A rapid screening method for estimation of total fermentable value of selected plant materials

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Abstract

Plant material is heated at 105°C in trifluoroacetic acid. After neutralisation and dilution, the reducing substances in this solution are determined by an automated colorimetric procedure, using 3,5 dinitrosalicylic acid. The resulting figure, described in this paper as the total fermentable value is used as an estimate of certain carbohydrate substances, for example starch and sugars in the sample. The method is manipulatively simple, and is suitable for screening of large batches of plant samples. The precision for a single determination is ±2.7%.

INTRODUCTION

Enzymatic (Haissig and Dickson 1979) or chromatographic (Marsden *et al.* 1982) methods are available for analysis of specific carbohydrates and related substances found in plant materials. Because these methods tend to be slow and tedious, other methods suited to automation are often preferred for screening purposes (Marais 1979), although there may be uncertainty in what they represent. For example, the total fermentable components of plant materials can include starches, sugars and other reducing substances. Results obtained using one acid hydrolysis procedure may not be compared with results obtained using a different acid hydrolysis procedure because of the non-specific nature of the organic compounds measured.

Specifically, there was a research need to develop and evaluate a method capable of providing reproducible estimates of total fermentable substances in large batches of samples. The method chosen is based on trifluoroacetic acid hydrolysis, followed by an estimation of reducing substances in the hydrolysate by an automated 3,5 dinitrosalicylate procedure (McCready *et al.* 1974; Miller 1959). Data so derived are referred to in this paper as total fermentable value. The effect of likely inorganic interferents, and the ruggedness of the acid hydrolysis step are tested. Procedural details and method performance criteria are presented.

The method has been used to assess the suitability of cassava roots and other starch sources for ethanol production by fermentation. Other research applications embrace screening for differential accumulation of fermentable substances in plants such as cassava, lucerne and macadamia as a consequence of variations in environmental and agronomic conditions.

MATERIALS AND METHODS

Apparatus

- Technicon AAI auto-analyser (see Figure 1).
- Aluminium heating block with temperature control unit to give±2°C.

•Vacuum oven with air drying system (Horwitz 1970*a*).

•McCartney glass culture bottles, 28 mL (United Glass, Cat. No. V667), screw cap with teflon inserts.

Reagents

Unless otherwise stated, all reagents are analytical grade and 'water' is deionized water.

D-glucose.

Diluent solution. Dissolve 0.5 g benzoic acid in 1000 mL of water and filter through Whatman No. 54 filter paper.

Reagent blank. Mix 2.0 g sodium hydroxide and 3.7 mL trifluoroacetic acid (LR) in 1000 mL of diluent solution. Solution is made just neutral to phenolphthalein using trifluoroacetic acid (LR) or sodium hydroxide and filtered through Whatman No. 54 filter paper.

1.00M trifluoroacetic acid. 75 mL conc. trifluoroacetic acid (LR) diluted to 1000 mL. Molarity is checked by titration against 1.00M sodium hydroxide and adjusted to 1.00M.

Colour developing reagent. The following are dissolved in water:

3,5 dinitrosalicylic acid (DNSA) (LR)	: 8.0 g
Sodium hydroxide	: 10.2 g
Ethylenediaminetetra-acetic acid disodium salt (EDTA)	: 10.0 g
Phenol	: 1.6 g
Sodium metabisulphite (added on day of run)	: 0.41 g
30% Brij 35	: 0.2 mL

Solution is made to 1000 mL and filtered through Whatman No. 54 filter paper.

Standard solutions

5000 µg/mL of glucose. Add 5.00 g glucose (vacuum dried at 60°C) to a 1000 mL volumetric flask, dissolve and make to volume with reagent blank.

5000 µg/ml of fructose. Add 5.00 g fructose (vacuum dried at 60°C) to 1000 mL volumetric flask, dissolve and make to volume with reagent blank.

Mixed calibration standards. The ratio of glucose to fructose used in calibration standards should correspond to that found in plant hydrolysates, as glucose and fructose give different responses to the colour-developing reaction used.

Calibration standards containing 100, 200, 300, 400, 500, 600, 800, 1000 and 1200 µg/mL of mixed sugars represent 10, 20, 30, 40, 50, 60, 80, 100 and 120% total fermentable value of a 50 mg sample when diluted to 50 mL. These solutions were prepared from the appropriate bulk standards in suitable ratios, diluted to volume with reagent blank.

