A SAMPLE APPLICATOR FOR CHROMATOGRAPHIC PAPER AND ITS USE FOR 5-HYDROXYMETHYL-2-FURALDEHYDE AND LEVULINIC ACID ANALYSIS

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INTRODUCTION

In a recent investigation into the kinetics of the decomposition of glucose in aqueous acidic media and its conversion to 5-hydroxymethyl-2-furaldehyde and levulinic acid[1], it was necessary to develop experimental techniques for the quantitative analysis of these compounds. To assure that isothermal conditions were obtained in these reactions where maximum yields were reached in a few seconds, it was necessary to work with reactant volumes as small as 0.03 ml, using paper chromatography to separate the materials of interest prior to quantitative measurement. Since the wide range of variables of interest in the kinetics study demanded the analysis of hundreds of samples, a new applicator was developed which was rapid, precise, and permitted the loading of paper to the limit of its capacity.

The design of this applicator is described below and, in addition, the data obtained through its use for the analysis of 5-hydroxymethyl-2-furaldehyde and levulinic acid are summarized.

DESIGN OF APPLICATOR

One of the main criteria of an applicator for quantitative paper chromatography is that it yields a reproducibly uniform streak of the sample solution on the paper. In order to extend its range of usefulness, it should also be capable of applying a different volume per lineal dimension to separate sheets. An applicator which satisfies these requirements and has the additional desirable feature of ease of operation is shown in Fig. 1.

This model was constructed from two ¼ × 2 × 24 in. machinist's straight edges. These were joined at their ends as shown by two cross pieces which were fastened to the lower straight edge permanently and to the upper straight edge by close fitting removable bolts. The angle between these two edges could then be varied in

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Fig. 1. Sample applicator for chromatographic paper.

discrete steps. All pieces of this model had been chrome-plated to reduce any corrosion and cleaning problems.

The second part of this applicator was made by securing a precision hypodermic syringe with adhesive to a maple sled as shown in Fig. 1. There is an overhang on the back of the sled to act as a guide when it is moved along the lower machinist's edge. The back of the syringe plunger had been given a hemispherical shape where it touched the upper bar. The hypodermic needle tip had been cut to an appropriate length, ground smooth, and given a slight backward curve to avoid tearing the paper. The whole assembly was then mounted on a wooden base and fitted with a bracket to hold the paper sheets as shown in the figure.

The operation of the applicator is carried out as follows: A sheet of chromatography paper is first placed on the bracket of the applicator and the sample solution loaded into the syringe. The sled with the plunger maintained in its extended position is then placed on the lower bar and moved slowly along until the back end of the plunger contacts the upper bar and forces out some solution. The needle tip is then wiped dry and the paper moved into position so that one edge touches the needle tip. The paper is then held firmly in position. As the sled is moved toward the apex of the angle of the two straight edges, the plunger is forced into the syringe and the solution is applied uniformly to the sheet.

The volume of solution applied per lineal dimension may be varied in three ways. The angle between the two bars may be changed, syringes calibrated to different sizes may be used, and multiple streaks may be applied. If multiple streaks are used, it is advisable to dry each application before a subsequent streak is made to prevent the formation of an unduly wide starting line.

In an effort to measure the linearity or uniformity of the applied streak of solution, the following trial was made. The syringe was filled with water and placed in streaking position on the lower straight edge. A stop was then clamped securely 1.5 in. down the bar. As the sled was moved slowly toward the stop, the ejected water was taken up in a tared glass capillary tube. The stop was then moved 1.5 in. further down the bar and the process repeated. This was continued in nine steps which reached from one end of the machinist's edge to the other.

The results of two such trials are summarized in Table I. A linear regression analysis of each data set was made in which the volume delivered per 1.5 in. increment was measured as a function of position on the applicator. The very small random values obtained for the slopes of these two regression lines, $-1.50 \cdot 10^{-5}$ and $+3.33 \cdot 10^{-6}$ ml/1.5 in. per increment, corroborate the linearity of the applied streak over the length of the applicator. A measure of the precision of this method may also be obtained from these data from the magnitude of the standard deviation, 0.00023 ml/1.5 in., of all the individual observations from the overall average. When replicate samples from one streak are averaged, as will normally be done, the standard deviation will, of course, be smaller (*e.g.*, the standard deviation of the average of each set in Table I from the overall average is 0.00006 ml/1.5 in.). It must be pointed out that a portion of this error can be ascribed to the collection and weighing of the delivered liquid.

