The TTZ (Triphenyltetrazolium Chloride) Reaction for Invert Sugars

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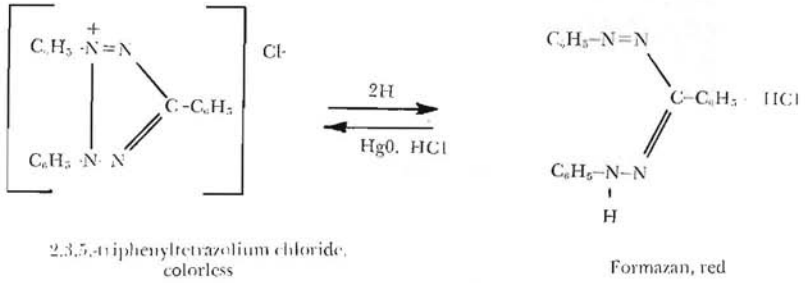
Introduction

The classical Lane-Eynon method for the determination of invert sugar has served well for many years. But the time consumed for the analysis places an extreme burden on the laboratory when studies are made that require evaluations on several samples.

The TTZ reaction with reducing sugars can provide an alternative procedure that is far superior to the Lane-Eynon for speed, and is no less accurate. The reagent 2,3,5-triphenyltetrazolium chloride is reduced to an insoluble red compound, formazan, under alkaline conditions, heat and in the presence of reducing sugars (1)[2]:



2,3,5-triphenyltetrazolium chloride, colorless

Formazan, red

Under certain conditions, the amount of formazan produced is proportional to the amount of reducing sugar. The compound is soluble in organic solvents, such as isopropanol, to give a clear, red solution that can be measured colorimetrically.

Mattson and Jensen (2) applied the reaction to the determination of glucose, fructose and lactose in nonsucrose solutions. Carruthers and Wootten (3) and, later, Bichsel (4) studied the TTZ reaction and established conditions for the rapid determination of invert sugars in factory juices with an accuracy at least as good as with the Lane-Eynon procedure.

The present studies have lead to basic modifications of the method that further reduces manipulation time and circumvents

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[2] Numbers in parentheses refer to literature cited.

certain potential sources or error: no pretreatment of juice sample is required; no turbid solutions develop to require filtration; careful timing of the reaction is not required; an acid barricade of the reaction at a specified time is not necessary; no extreme care is required in preparing reagents; all simple reducing sugars give the same amount of reaction to simplify preparation of standards; and addition of sucrose to standards is not required since a permanent correction curve for sucrose is established with such accuracy that "pure" sugars can be examined for contamination with reducing materials.

The Method

Materials

6" × ¾" pyrex test tubes.

#2 rubber stoppers.

Metal test tube racks. Preferably for two rows of test tubes.

Boiling water bath.

Suitable flasks. For reagent, standards and pre-dilution of sample if required.

Suitable burettes. 25 or 50 ml. For reagent addition and for addition of water when dilutions are made directly in the reaction tube.

Suitable pipettes. 5 ml pipettes are suitable if a full 2 ml of sample is being used. For most fractional deliveries a 2 ml pipette is satisfactory. For very small deliveries, a 0.2 ml pipette is preferred.

Colorimeter.

Reagents

TTZ stock sol'n—1.0% solution in about .005N HCl. Stable for several weeks.

NaOH stock—0.5N NaOH in a 1-2% disodium versenate solution. Stable.

Working reagent—Make a 1:1 mixture of the TTZ stock and NaOH stock. Stable about 8 hrs.

Solvent—Acidified, dilute isopropanol. Mix 1000 ml of isopropanol with 200 ml of distilled water and 10 ml of concentrated HCl.

These components can be varied somewhat. Requirements are that there be sufficient isopropanol to dissolve the formazan; sufficient water to keep sugar in solution; sufficient acid for decided acidification.

Standard invert stock—The usual Lane-Eynon stock standard: 9.5g sucrose in 100 ml distilled water plus 5 ml concentrated HCl; allowed to stand 2 days at room temperature; then made to 1000 ml. Contains 10 mg/ml of invert.

Pure crystalline dextrose or levulose may be used if desired.

Working standard—Make a 1/100 dilution of the stock standard. Contains 0.1 mg/ml.

If the working standard is used to "spike" unknown sugars or sugar solutions it is preferred that it be neutral: Dilute the acid Lane-Eynon stock 1:1 with 0.062N NaOH; dilute this neutral solution 1/50.

Procedure

1. Establish standard curve.

Into a duplicate series of racked test tubes deliver a series of aliquots of the working standard to provide 0.05 to 0.2 mg of invert—i.e., from ½ ml to 2 ml.