Hydrolysis

Sample material (<0.5 mm) was dried in a vacuum oven at 60° to 70°C using an air drying system (Horwitz 1970*a*). A dried sample, 50 mg, was transferred into a 28 mL McCartney bottle and 2.5 mL of 1.00M trifluoroacetic acid was added. The bottle was sealed, heated at 105± 3°C for 2.5 hours in an aluminium heating block and cooled. The lid was removed and 2.5 mL of 1.00M sodium hydroxide added. The solution was quantitatively transferred to a 50 mL volumetric flask, made to volume with diluent solution and filtered into a test tube through Whatman No. 41 filter papers before determination.

Determination

The automated system was assembled as shown in Figure 1 and operated using the parameters indicated. Note the use of nitrogen instead of air in the manifold. Calibration curves were prepared by running the standards described. Results were calculated as per cent total fermentable value of dry samples.
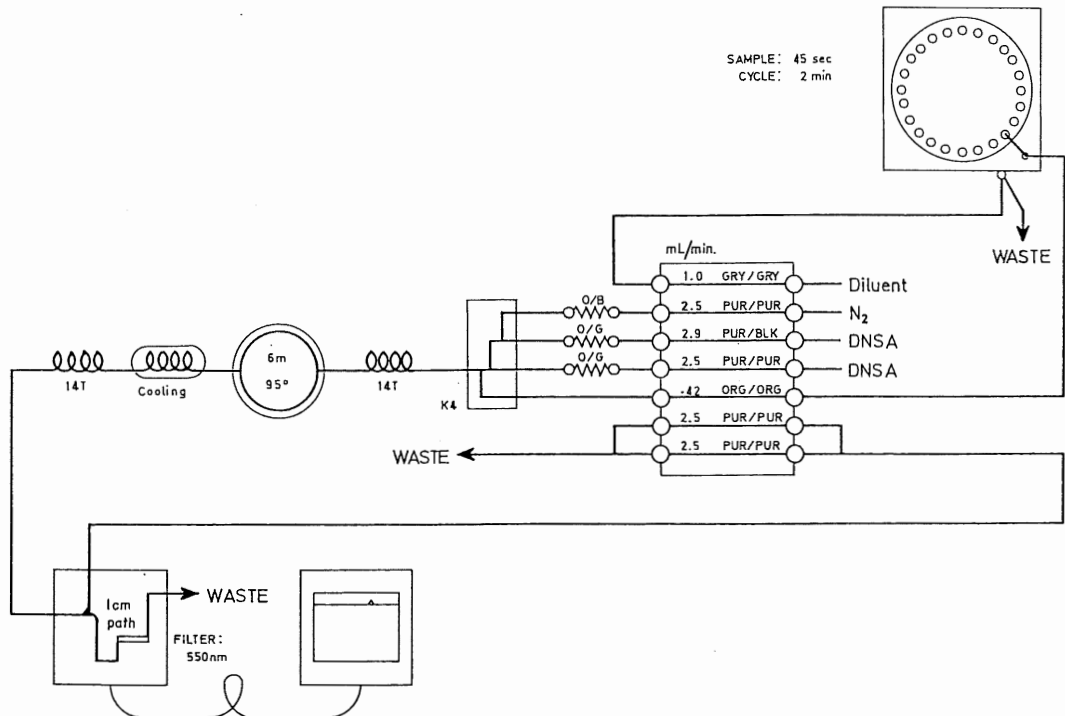

Figure 1. Auto-analyser flow chart for total fermentable value of plant material.

Ruggedness of method

Ethanol-washed maize flour was considered suitable for testing the ruggedness of hydrolysis conditions as the total fermentable procedure was mainly intended for analysis of high starch plant samples, for example, cassava.

Analysis of vacuum-dried ethanol washed maize flour by a polarimetric procedure (Horwitz 1970*b*) gave 98.5% starch. The effects of variations in hydrolysis times and temperatures were determined by hydrolysing ethanol washed maize flour material for various times between one and three hours at temperatures between 95°C and 115°C.

The use of a laboratory oven instead of an aluminium heating block for sample hydrolysis was tested.