TABLE I

TESTS FOR APPLICATOR LINEARITY

Sample position	*Volume delivered ml/1.5 in.*	*Deviation from average ml/1.5 in.*
1	0.0260	—0.00008
2	0.0262	+0.00012
3	0.0260	—0.00008
4	0.0263	+0.00022
5	0.0260	—0.00008
6	0.0262	+0.00012
7	0.0263	+0.00022
8	0.0256	—0.00048
9	0.0261	+0.00002
Average	0.02608	
1	0.0260	+0.00004
2	0.0261	+0.00014
3	0.0254	—0.00056
4	0.0263	+0.00034
5	0.0259	—0.00006
6	0.0259	—0.00006
7	0.0261	+0.00014
8	0.0261	+0.00014
9	0.0258	—0.00016
Average	0.02596	

Quantitative tests were also made to measure the effect of the rate of sample application. In these tests, streaks of 2*N* potassium iodate solution were applied to sheets of chromatographic paper very rapidly in one case, very slowly in another, and alternately rapidly and slowly in still another. When the streaks had dried, quadruplicate samples precisely 3 in. long were cut from each streak, placed in individual 40 ml beakers of distilled water and titrated with a standardized sodium thiosulphate solution. In Table II are summarized the average recoveries for each trial which fail to indicate a significant dependence on the rate of application. A measure of the precision of this method of sample application, including the analytical errors of the potassium iodate titration, may also be obtained from these data in

TABLE II

TESTS FOR DEPENDENCE ON RATE OF APPLICATION

Sample	*Average potassium iodate concentration recovered from paper*		
	Rapid streaking mequiv./3 in.	*Slow streaking mequiv./3 in.*	*Intermittent streaking mequiv./3 in.*
1	6.893	6.842	6.840
	6.862	6.855	6.775
2	7.430	7.478	7.385
	7.470	7.475	7.465

which the standard deviations from the overall average of each sample set are both approximately 0.034 mequiv. per 3 in.

EXPERIMENTAL

Analysis for 5-hydroxymethyl-2-furaldehyde

An extensive investigation of various solvent systems for irrigating the papers did not result in one system which could handle both 5-hydroxymethyl-2-furaldehyde and levulinic acid satisfactorily. Consequently, separate systems were employed in the analysis of each. The solvent system used in the analysis of 5-hydroxymethyl-2-furaldehyde was suggested by BUCH, MONTGOMERY AND PORTER[3] and was the organic layer which results from the mixing of equal volumes of 5 M formic acid and pentanol. This irrigating system resulted in a compact band of 5-hydroxymethyl-2-furaldehyde with an R_F value of 0.75. Quantitative tests performed to determine its distribution over the entire sheet indicated that less than 1 % of the material was located outside of the indicated band.

The analytical procedure employed involved streaking the solutions onto 18 by 22 in. sheets of Whatman No. 3 MM chromatographic paper, irrigating them at 30° for 15 hours with the formic acid–pentanol solvent, drying them, and locating the band of 5-hydroxymethyl-2-furaldehyde with the aid of an ultraviolet lamp. Four samples, each 75 mm wide, were then cut from this band and placed into individual beakers containing predetermined quantities of distilled water to elute the 5-hydroxymethyl-2-furaldehyde. Since solutions of this material follow Beer's law, their concentrations were determined from the optical absorbence with a Beckman Model DU spectrophotometer at 284 mμ. It should be noted that the very dilute solutions of 5-hydroxymethyl-2-furaldehyde required by the spectrophotometer show a tendency to degrade when stored in sunlight at room temperature. This degradation will not occur significantly if they are kept under refrigeration and in the dark. In practice, direct sunlight and long delays in analysis should be avoided.

This method was calibrated with carefully recrystallized samples of 5-hydroxymethyl-2-furaldehyde prepared in this Laboratory and the results of this calibration are shown in Fig. 2. A linear regression analysis made on these data employing the method of least squares indicated a correlation coefficient of 0.99 (significant well below the 1 % level) with a standard error of estimate from the regression line of 0.031 mg per 75 mm. The slope of this line is 0.919 and the intercept value of 0.006 agreed closely with the experimentally determined paper blank.

From these results it is evident that approximately 8 % of the material applied was not covered. A detailed investigation into the possible sources of this loss, including several chromatographic separations of the same sample of 5-hydroxymethyl-2-furaldehyde, seemed to indicate that the loss is due to its volatilization during the removal of water after streak application, prior to irrigation. Since the loss is constant over a several-fold change in concentration and is satisfactorily

compensated for, the indicated calibration may be used with confidence over the range indicated.

