# ANALYSIS OF WOOD SUGARS

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# ANALYSIS OF WOOD SUGARS[1, 2]

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## Summary

Experimental work in wood saccharification requires methods for the analysis of reducing sugar, fermentable sugars, and alcohol that are dependable, simple, rapid, and capable of being run in large numbers.

Two methods were used for the determination of reducing sugars -- one an electrometric titration method and the other a micro method due to Shaffer and Somogyi. The electrometric titration method was used when it was desirable to have a sugar analysis in as short a time as possible; the micro method when large numbers of sugars were run or when concentrations and quantities available were too low for the macro methods.

Fermentable sugars were estimated by determining the amount of sugar sorbed from a 1.5 milligram-per-milliliter solution by a 5.0 percent yeast suspension.

Alcohol was determined by the specific gravity method using a Westphal balance with a thermostated container for the beer distillate. It was found that the surface tension error on the wire supporting the bob could be eliminated by the addition of a very small amount of a wetting agent.

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[1] Based on studies of the U. S. Forest Products Laboratory at Madison, Wis., in cooperation with the Office of Production Research and Development of the War Production Board.

[2] Presented before the Cellulose Division of the American Chemical Society, New York City, September 11-15, 1944.

[3] Maintained at Madison, Wis., in cooperation with the University of Wisconsin.

## Introduction

In studies of wood saccharification it is necessary to have available analytical methods for the determination of the sugars produced, the amount of fermentable sugars, and the amount of alcohol produced when the wood sugars are fermented. Because wood sugars consist of mixtures of several sugars as well as dissolved lignin, furfural, formic and acetic acid, various oils, and other products in the wood, their analysis presents special problems. To be of most use in following the work, the analysis should be simple, rapid, and capable of being run in large numbers.

Two methods were used for the sugar determinations made at the Forest Products Laboratory: an electrometric titration method, where it was desirable to obtain analytical results as soon as possible; and the Shaffer and Somogyi method, where a large number of determinations were made simultaneously and small quantities were involved, but where it was not important to have the determinations completed in a short time.

Other methods were studied but rejected because of the time involved, the difficulty in observing the end point, or unreliable results.

Benedict's volumetric method (1) proved unsatisfactory because of the obscure end point. A volumetric hypoiodite method (3) was rapid but gave results that were frequently 50 percent higher than the Munson-Walker method (8), which, in turn, gave reliable results, but was time-consuming. An attempt was also made to use the Lane and Eynon volumetric method (9), but the color of the wood hydrolysate in the presence of strongly alkaline Fehling's solution made it impossible to see the point at which the color of the methylene blue indicator disappeared.

## The Determination of Reducing Sugars in Wood Hydrolysis by Electrometric Titration

The determination of reducing sugars in wood hydrolysis by electrometric titration utilizes the titration of a standard Fehling's solution with reducing-sugar solutions as in the Lane and Eynon procedure (9), but avoids the difficulty with the determination of the end point. Britton and Phillips (2) have shown that the electromotive force across a normal calomel cell and a platinum electrode can be used to determine the end point in the titration of glucose with Fehling's solution. In their experiments the point of maximum inflection was the same as that point at which methylene blue is decolorized. Because they worked at a temperature of 90° C., however, they experienced difficulty with re-oxidation by air, and the reaction was slow. In the Lane and Eynon procedure all but 1 or 2 milliliters of the solution to be treated are added to the Fehling's solution, the mixture brought to a boil and boiled for 2 minutes, and the titration completed in a total boiling time of 3 minutes. Such a reaction was carried out in experiments at the Laboratory, and the electromotive force across saturated calomel-platinum electrodes immersed in the solution was determined. The values for the electromotive

force were plotted against the volumes of sugar solution to give the curve shown in figure 1. The point of maximum inflection (-550 millivolts) was found to agree with the end point obtained with methylene blue when pure glucose solutions were used.

The reducing sugar content of dark-colored solutions may therefore be determined in the manner described by Lane and Eynon, except that the end point is determined electrometrically. This requires no more time than the original method in which an internal indicator is used because the potentiometer readings may easily be taken in the intervals between additions of the sugar solution.

A more rapid method than this has been developed in which it is not required to clean and refill the burette for each determination nor to make an accurate dilution of the sample if the concentration is below 12 grams per 100 milliliters.

The apparatus for this determination is shown in figure 2. The burette is arranged for automatic filling with a 5-milligram-per-milliliter solution of glucose. Calomel and platinum electrodes are held firmly in place by the rubber stopper. After each determination, the ground glass plug in the calomel cell is momentarily loosened to rinse it. A motor is provided for rapid stirring. The titrations are carried out in tall form beakers held to the stopper by means of springs. A blank titration is made using 25 milliliters of Soxhlet's modification of Fehling's solution. This amount of sugar reagent requires approximately 120 milligrams of glucose. An estimate is then made of the reducing sugar content of the unknown sample. This can be conveniently done with wood sugars by multiplying the degrees Brix by 0.7. A volume of the unknown solution containing less than 120 milligrams of reducing sugar is put in the beaker and diluted to approximately 5 milligrams of reducing sugar per milliliter by the addition of water. This addition of water need not be done precisely since an error of $\pm$ 5 milliliters will result in an error of only 0.3 percent in the apparent sugar concentration. Fehling's solution is then added and the titration completed with the standard glucose solution in the manner described by Lane and Eynon except that the end point is determined electrometrically.

The calculations are simple. The milligrams of glucose required for a blank titration minus the milligrams of glucose added in the standard solution to complete the titration divided by the milliliters of unknown samples used equals the milligrams per milliliter in the unknown sample.

In table 1 is shown a comparison of the apparent reducing sugar content in the first, third, sixth, and ninth cycles from a multistage hydrolysis as measured by the Munson-Walker, electrometric titration, and Shaffer and Somogyi methods. The Munson-Walker and electrometric titration methods show good agreement; the Shaffer and Somogyi method gave lower values.