The effect of variations in trifluoroacetic acid strength on analytical results was determined by hydrolysing ethanol-washed maize flour material with varying acid strengths. In each case, the volume of sodium hydroxide solution required to neutralise the acid was varied accordingly. In another experiment, excess amounts of either trifluoroacetic acid or sodium hydroxide were added to ethanol washed maize flour samples after hydrolysis to test the effect of variations in hydrolysate solution pH on colour development.

A series of experiments were conducted to determine the optimum reagent concentration for the manifold described.

Interferences

Interferent effects due to 50 μg/mL of potassium, 40 μg/mL of calcium, 10 μg/mL of magnesium, 1 μg/mL of iron, 16 μg/mL of phosphorus, 6 μg/mL of manganese and 8 μg/mL of sulphate in the presence of 1000 μg/mL of glucose were monitored. Levels tested were more than double average levels found in plant hydrolysates.

Calibration

Samples, 50 mg, of glucose, sucrose, fructose and maltose were hydrolysed and determined by this procedure to compare responses.

A calibration curve was prepared from nine standards (100 to 1200 μg/mL glucose).

Detection limit was estimated by analysing a near blank solution followed by a blank solution ten times, and calculated as twice the standard deviation of the near blank solution.

Recovery of added starch

To 50 mg samples of finely ground cassava rind material, additions of 0, 10, 20, 30 and 40 mg of ethanol-washed maize flour were made in triplicate. The starch content of this ethanol-washed maize flour was known from polarimetric analysis of $CaCl_2$ extracts (Horwitz 1970b) to be 86.2%. The moisture content determined by drying at 60°C under vacuum (Horwitz 1970a) was 12.5%.

Precision

Five plant materials were analysed in triplicate on each of three days by the same analyst.

Statistical analyses were done by the methods of Youden and Steiner (1975). Repeatability is defined as the error between replicates and reproducibility as the error between days. Reproducibility relative confidence interval ($P>0.05$) is the expression of twice the reproducibility standard deviation as a percentage of the grand mean.

RESULTS AND DISCUSSION

Ruggedness of method

Variations in hydrolysis times between 2 and 3 hours and variations in temperatures between 100°C and 110°C had no significant effect ($P>0.05$) on analytical results.

As a result, 105±3°C for 2.5 hours was considered to give the most rugged hydrolysis conditions. Temperatures higher than 110°C or lower than 100°C, or times longer than 3 hours or shorter than 2 hours all gave lower recoveries of maize starch.

The use of a laboratory oven was found to give less reproducible conditions than an aluminium heating block. After 60 samples were introduced into an oven at 105°C, the temperature dropped by up to 20°C. Time taken to regain this temperature was considerable, giving rise to batch variations in sample analyses.

Variations in trifluoroacetic acid strength between 0.7 and 1.3M did not affect analytical results. Addition of 0.5 mL more than the recommended volume of either acid or alkali after hydrolysis affected results by less than 2%. From these results it can be concluded that normal errors involved in the dispensing of both acid and alkali may be discounted.

Tests on colour reagent concentration showed that compared to the specified conditions in this method, an increase in reagent concentration of 25% increased chart recorder response of the 1200 µg/mL glucose standard by 1%, while a decrease in the reagent concentration of 25% reduced response of the 1200 µg/mL standard by 5%. Hence, for the manifold and reagent concentrations described, differences between batches of reagent due to normal errors involved in reagent preparation will have little effect on the sensitivity of the determination. Similarly, it is reasonable to expect that the reagent: sample volume ratio is not critical to the determination.

Interferences

Preliminary testing showed calcium, magnesium and manganese affected the absorbance of a 1000 µg/mL glucose solution by more than 10%, at the levels described. This was due to effects on the colour development reaction. After incorporation of 1% EDTA in the colour development solution to complex these cations, none of the elements tested affected the absorbance of a 1000 µg/mL glucose solution by more than 1%. The EDTA increased the method's limit of detection by increasing absorbance on reagent blank, but the reduction in interferences justified this.

Samples, 50 mg, of powdered cellulose (Whatman, chromatographic grade) were hydrolysed and analysed as normal samples. The results (which were less than the detection limit) showed that structural cellulose is not hydrolysed under the conditions of this procedure and therefore does not interfere with a total fermentable value determination.