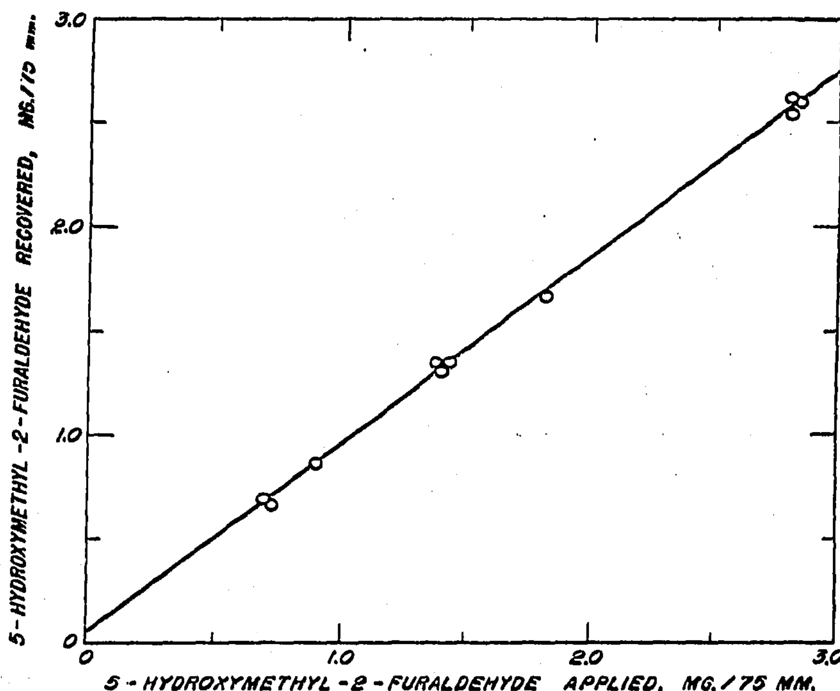

Fig. 2. Calibration curve for chromatographic recovery of 5-hydroxymethyl-2-furaldehyde.

A study was also made of several additional similar compounds which might result from the acid-catalyzed degradation of glucose. Since the chromatographic and spectroscopic behavior of these materials may be of value to those working in this field, the properties measured in this investigation are summarized in Table III and Fig. 3. It will be noted that the chromatographic behavior is quite similar for all the compounds, but that fortunately the molecular absorption spectra differ sufficiently to allow differentiation. Quantitative calibrations for these other materials were not established.

TABLE III

PROPERTIES OF 5-HYDROXYMETHYL-2-FURALDEHYDE AND RELATED COMPOUNDS

Compound	*Melting point* °C	*Wave length at major peak* $m\mu$	*Molecular extinction coefficient* *l/mole/cm*	R_F*
5-Hydroxymethyl-2-furaldehyde	33.4	284	16,920	0.75
Oxy-bis-(5-methylenefurfural)	114.5	282	28,600	0.84
5-Hydroxymethyl-2-furoic acid	160–161 (dec.)	251	12,420	0.74
2-Hydroxyacetylfuran	82.5	276	14,090	0.73
Levulinic acid	33.5	270	25.1	0.75

* Irrigation solvent system is the organic layer from an equi-volumetric mixture of 5 *M* formic acid and pentanol.