## Effect of Formic Acid on the Electrometric Titration of Wood-sugar Solutions

Kressman (5) found that formic acid is one of the decomposition products obtained in wood hydrolysis. When formic acid was added in varying quantities to the mixtures being titrated, it was found that even in solutions containing equal parts of sugar and formic acid the volume of glucose solution required was not affected, but that it did affect the shape of the titration curve, causing a more abrupt break from the horizontal to the vertical portion. The curve for the titration of the wood-sugar solutions also shows a more abrupt break than does pure glucose.

## The Effect of Calcium on the Electrometric Titration

In certain samples of wood sugar that had been neutralized with lime it was found that the sharp breaks characteristic of pure glucose or raw wood hydrolysates were lacking. Lane and Eynon (6) called attention to the fact that calcium, even in small quantities, adversely affects this titration, invariably resulting in low sugar values. A number of titrations were made in the presence of calcium to determine its effect on the titration curve. The curves are plotted in figure 3. Curve 1 is the curve characteristic of pure glucose; curves 2, 3, 4, and 5 show the effect of 1, 3, 5, and 10 parts of calcium per 100 parts of glucose, respectively. These curves indicate that calcium must be removed from solutions containing more than 1 percent as much calcium as glucose before a satisfactory analysis can be made.

Lane and Eynon found that strontium and barium also interfere with the volumetric sugar determination when methylene blue is used as an internal indicator. This work confirms their findings and explains to a certain extent the reason for their difficulties. The decoloration of methylene blue is dependent on reaching a certain oxidation-reduction potential. Since the presence of calcium reduces the slope of the electromotive force versus sugar curve, it is apparent that it must make the end point less sharp. It is also apparent that if decoloration occurs at a point corresponding to an electromotive force more negative than -500 millivolts, the volume of sugar required is increased and the apparent sugar content is decreased, since the curves above this point are shifted to the right by the presence of calcium. The experience has been that the presence of calcium results in low sugar values. Calcium may be removed from neutralized worts by shaking the sample as received with 1/2 percent of its weight of powdered sodium or potassium oxalate and filtering or centrifuging out the precipitated calcium oxalate. The filtrate is then tested for calcium with more oxalate. If no precipitate forms, the sample is analyzed in the usual way. It has been determined that a moderate excess of oxalate is without effect on the reaction.

The volumetric titration for reducing sugars, using an electrometric end point rather than the usual internal indicator, has been used in

approximately 3,000 determinations in the work on wood hydrolysis and found to be dependable and very valuable where it is necessary to obtain an accurate analysis in a short time.

## The Determination of Reducing Sugars in Wood Hydrolysates by the Shaffer and Somogyi Method

The sugar analysis developed by Shaffer and Somogyi (10) has been found in other laboratories to give good service in the analysis of such materials as biological fluids and fermentation products. In recent work at the Forest Products Laboratory, the method has been found suitable for wood-sugar solutions and may be easily adapted to sugar concentrations as low as 0.2 milligram per milliliter.

### Method Adopted for the Analysis of Wood Sugars

The method adopted for the analysis of wood sugars is identical with that of Shaffer and Somogyi, using their reagent No. 50, and a 30-minute boiling time. The boiling time required was established by the following experiment.

Two-milliliter samples of the wood hydrolysate obtained in the first cycle of a multiple-stage hydrolysis of spruce and of a composite sample from the same run were each diluted to 100 milliliters. One-milliliter samples of the diluted solutions were treated with the sugar reagent No. 50 according to the Shaffer and Somogyi method and boiled for varying lengths of time.

In figure 4 is shown on a percentage basis the reducing value plotted against time, using as 100 percent the value obtained in 30 minutes of boiling. It may be seen from these curves that the reducing value of the composite samples reached a maximum after 30 minutes of heating, while the value of the cycle 1 sample was still rising slowly. Due to the fact that the slowly reducing materials are concentrated in the first cycle, the reaction was not complete but sufficient for the work on saccharification and fermentation, and, therefore, a 30-minute heating period was used in all work involving the Shaffer-Somogyi method at this Laboratory

### Determination of Reducing Power of Various Sugars Toward the Reagent

Wood sugar is a mixture containing primarily glucose mixed with smaller amounts of xylose, mannose, galactose, and arabinose. The relative amounts of these sugars vary with the species. In the work on hydrolysis, all wood-sugar yields are expressed in terms of d-glucose without taking account of differences in reducing power toward the sugar reagent.

Values for the relative reducing power of various sugars expressed as d-glucose as obtained by two different analysts are shown in table 2. Glucose has the highest reducing power of the sugars tested in spite of the fact that the pentoses have a higher percentage of reducing groups.

## The Determination of Fermentable Sugars in Wood Hydrolysates

Wood hydrolysates contain various hexoses, pentoses, sugar decomposition products, furfural, wood extractives, and lignin. Their value for alcohol production depends, however, on the fermentable sugar content and therefore it is essential that fermentable sugars be differentiated from unfermentable sugars and other reducing material. This may be done by the fermentation of a sample of the sugar followed by an alcohol determination, or by a determination of the sugar consumed in the process. These methods, however, are time-consuming, especially when applied to a large number of samples.

### Methods of Estimating Fermentable Sugars in Dilute Solutions

It is well known that a high concentration of yeast will quantitatively remove sugar from dilute solutions at a rapid rate. In the examination of complex mixtures, such as blood or urine, fermentable sugars are estimated by determining the loss in reducing power of the sample after treatment with a high concentration of yeast (11). Menzinsky (7) describes the use of this rapid yeast sorption method for the evaluation of the sugars contained in sulfite waste liquors. Hagglund (4) has found sulfite liquor from pine to contain: 17.0 percent pentoses, 42.7 percent mannose, 4.2 percent galactose, 3.2 percent galacturonic acid, 4.0 percent fructose, and 28.9 percent glucose. This distribution varies between species. Because galactose is not readily fermented unless the yeast has been previously acclimatized to its utilization, Menzinsky recommends that yeast for sulfite liquor analysis be acclimatized to galactose, or that the yeast be taken from a vat in which the fermentation of sulfite liquor is taking place.