Calibration

Table 1 shows the response of some sugars when hydrolysed and determined by this procedure. Different sugars give different responses due to the complex, non-stoichiometric nature of the chemistry involved (Marsden *et al.* 1982; McCready *et al.* 1974; Miller 1959). Therefore, standards should contain a similar composition of sugars to that contained in plant hydrolysates. The average ratio of glucose to fructose for each sample type should be determined by a suitable method (McCready, *et al.* 1974; Englyst 1981). The standards for analysis of samples of that type should then be prepared according to that ratio. When glucose comprises at least 95% of the sugars in plant hydrolysates, for example, high starch samples, glucose may be used as the sole standard material.

Table 1. Colorimetric response of various sugars after hydrolysis

Sugar	Response relative to glucose
Glucose	100
Sucrose.	74.9
Fructose	51.2
Maltose	99.9

The calibration curve showed a loss of linearity at levels below 100 µg/mL glucose, due to oxidation of glucose by dissolved oxygen (Marsden *et al.* 1982). The omission of nitrogen from the manifold would cause much greater losses of glucose, reducing sensitivity at all levels.

The detection limit was estimated to be 16.2 µg/mL of glucose. The limit of quantitation (five times the detection limit) was calculated at 81 µg/mL of glucose, or 8.1% total fermentables in a 50 mg sample.

Recovery of added starch

Starch values were calculated on the assumption that 1.00 g starch yields 1.105 g glucose when hydrolysed. This is based upon the formula:

$$\text{starch:glucose conversion ratio} = \frac{\text{mol. wt. glucose} + (\text{mol. wt. glucose} - \text{mol. wt. H}_2\text{0})(n-1)}{\text{mol. wt. glucose} \times n}$$

Where n=no. of glucose units per molecule of starch.

For example, when n=10, starch:glucose ratio=0.910, and when n= ∞ starch:glucose ratio=0.900.

As the ratio could vary between 0.900 and 0.910, a value of 0.905 is arbitrarily chosen. Hence, 1.00 g starch is expected to yield 1.105 g glucose.

When calculated starch values were regressed on starch added (Table 2), the F test for lack-of-fit of the linear model to the regression data was not significant ($P>0.05$). The recovery estimate of 100%±1.1% suggests there are no sample losses during hydrolysis and no significant interferences due to the plant material used.

Table 2. Recovery of added maize starch from cassava rind material

Maize flour added (mg)	Calculated mass of dry starch added (mg)	Glucose found (mg)	Corresponding calculated starch founded (mg)	Added starch recovered (%)
0	0	28.8±0.1	26.1±0.1	n.a.
10	8.6	38.0±0.8	34.4±0.7	96.5
20	17.2	47.7±0.7	43.2±0.6	99.4
30	25.9	57.2±0.5	51.8±0.5	99.1
40	34.5	66.9±0.1	60.5±0.1	99.7

* Mean±2 standard deviations.

†Starch values were calculated on the assumption that the starch:glucose conversion ratio is 0.905.

n.a.=not applicable.

Precision

A 2-way analysis of variance (Youden and Steiner 1975) of the data in Table 3 gave a repeatability standard deviation of±0.672% total fermentable value and reproducibility standard deviation of ±0.785% total fermentable value. The reproducibility relative confidence interval ($P>0.05$) of ±2.7% for a single estimate shows the method has good precision.

Table 3. Precision test data

Sample	Total fermentable values (%)*†		
	Day 1	Day 2	Day 3
Cassava root	92.3±1.0	92.3±0.5	92.8±0.8
Cassava rind	65.8±0.4	66.2±0.8	66.4±1.9
Macadamia wood	26.2±0.8	27.4±0.4	27.2±0.5
Lucerne taproot (A)	60.7±2.3	61.6±1.8	61.4±2.2
Lucerne taproot (B)	47.7±0.7	48.7±0.3	48.7±1.0

* Samples were analysed using glucose standards.   †Mean±2 standard deviations.

CONCLUSIONS

The method has been applied to large batches of cassava plant parts, lucerne taproot, macadamia wood and bark. It provides a rapid, relative assessment of total fermentable components. Advantages are that interferences due to inorganic substances have been overcome, and precision has been improved by optimisation of digestion techniques. Recovery of added starch is 100%.

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