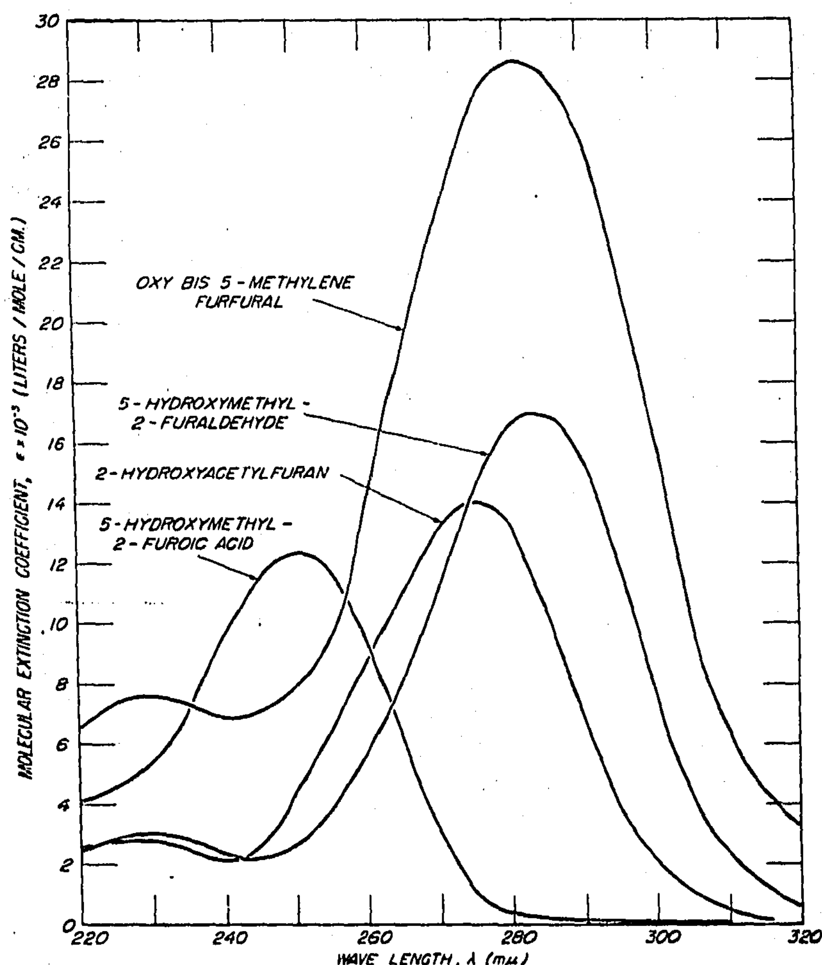

Fig. 3. Ultraviolet absorption spectra for 5-hydroxymethyl-2-furaldehyde and related compounds.

Analysis for levulinic acid

Free levulinic acid is too volatile to yield a reproducible, quantitative chromatographic separation. Therefore, the possibility of separating it as a salt was considered and it was found that ammonium levulinate would chromatograph into compact, quantitative bands when a mixture of ethanol (95 %), aqueous ammonia (29 %), and water in a volumetric ratio of 100:5:5, respectively, was used as the irrigating solvent. This system resulted in the following R_F values for these major components of the reacted glucose solutions:

Component	R_F
Glucose	0.30
Ammonium formate	0.35
Ammonium levulinate	0.40
5-Hydroxymethyl-2-furaldehyde	0.75

The method employed to measure the amount of the separated levulinic acid was suggested by PLOETZ AND BARTELS[4] and FROST AND KURTH[5], and depends on the

presence of the acetyl group which undergoes the iodoform reaction in the presence of alkaline iodine solutions. Since the reaction is not stoichiometric, a standardization was made by analyzing aqueous levulinic acid solutions of known concentrations varying from 0 to 5 mg/ml. 1 ml aliquots of these solutions were analyzed by adding 5 ml of a 0.1 *N* iodine solution followed by 5 ml of 1.0 *N* potassium hydroxide. They were then swirled, stoppered, and placed in a thermostated water bath for 10 min, during which time a yellow flocculent precipitate of iodoform was produced. The samples were then removed, 5 ml of 1.1 *N* hydrochloric acid added to render the solution acid, and the free iodine remaining in the samples titrated with a standardized 0.1 *N* sodium thiosulfate solution, using a starch solution as an indicator. A blank sample containing only water was also run through this procedure as a measure of the amount of iodine originally added. Since this analysis is somewhat sensitive to reaction conditions, the specified procedure must be carefully followed. From the above standardization, the calibration of Fig. 4 was obtained. It may be noted that