In experiments at the Forest Products Laboratory on liquors obtained by the hydrolysis of wood, no effort was made to use acclimatized yeast for analysis because the proportion of galactose present is much smaller than in sulfite liquor; and in the industrial utilization of these liquors, an unacclimatized inoculum may be used.

### The Adjustment of the pH of the Sugar Solutions

In using the yeast sorption method for fermentable sugar analysis, it is desirable to have a pH slightly on the acid side. Menzinsky neutralized undiluted sulfite liquor with excess precipitated chalk and diluted the sample after filtration. This procedure is also satisfactory for use with wood hydrolysates, but in order to avoid time required for the filtration, approximately 0.1 milliliter of concentrated sulfuric acid was added to 100 milliliters of the diluted liquor to insure the presence of more than 0.1 percent acid and then precipitated chalk was added in excess. Large variations in the amount of added acid or chalk have little effect on the pH of the resulting solution. In all the solutions tested, the pH in the presence of excess chalk was $6.3 \pm 0.2$

## Determination of Rate of Sorption of Fermentable Sugars from Diluted Wood Hydrolysates by Yeast in High Concentrations

An experiment was devised to determine the rate at which the fermentable sugars in diluted wood hydrolysates are "sorbed" by high yeast concentrations. Other workers have shown that sugar sorption is rapid and complete in the case of blood, urine, and sulfite liquor, but because of their complex nature it was desirable to determine this rate using acid-hydrolyzed wood sugars.

Four milliliters of a sample of a composite liquor from the Marquette pilot-plant operations[4] containing 3.95 grams of reducing sugar per 100 milliliters were diluted to 100 milliliters. To this was added approximately 0.1 milliliter of sulfuric acid from a calibrated dropper. Twenty milliliters of this solution were treated with excess calcium carbonate and 1 gram of a commercial compressed baker's yeast,[5] shaken at 30° C. for various times, centrifuged, and analyzed. The time allowed for sorption and the apparent fermentable sugar are plotted in figure 5.

The sorption is complete in 1 hour and 1 hour, therefore, was chosen as the standard time of sorption.

## The Determination of Fermentable Sugar by Yeast Sorption

The sample to be analyzed for fermentable sugar is diluted to approximately 1.5 milligrams of reducing sugar per milliliter. The reducing sugar content is determined by the Shaffer and Somogyi method, using reagent No. 50 with a heating period of 30 minutes, sulfuric acid is added to the diluted solution from a microburette in the proportions of approximately 0.1 milliliter per 100 milliliters. Twenty milliliters of this solution are then put into a 30-milliliter vial, and a sufficient quantity of precipitated chalk is added to leave a small amount of undissolved excess. Approximately 1 gram of compressed baker's yeast (Saccharomyces cerevisiae) is then added, and the vials are stoppered and shaken for 1 hour at 30° C. At the end of this time the vials are centrifuged, and a sample of the supernatant liquor is pipetted off and analyzed for sugar by the Shaffer and Somogyi method. If unwashed yeast is used, the value is corrected by subtracting the apparent sugar found in a blank determination. The difference in the sugar content of the solution before and after treatment with yeast corresponds to the fermentable sugar present.

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[4] Marquette run No. 75.

[5] "Red Star" compressed yeast was used in all the experiments reported in this paper.

## Comparison Between the Fermentability of Wood-sugar Worts as Determined by Yeast Sorption and by Fermentation

A comparison was made between the fermentability of wood-sugar worts as determined by yeast sorption, and by fermentation of an 8-liter batch. The results are given in table 3.

Over 100 fermentations in 8-liter batches using hydrolysates from 15 different species of wood were checked by the yeast sorption method with essentially the same results.

## The Determination of Alcohol in Fermented Wood-sugar Worts

The fermentation of wood hydrolysates or waste sulfite liquor produces a beer containing between 0.5 and 2.5 percent alcohol by weight. When analyzing solutions of this concentration, it is necessary to use controlled methods in order to get results of satisfactory accuracy.

### The Quantitative Removal of Alcohol from Beer by Distillation

In developing a procedure for the analysis of dilute alcohol solutions, it is important to know the minimum percentage of a beer that must be distilled in order to quantitatively remove the alcohol. By taking off this minimum percentage of the beer, a distillate of maximum concentration is obtained, resulting in greater accuracy in the final measurement.

By means of the equation of Virtanen and Pulkki (12) it can be shown that distilling off 45 percent of the initial volume of a beer will result in in the removal of 99.9 percent of the alcohol.

### The Quantitative Determination of Alcohol

A number of techniques are available for the determination of alcohol in dilute solutions. A determination of the specific gravity, refractive index, or of the amount of dichromate required to oxidize the alcohol to acetic acid have all been used satisfactorily.

In work on wood sugars, the use of a refractive index method was avoided because of the interference by furfural. The difference between the refractive index of furfural and water is eight times as great as the difference between the refractive index of alcohol and water. The error due to extraneous materials in the refractive index determination is seriously high. In the specific gravity method, uncorrected values are obtained that are slightly low. The dichromate oxidation method for the determination of ethanol is useful where small quantities of sample are available at low concentration, but the method requires more time per determination than does the specific gravity method.

In the official method of the Association of Official Agricultural Chemists for alcohol, an unjacketed pycnometer is filled at a temperature below that at which it is to be thermostated, thermostated for 20 minutes, and then handled in the usual way. Although vacuum-jacketed pycnometers have been used in an effort to speed up the determination, they have not proven entirely successful. The technique in this method is to rinse several times, and then fill th pycnometer with thermostated liquid, assuming that the insulating ability of the vacuum jacket will avoid volume changes of the liquid while the stopper is adjusted and wiped. It was found, however, that when dilute solutions were used and the room temperature differed from the thermostat temperature by 5° or 10°, it was impossible to get reproducible results due to temperature changes occurring during the filling of the pycnometer.