Make to a final volume of 2 ml with distilled water.

Add 1 ml of working reagent to each tube. Mix by shaking.

Stopper the tubes loosely to minimize evaporation.

Place the racked tubes in the boiling water bath for five minutes.

Remove to a cold water bath until cool.

Add 20 ml of solvent when convenient.

Read % transmittance at 480 millimicrons, using distilled water to set 100% transmittance. This may be done at leisure.

Plot standard curve on 2 cycle semi-log paper. Deviation from a straight line will occur only in a region below about 0.05 mg. Figure 1 shows a typical standard curve with mg of invert (right hand scale) plotted against % transmittance. One or two points on the curve should be rechecked each day.

A new curve should be established for each batch of TTZ purchased.

2. Unknown juice samples.

Follow the same steps as described above for standards. Aliquots may be as little as 0.1 ml on some raw juices. Final sample volume must always be made to 2 ml.

Translate transmittance to mg of invert from the standard curve.

Make the proper sucrose correction if highest accuracy is desired. The correction curve for sucrose is found in Figure 5 of this paper, and should be imposed on the same graph as your standard curve.

Reproducibility

Single tubes sometimes show more variation than desirable; hence this lab. makes all analyses in duplicate.

Reproducibility depends almost entirely on the accuracy of sample delivery. Where deliberate errors in sample delivery was

5%, the values indicated were in error by about 10%. However deliberate errors of 10% in the reagent volume had no apparent effect on the determination.

In a series of 38 duplicated tests, deviations from the standard curve were greater and more frequent when standards were delivered from a 50 ml burette than when delivered from a 2 ml pipette. Maximum deviation was 2.5% to either side of actual value.

Despite this, surprisingly consistent results can be obtained with 0.1 ml samples delivered from 0.2 ml pipettes.

Accuracy

Other workers have found the accuracy of the TTZ determination comparable to that of the Lane-Eynon (3).

In the present investigations values found by this procedure were in good agreement with Lane-Eynon determinations provided comparisons were made on juices with identical pretreatment.

Leading and deleading, as stipulated in the Lane-Eynon procedure, results in lowered values for both the Lane-Eynon and TTZ methods. Whether this is because of elimination of reducing substances other than sugars might be debated. The amount of lead used influences the extent to which values are lowered. Heavy leading of diluted molasses, or other juices, has caused up to 50% reduction in invert value as determined before treatment.

When a known amount of standard was added to molasses and followed with heavy leading and deleading, the entire amount of originally indicated invert was nullified in addition to 4% of the added invert.

It seems probable that invert content may be more accurately represented by values found on untreated juices.

Developmental Observations

Reaction Time

The family of curves in Figure 1 shows the relative rates at which various reducing sugars attain peak color. When time of reaction (left-hand scale) was plotted against % transmittance, all simple reducing sugars had reached the same point of minimum transmittance in 3-4 minutes. Each monasaccharide reaches this equilibrium point at a different rate.

By translating from time to percent transmittance (for a selected sugar) then to the standard curve in Figure 1, it is seen that mannose is only about 40% reacted in 90 seconds and 61% reacted in 2 minutes. The fructose reaction is much more

rapid, being 84% complete in 90 seconds and 97% complete in two minutes. Other reducing sugars, or their mixtures, show intermediate rate paths.

After minimum transmittance is reached, the color equilibrium is maintained for some time. Figure 1 shows equilibrium up to 10 minutes. However, color reversion can begin after about 7 minutes with low concentrations of invert; weaker concentration of alkali; or weaker concentration of TTZ.

Nevertheless, the time element is far from critical; and different ratios of reducing sugars cannot effect the determination.

Other Sugars

Melibiose, the galactosyl glucose component of raffinose, shows —on a weight basis—57% as much reaction as the reducing monosaccharides. Lactose is also shown, in Figure 1, as a matter of interest. This is also a galactosyl glucose, but it gives a 70% reaction. Apparently there is a difference in ease of hydrolytic cleavage of the two linkages.

Free galactose shows some reducing power. Its reaction equilibrium is reached after a full five minutes with 60% reaction.

The existence of galactose or melibiose in juices—at least in interfering amounts—would not be expected unless raffinose has undergone cleavage during the reaction. If raffinose cleavage should occur, however, fructose would be split off first and contribute to the reaction.

However, raffinose does not react significantly. 20 - 100 mg of raffinose—an amount many times that ever encountered in juice samples—had to be "spiked" with invert to reveal a reaction of 0.03%.