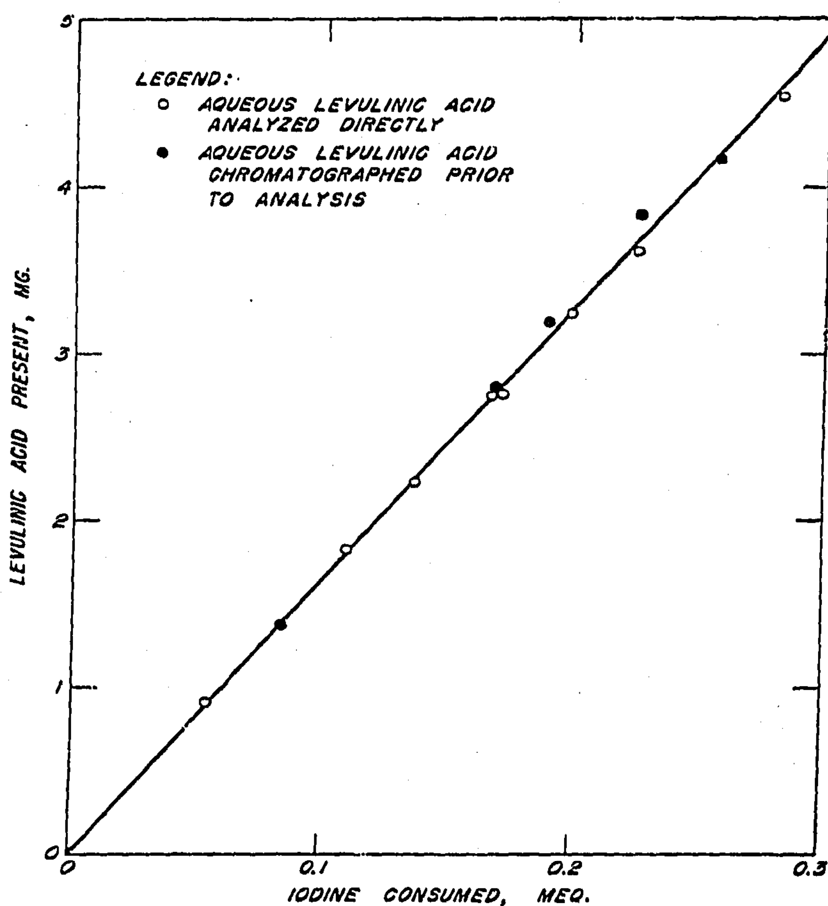

Fig. 4. Standardization curve for analysis of levulinic acid.

7.18 moles of iodine are consumed per equivalent of levulinic acid indicating that the overall reaction is not a simple iodoform reaction.

To determine if the separation step was also quantitative, the calibration was repeated on samples of levulinic acid recovered from 18 × 24 in. sheets of Whatman

No. 3 MM chromatography paper. In these trials, a streak of 1.0 N ammonium carbonate was first applied to the paper and dried. The solution of levulinic acid was then applied over the first streak and thus converted to ammonium levulinate *in situ*. After a 15 hours irrigation with the designated solvent, the papers were dried and four 75 mm vertical strips removed for analysis. The remaining guide strips were sprayed with a 2,4-dinitrophenyl-hydrazine indicator (2 g dissolved in 400 ml of 10 % hydrochloric acid) to locate the position of the levulinate band. This position was marked on the 75 mm strips and the corresponding area containing the levulinate removed for analysis.

The ammonium levulinate was eluted from the paper tabs in a modification of the method described by SAEMAN *et al.*[2]. To accommodate the thick 3 mm paper, the lower glass plate was substituted for by a stainless steel plate with a notch 0.125 in. wide by 0.008 in. deep milled into the front edge. In this method, the material is eluted from the paper tab and collected completely in a 0.75 ml capillary pipette. The solution was then transferred to a 50 ml Erlenmeyer flask for analysis. Since the ammonium ions interfere with the iodometric analysis, it was necessary to remove them by adding 0.25 ml of 0.5 N sodium carbonate to the flask and evaporating the solution to dryness. This was done at room temperature under a reduced pressure aspirator in a vacuum manifold arrangement capable of handling several samples at one time[2]. The dried sodium levulinate was then put back into solution with 1 ml of distilled water and the analysis carried out as described above. The results of these analyses are also shown in Fig. 4 where the amount of levulinic acid applied to the paper is plotted as a function of the iodine consumed.

The data of Fig. 4 indicate that the chromatographic separation and recovery of levulinic acid in the manner described is quantitative and that the proposed technique may be employed with confidence within the range studied. A linear regression analysis of all the data shown in Fig. 4 was made and a correlation coefficient of 0.99 was obtained. The slope of the line is 16.27 mg of levulinic acid per mequiv. of iodine consumed and the data show a standard error of estimate from the regression line of 0.059 mg.

Application of chromatographic techniques

The techniques described have served effectively in an extensive study of the acid-catalyzed decomposition of glucose. The sample applicator aided significantly in analytical scheme by reducing the time-consuming step of sample application, and the analysis schemes for 5-hydroxymethyl-2-furaldehyde and levulinic acid proved very reliable.

SUMMARY

In the course of a kinetics study into the production of 5-hydroxymethyl-2-furaldehyde and levulinic acid from aqueous acid solutions of glucose, a sample applicator was developed for use in paper chromatographic separations. This applicator is capable of delivering a continuous, uniform streak of the sample solution to the paper

and has the additional advantages of versatility of range and ease of operation. Also included are the analysis schemes and standardizations developed for the determination of 5-hydroxymethyl-2-furaldehyde and levulinic acid. These materials are first separated chromatographically from the other reaction products and then the concentration of 5-hydroxymethyl-2-furaldehyde is measured by its ultraviolet absorption and the levulinic acid concentration determined by means of the iodoform reaction.

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