## The Determination of Alcohol Using the Westphal Balance

The method chosen for alcohol analysis of fermented wood sugars was one involving the determination of the specific gravity with a Westphal balance. The apparatus used is shown in figure 6. One of the balance pans of a high-quality analytical balance was removed, and in its place was suspended a mercury-filled glass bob 3-3/8 inches long by 9/16 inch in diameter in such a way that it hung freely in a jacketed glass container of 4-1/4 inches by 7/8-inch inside dimensions. Thermostated water was pumped through the jacket from a bath maintained at 20.00° C. ± 0.01° C. On hot days there was a difference of about 0.1° C. between the temperature of the bath and the outlet from the jacket, but this differential was constant throughout the day's operations.

In the tests, the bob is weighed in air, in freshly boiled water, and in the solution that is to be analyzed. The loss in weight of the bob in the solution divided by the loss in weight in water gives the specific gravity. Due to a surface tension effect on the supporting wire the balance works less smoothly and a less reproducible rest point is obtained when water is used than when alcohol solutions are used. To overcome this difficulty with water and very dilute alcohol solutions, 1 milliliter of a 0.8 percent solution of a wetting agent, Nacconol NR, is added to the water used for standardization and to the beer distillate before final adjustment of the volume. The effect of the Nacconol NR on the density of the alcohol solution is neglected in the calculations, since it is added in the same concentration to both the unknown solution and to the water used for standardization.

## Procedure

Distillation.--A 100-milliliter sample of beer is pipetted into a 500-milliliter round-bottom flask, neutralized using a spot plate and brom thymol blue indicator, and distilled into a 50-milliliter volumetric flask. After about 48 milliliters have distilled, the distillation is interrupted and 1.00 milliliter of 0.8 percent Nacconol NR is added. The volume is then adjusted to the mark. The volumetric flask containing the distillate is thermostated at 20° C.

Determination.--The apparatus is standardized against water before each day's operations by weighing the bob in freshly boiled distilled water to which a wetting agent has been added in the same proportions as in the beer distillate. The container is always filled to the same level so that the same amount of liquid is always displaced by the wire supporting the bob. To initiate a gentle swing of the balance pan on release, a rest point is chosen about 6 millimeters off center. The determination of the weight of the bob in the unknown solution is carried out in the same manner.

Calculations.--The specific gravity may be calculated by the following formula:

$$\text{Specific gravity} = \frac{\text{Weight of bob in air} - \text{Weight of bob in unknown solution}}{\text{Weight of bob in air} - \text{Weight of bob in water}}$$

The concentration of the alcohol in the beer in terms of grams per 100 milliliters is determined by reference to a table showing the relationship between specific gravity and alcohol concentration. Since the concentration in the distillate is double that in the beer, the value obtained must be divided by two.

To make routine calculations more rapid, the value of the weight of the bob in alcohol solutions minus the weight of the bob in water may be plotted as a function of the alcohol concentration in the beer to which it corresponds. In this way calculations are reduced to a simple subtraction and reference to a graph.

In order to correct for volatile material other than alcohol in the beer, an unfermented sample of the wood-sugar solution may be distilled and the percentage of apparent alcohol determined in the usual way. The alcohol concentration of the beer is then corrected by subtracting the apparent alcohol concentration of the wort before fermentation from the value obtained after fermentation. In the samples tested, this apparent alcohol concentration of the wort before fermentation has proven to be a small negative value.

The alcohol yield as determined by the specific gravity method may be checked by the use of an immersion refractometer, using the tables provided by the Association of Official Agricultural Chemists.

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(9) Ibid. p. 498.

(10) Shaffer, P. A. and Somogyi, M., J. Biol. Chem. 100:695 (1933).

(11) Somogyi, M., J. Biol. Chem. 75:33-43 (1927).

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Table 1.--Comparison of the values for the percent of reducing sugars in wood-sugar solutions determined by different methods

| Sample | Munson-Walker | Electrometric titration | Shaffer-Somogyi 30-minute heating |
|---|---|---|---|
| | Percent | Percent | Percent |
| Untreated | | | |
| 19-1 | 5.77 | 5.70 | 5.40 |
| 19-3 | 3.48 | 3.47 | 3.17 |
| 19-6 | 2.31 | 2.31 | 2.16 |
| 19-9 | 1.82 | 1.80 | 1.71 |
| Neutralized and clarified | | | |
| 19-1 | 5.65 | 5.72 | ..... |
| 19-3 | 3.41 | 3.45 | ..... |
| 19-6 | 2.26 | 2.26 | ..... |
| 19-9 | 1.77 | 1.74 | ..... |

Table 2.--Relative reducing powers of various sugars toward the sugar reagent

| Sugar | Sample used | Reducing sugar calculated as glucose | | |
|---|---|---|---|---|
| | | Found by Analyst A | Found by Analyst B | Average |
| | Grams | Grams | Grams | Grams |
| d-glucose..... | 1.000 | 1.000 | 1.000 | 1.000 |
| d-xylose..... | 1.000 | .930 | .926 | .928 |
| d-arabinose..... | 1.000 | .760 | .760 | .760 |
| d-mannose..... | 1.000 | .926 | .928 | .927 |
| d-galactose..... | 1.000 | .814 | .816 | .815 |

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Table 3.--Comparison between fermentability of wood-sugar worts as determined by fermentation and by yeast sorption

| Run No. | Species | Fermentability, 8-liter fermentation | Fermentability, yeast sorption method |
|---|---|---|---|
| | | Percent | Percent |
| 3 | Spruce | 72.9 | 74.6 |
| 4 | do | 74.0 | 73.4 |
| 5 | do | 72.5 | 71.6 |
| 8 | do | 76.2 | 74.2 |
| 9 | do | 75.1 | 73.5 |
| 19 | do | 76.0 | 76.4 |
| 20 | do | 75.5 | 75.3 |
| 21 | do | 76.5 | 75.0 |
| 47 | Douglas-fir | 77.9 | 77.4 |
| 48 | do | 81.0 | 81.1 |
| 49 | do | 81.3 | 80.6 |
| 50 | do | 81.4 | 82.3 |
| 51 | do | 81.2 | 80.8 |
| 52 | do | 82.0 | 80.0 |
| 53 | do | 80.8 | 79.3 |
| 54 | do | 80.5 | 79.2 |

