separation of glycerine from hexitols, pentitols, tetritols, and glycols. After separation of the glycerine on paper the glycerine is eluted from the indicated zone with water. The formaldehyde, which is a product of periodic acid oxidation, is determined colorimetrically. A suggested procedure and the results of the analyses of standard glycerine and polyhydric alcohol mixtures are presented in this paper. The results indicate that the glycerine content of the mixtures (glycerine content ranging from 15% to 100% glycerine) may be determined with an average relative precision of 0.7% and with an average recovery of 101.0%.

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