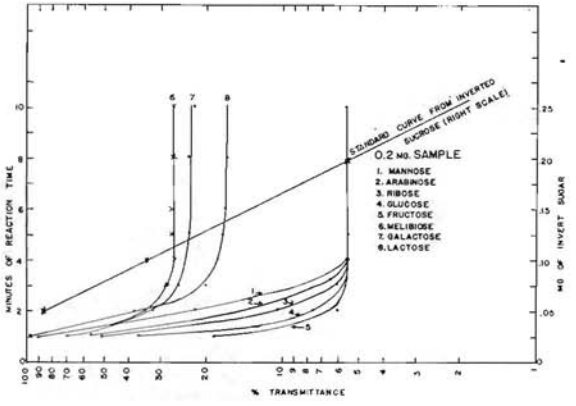

Figure 1.—Relative reaction rates of reducing sugars at 95°C (left scale).

Small amounts (0.2mg) of mannitol or inositol showed no detectable reaction.

Sucrose gives a slight reaction and usually should be considered. The sucrose subject will be discussed in more detail later.

The Alkali

All alkalies appear to have a concentration range that give the same amount of color for a given invert and TTZ concentration. Figure 2 shows that NaOH concentration can be varied from about 0.15 to 0.3N (as 1/3 of the total reaction mixture) without greatly effecting color intensity—regardless of invert concentration or quality of TTZ used.

Both Na_2CO_3 and Na_3PO_4 show the same phenomenon. Again used as 1/3 of the total reacting volume, sodium carbonate had a minimum effect range of 0.3 to 1.0N and required about 7 minutes reaction time. Trisodium phosphate had an optimum range of 0.3 to 0.6N with 4-5 minutes required to attain peak color.

The same standard curve was obtained with each of the three alkalies individually or in combination (NaOH used as 0.2N; Na_2CO_3 and Na_3PO_4 used as 0.45N). Color intensity was not effected when small amounts of $NaHCO_3$ were added to the NaOH or Na_2CO_3 - NaOH combination.

Weak alkalies require much longer heating periods to attain peak color. Sodium bicarbonate, for example, required about 30 minutes, and results were not as reproducible. When weak alkali concentrations exceeded the optimum range, the peak color attained was less than that produced with the optimum concentration—the effect was the same as when too little alkali is present.

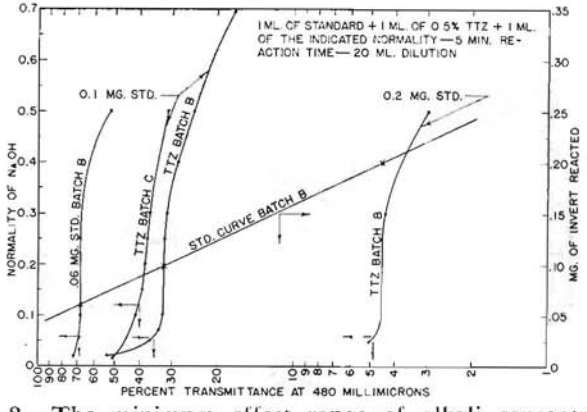

Figure 2.—The minimum effect range of alkali concentration with two different batches of TTZ.

The minimum effect range can be influenced somewhat by the particular batch of TTZ being used. This is also demonstrated in Figure 2. Batch C, used with a 0.1 mg standard, allowed a narrower optimum range and increased the slope of the curve.

However, by translating the transmittance values of different normalities to the standard curve, it is seen that a sizeable deviation in normality can be tolerated without appreciably effecting final results of the determination.

The TTZ Reagent

Stability

TTZ in aqueous solution produces formazan rather rapidly on exposure to ultraviolet or even direct sunlight. This vulnerability is increased under alkaline conditions. In fact, strongly alkaline conditions cause very rapid degradation of TTZ without U. V. catalysis. On the other hand, slight acidification with HCl maintains the tetrazolium structure and no formazan is produced. Consequently a very small amount of HCl in the TTZ stock solution (about 0.005N) will stabilize the solution. The minute change in NaOH normality that results in the final reaction mixture is too small to effect the reaction.

Behavior

The TTZ reagent was soon found to vary in strength from one purchase to another. The most extreme deviation was found in "Batch C"—shown in Figure 2 to be rather weak. Repeated attempts at recrystallization failed to alter its reactivity even slightly.

It was found necessary to establish a new standard curve for each purchase of TTZ.

Figure 3 shows that large increments in TTZ concentration (left-hand scale) cause only small increments in maximum color intensity with any concentration above about 0.3% TTZ in 1/3 of the reaction mixture.