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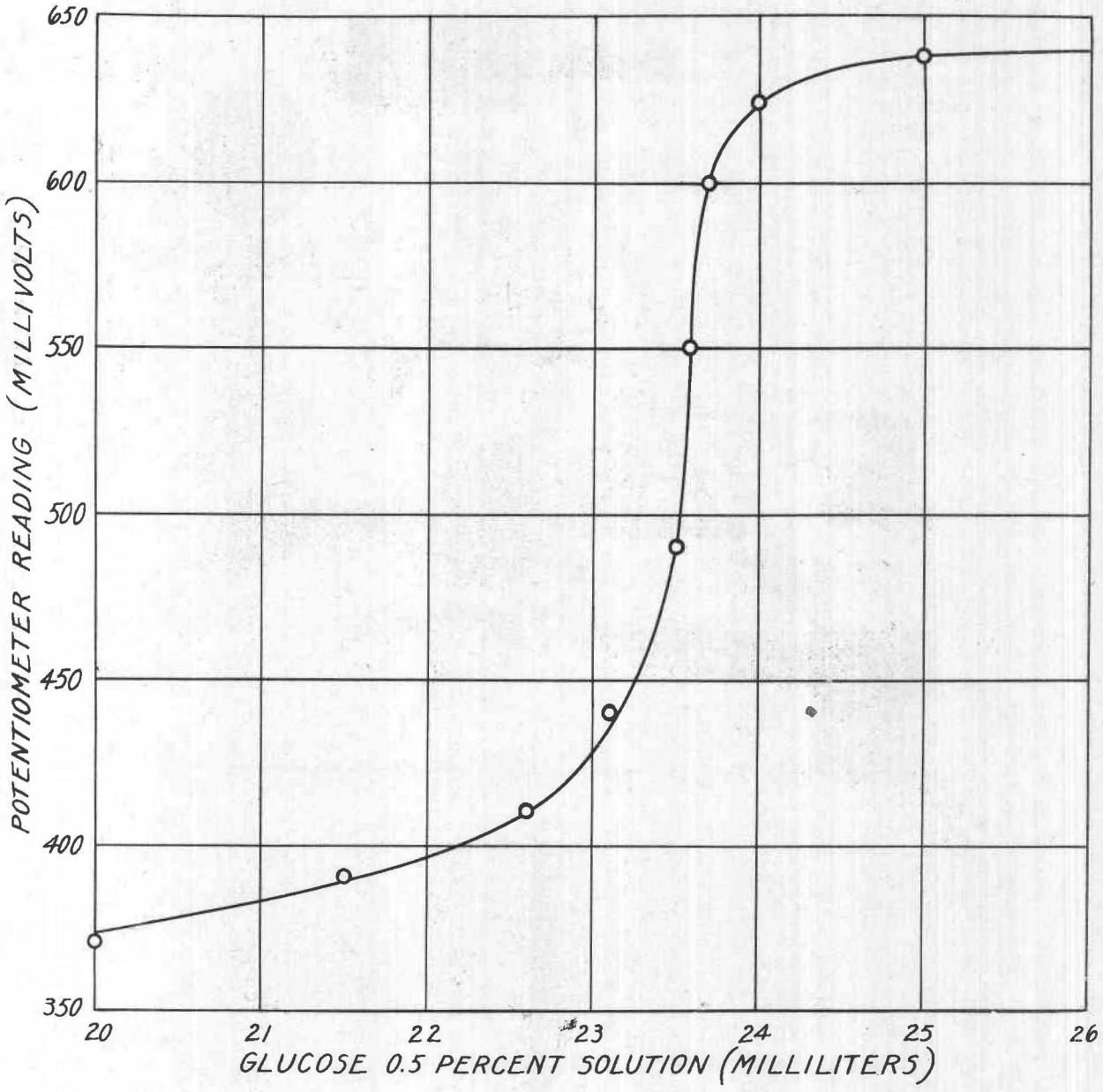


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Figure 1.--Electrometric titration curve for glucose.

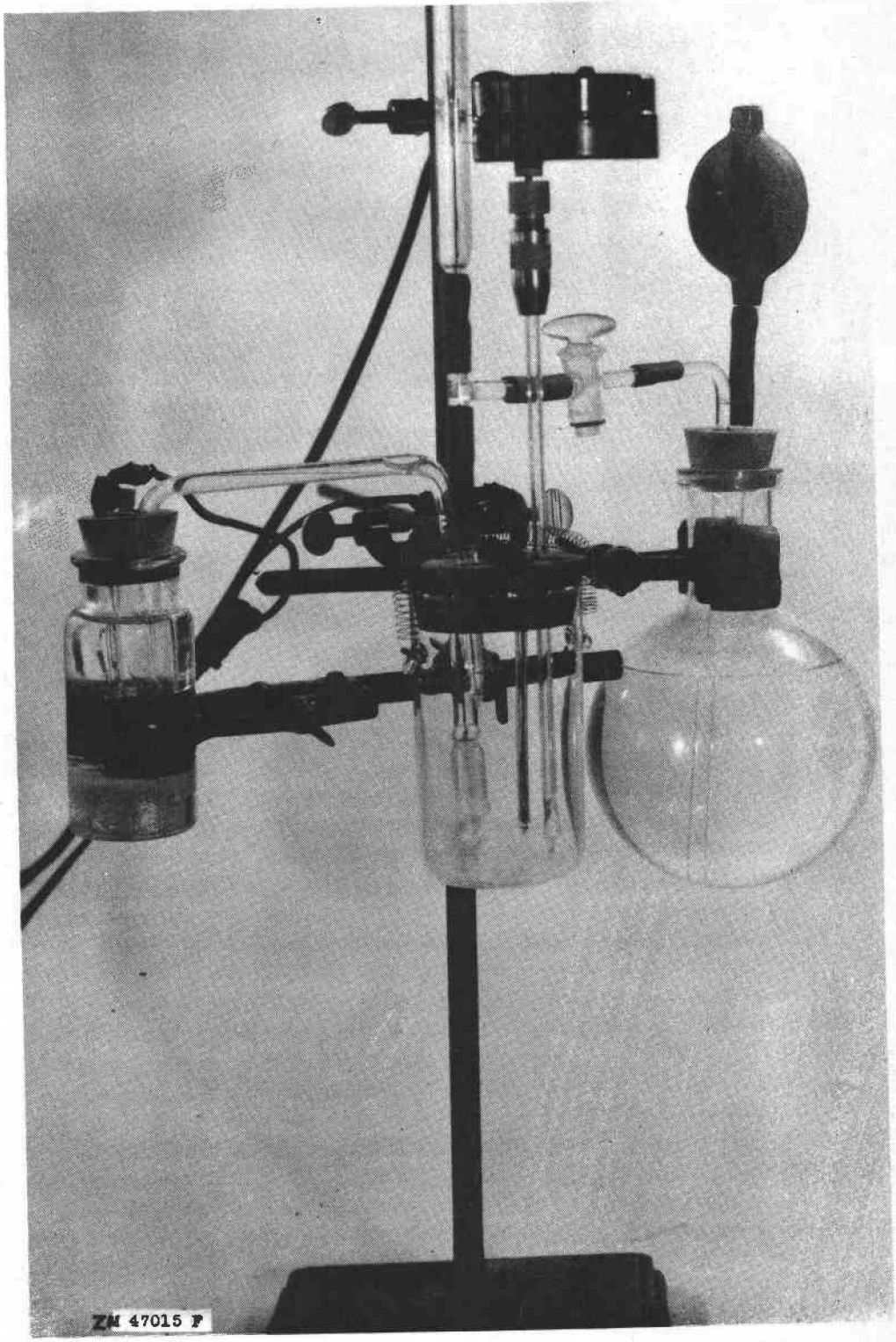


Figure 2.--Electrometric titration apparatus

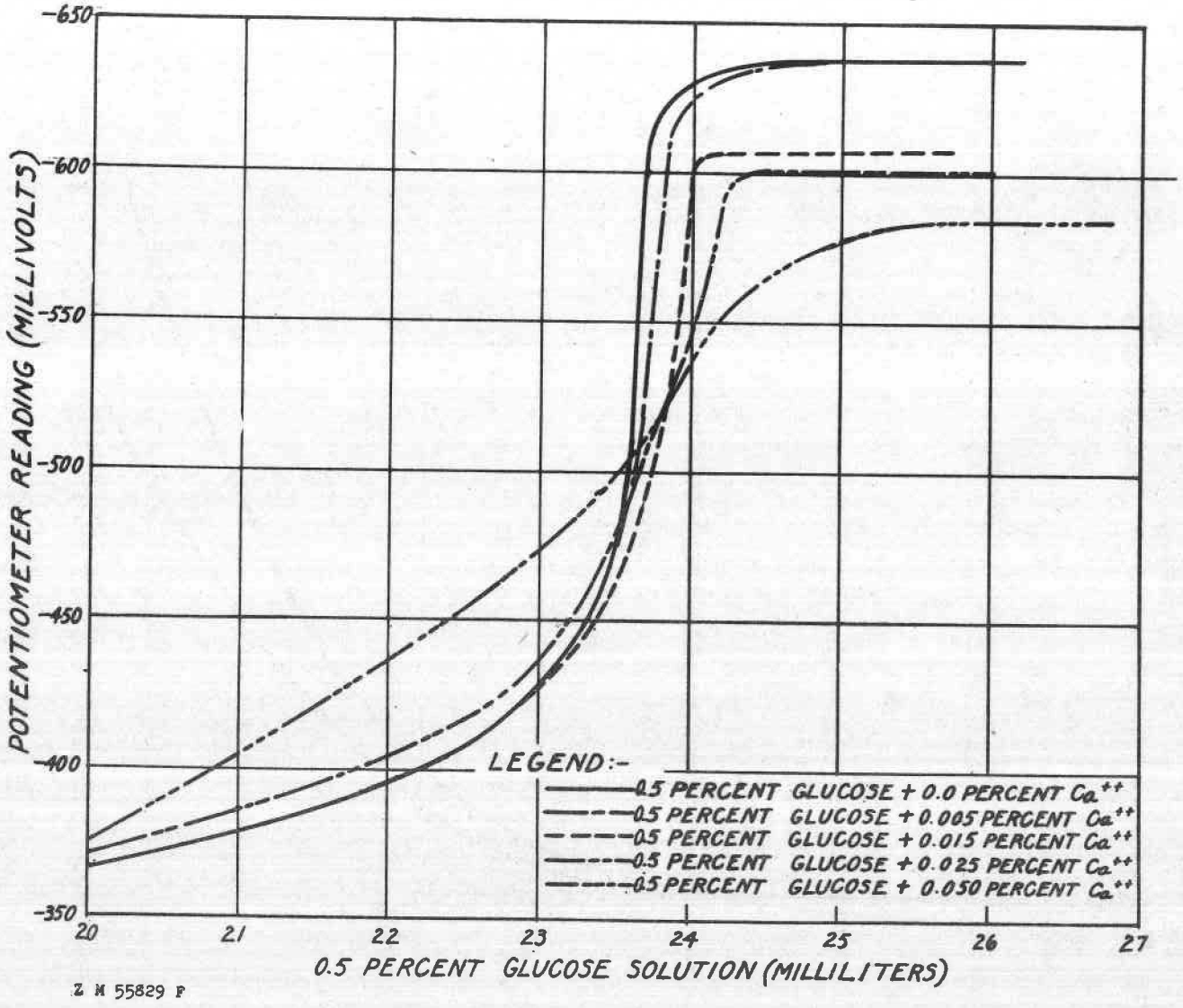


Figure 3.--The effect of calcium on the electrometric titration of Fehling's solution with glucose.