A standard curve can obviously be constructed for any TTZ concentration, as shown in Figure 3. However, continued heating at the weaker concentrations of TTZ causes color reversion to begin more quickly—especially with small amounts of invert. Aside from this, Figure 3 shows that a difference of 0.1% in TTZ concentration effects transmittance by only about 1.0%. Error in indicated invert value would be about 2.0%. Obviously no great care is required in making up the TTZ reagent.

The level selected for the method corresponds to the 0.5% level in Figure 3.

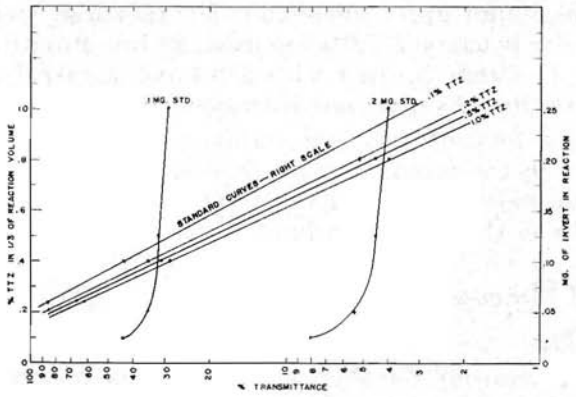

Figure 3.—Effect of TTZ concentration—1 ml std. + 1 ml 0.25N. NaOH + 1 ml TTZ reacted 6 min, diluted with 20 ml isopropanol—HCl.

Ionic Interference

During 1963 when the method was being used in the field, a marked inhibition of the reaction was noted. This effect was traced to commercially bottled distilled water that was being used to make up standards, reagents, and sample dilutions and found to be caused by minute traces of copper. Disodium versenate added to the reaction restored it to full intensity.

It was found that traces of Cu^{++} could completely inhibit the reaction but that essentially total reaction could be restored with versenate.

Pb^{++} or Zn^{++} gave small inhibitions at 25 ppm that were also completely eliminated with disodium versenate.

At 25 ppm, Fe^{+++} showed no interference, but Fe^{++} gave a slight positive reaction that was prevented with the sequestering agent. Larger amounts of Fe^{++} gave intense, and immediate, positive interference that could not be eliminated.

However, the incorporation of 1-2% disodium versenate in the caustic fraction of the reagent mixture would apparently overcome any interferences that might be encountered in beet juices.

Temperature

At low temperatures the reaction equilibrium occurred at lower color peaks and more time was necessary to reach the peak.

In Figure 4, 0.1 mg of invert was used with different amounts of sucrose. In the left half of the figure the equilibrium peak—with no sucrose present—was at 58% transmittance when the reaction temperature was at 70°C. At 95°C the peak was at 34% transmittance. The peak was at 49% transmittance with a 75°C temperature.

As high temperatures were approached, the effect became less. Small variations around the 95°C point had no measurable effect. It appears that normal barometric fluctuations would not change the boiling bath temperature sufficiently to distort results.

Sucrose

In Figure 4 it is seen that large amounts of sucrose, in solution with 0.1 mg of invert, have strong initial inhibiting effects. The inhibition, in the first seconds of reaction, is stronger with 500 mg of sucrose than with 100 mg. As time of reaction progresses, the inhibition is overcome. It is overcome more rapidly at high temperature than at low temperature. And it is overcome more rapidly at higher alkalinities.

Figure 4 shows that alkaline hydrolysis of the sucrose continues to contribute additional invert to the reaction, but at a decreasing rate. At the higher caustic normality and higher temperature, values with different sucrose concentrations are beginning to plateau in their proper relative positions after 5 minutes reaction time. The plateau is not as sharp with high sucrose levels; more attention should be given to timing the reaction where large amounts of sucrose are involved.

It is also apparent that the color contribution per unit of sucrose would not be arithmetically linear. Indeed it was found that apparent invert formation from sucrose, under conditions of this test, is a linear function of the log of sucrose concentration.

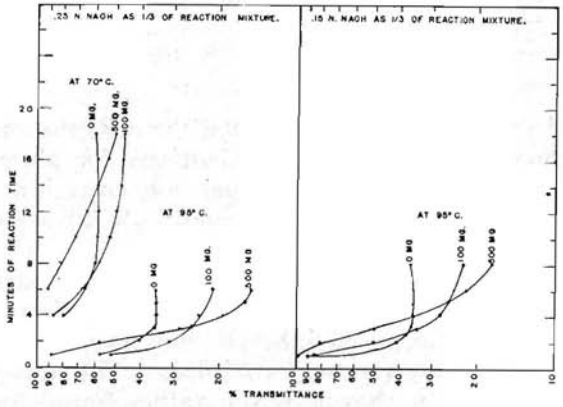

Figure 4.—Sucrose effect on color under conditions of varying time, concentration, temperature and alkalinity.