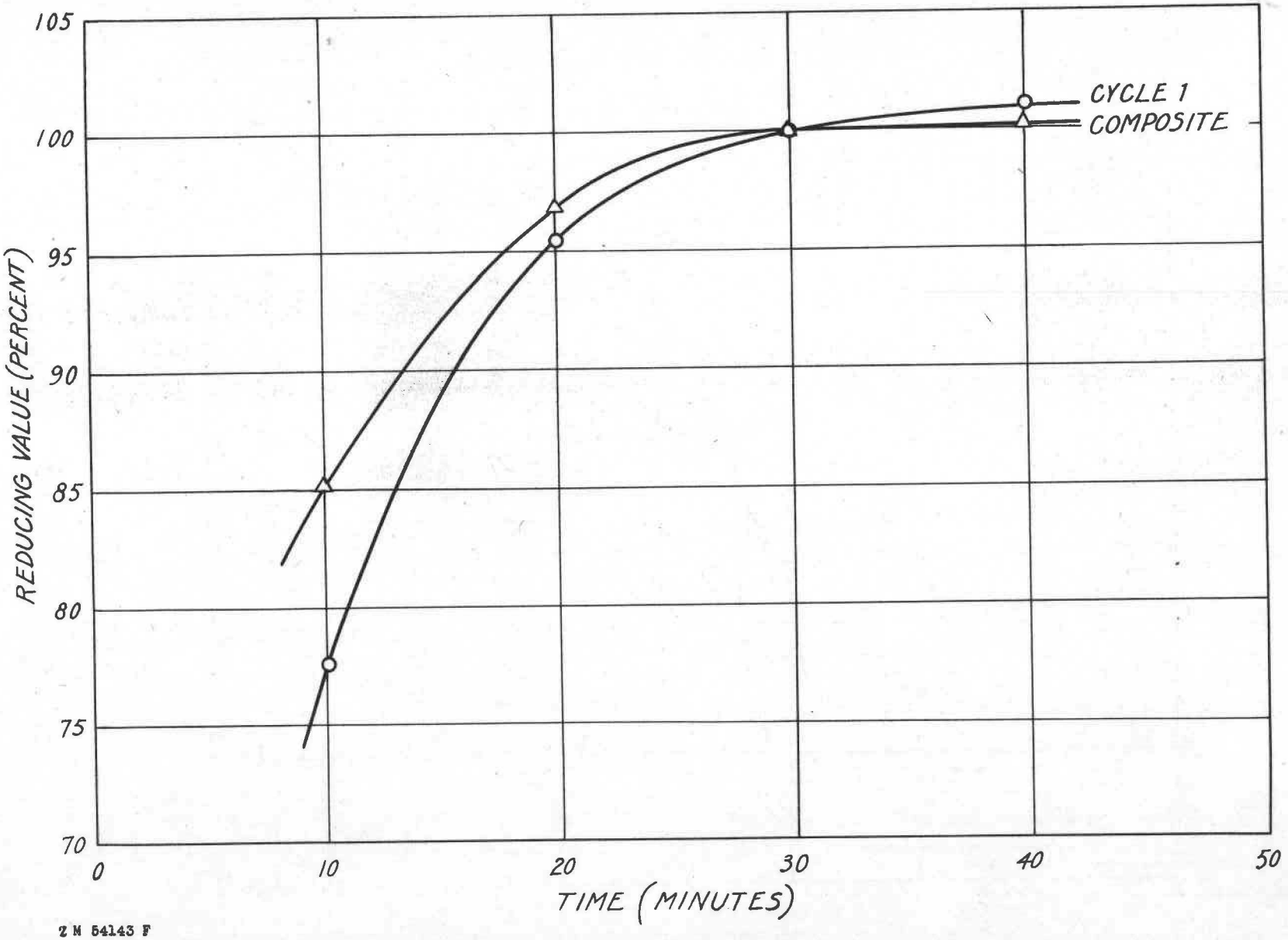


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Figure 4.--Reducing value of wood hydrolysates heated with Shaffer and Somogyi reagent 50.

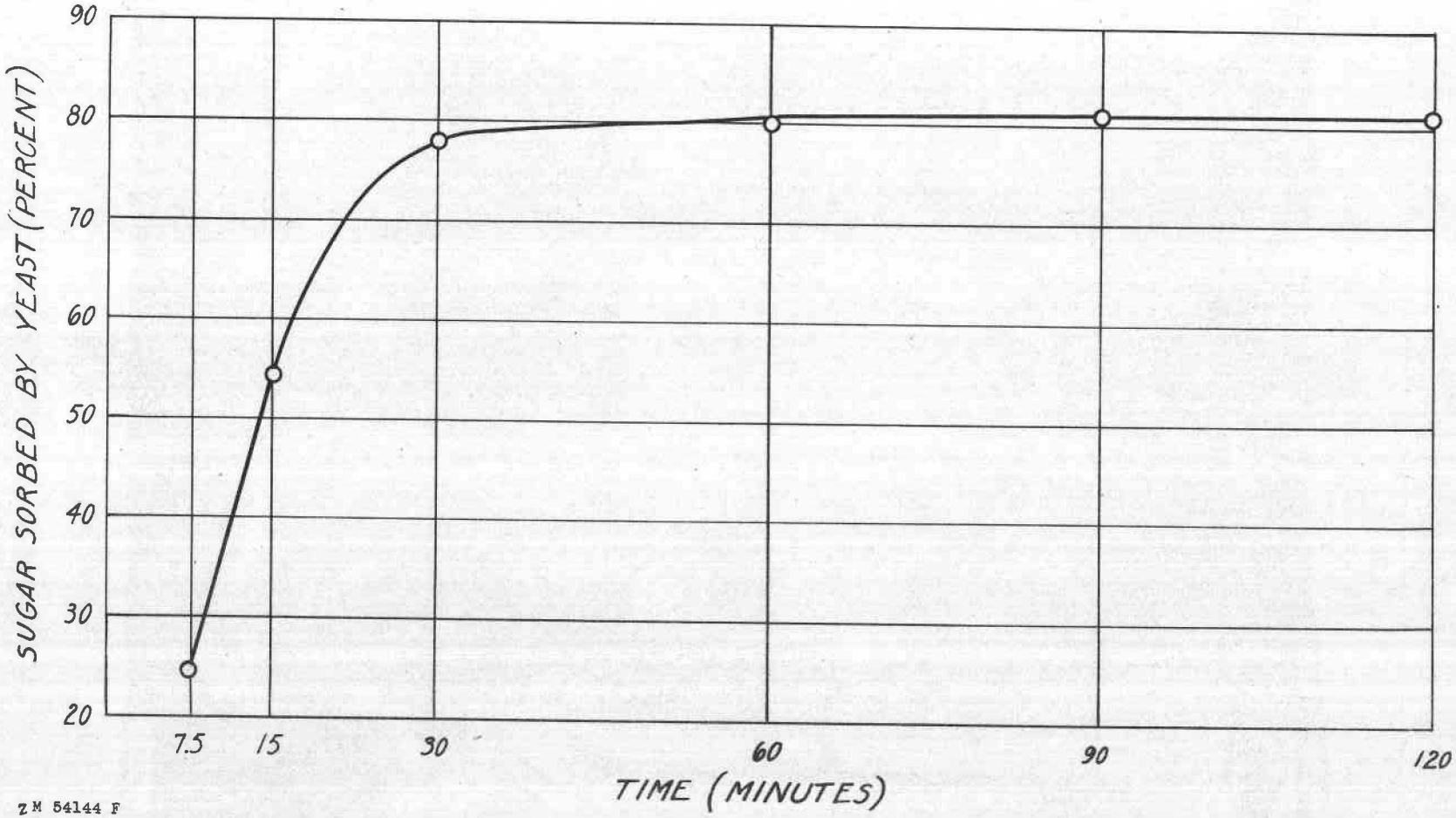


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Figure 5.--Sorption of fermentable sugars from wood hydrolysates by a 5 percent suspension of yeast.

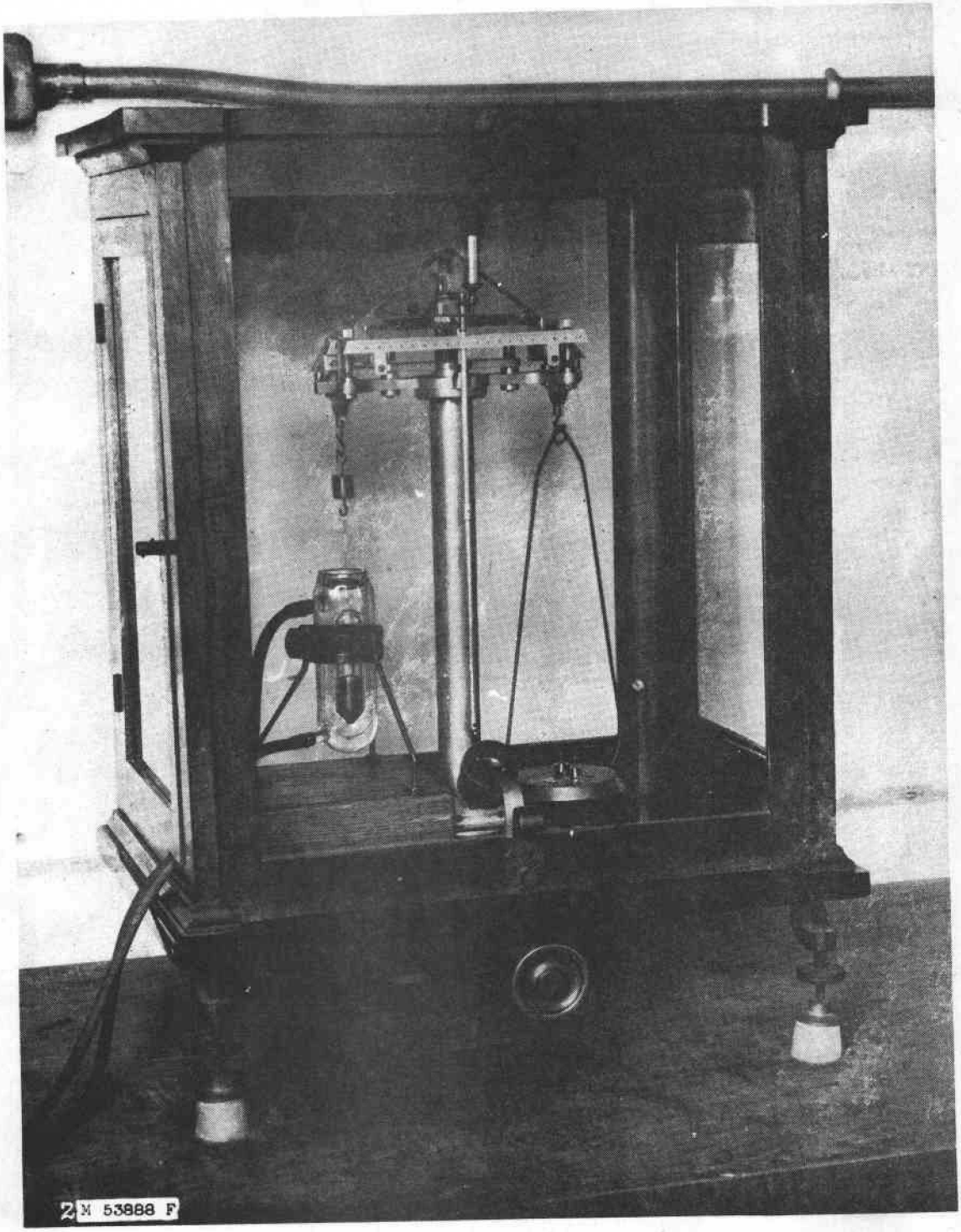


Figure 6.--Westphal balance and jacketed container.