Many sugars were evaluated to establish this and only two absolutely invert-free sugars were found. One of these, Sugar D in Figure 5, is seen to give a straight line when apparent mg

of invert was plotted against the log of concentration from 10 to 1000 mg. (Obviously all samples were carefully "spiked" with invert since values are below the 0.05 mg limit for accurate evaluation).

Figure 5 also shows several other sugar samples evaluated at two or more concentration levels. The most striking of these is Sugar A—a "deteriorated" silo sample.

This sample was evaluated at various concentrations—with or without added standard. The invert values obtained were calculated as % on sucrose. The apparent percent invert on sugar decreased as concentration increased. For example, 300 mg of Sugar A showed about 0.176 mg of invert—as interputed from the standard curve—which would be 0.059% on sugar. At 100 mg the % indicated would be 0.072; 30 mg of sugar showed 0.027 mg of invert—or 0.09%.

When Sugar D was found, this behavior could be resolved. If Sugar D is invert free—its invert value at any concentration represents the contribution of sucrose itself to the reaction. Any value from a sugar that is additional to this is actual reducing sugar in the sucrose and would calculate to the same percentage value regardless of concentration used.

Thus:

1. Apparent invert from unknown − apparent invert from Sugar D = true invert.

2. $$\frac{\text{wt True invert} \times 100}{\text{wt Sucrose}} = \% \text{ invert on sugar.}$$

Figure 5 shows a graph constructed through the mean values found for Sugar A in repeated evaluations for about a dozen different concentrations. At any sugar concentration along this line, the true invert calculated to an essentially unvarying 0.047% on sugar.

As further proof, percent invert on Sugars B and C could be calculated at any concentration by the same simple formula.

Obviously there are more inherent sources of error in applying the method to sugars than to juices. The reproducibility is a bit less precise, as shown by the values found for Sugar A in Figure 5. Nevertheless a very reasonable accuracy is achieved, and the sensitivity of the method is such that differences between internal and external composition of sugar crystals can be measured with confidence.

For example, Sugar A was washed, in a fritted funnel, to remove the outer 43% of the crystals. The filtrate (Aw in Figure

5) was evaluated at two concentrations—260 mg and 130 mg in the reaction tubes. The corrected invert values showed .079 and .076% on sugar.

The residual material, Ar, was then dissolved and evaluated at concentrations of 340 and 170 mg in the reaction. The true invert at these levels was .052 and .031 mg—or .015% and .018% on sugar. Although the deviation for such unreplicated determinations can be large, the accuracy is still sufficient to be of practical value.

It is not impossible that application of the method in a study of deteriorated sugars might help give an insight into causes of deterioration.

FIGURE 5

SUCROSE DIFFERENCES IN INVERT CONTENT—SUCROSE CORRECTION CURVE

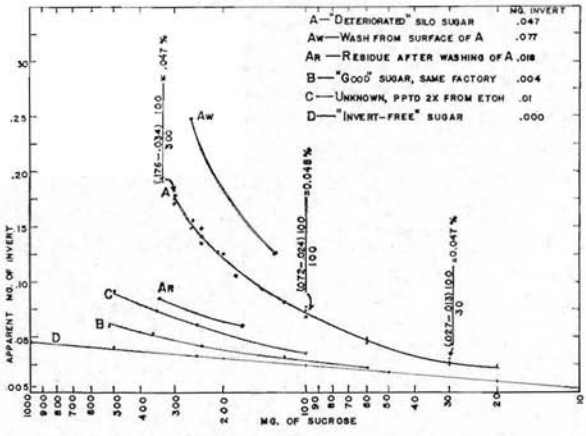

Figure 5.—Sucrose differences in invert content—sucrose correction curve.

Summary

Factors influencing the TTZ reaction for invert sugars were investigated. Optimum conditions for each factor were established to arrive at a simplified method giving results comparable to those of the Lane-Eynon procedure. Sensitivity of the method, and evaluation of interferences, permits reasonably accurate evaluation of factory sugar for invert content.

Acknowledgement

Particular appreciation is expressed to Joe Crowley, of this laboratory, for invaluable technical assistance in exploring many facets of this investigation.

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