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EVALUATION OF SOME METHODS OF DETERMINATION OF STARCH FOR LEGISLATIVE PURPOSES IN THE EUROPEAN COMMUNITY.

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Abstract: A newly developed enzymic method for starch purity determination has been designed in response to the search by the European Community (EC) for a reliable and accurate official method for direct total starch measurement of high purity starches. This new method, the Birmingham method, utilizes a multi-enzyme regime, consisting of an α-amylase (E.C. 3.2.1.1), a glucoamylase (E.C. 3.2.1.2) and a pullulanase (E.C. 3.2.1.41), for the complete and specific hydrolysis of starch to glucose. Greater than 99.5 % conversion of most starches to glucose is achieved which is higher than that afforded by other enzymic methods being considered for adoption. In this communication the reliability and accuracy of this method are assessed and demonstrated. Also, some results during its development are presented. Advantages and drawbacks of the other methods for starch purity measurement being investigated/assessed for permanent adoption as an official method of the EC are also discussed.

INTRODUCTION

While initial investigations on starch chemistry date back more than a century and a number of researchers have spent their lifetimes' work on starch (albeit, not exclusively), recent times still see studies on the development and improvement of methods for starch measurement. This is due to advances in our knowledge of the constituents of starch granules, in the understanding of the mechanisms and/or principles of the methods employed, in enzyme technology, in purification and isolation processes and in instrumentation and is also due to new legislation.

Measurements of starch content in a variety of sample matrices have been of importance to scientists interested, for example, in the biological aspects (synthesis and catabolism/degradation) of starch and in the nutritional qualities of food products.

The need for a reliable method for starch purity measurement of high purity starches in the European Community (EC) only became apparent for the purpose of manufacturers' declarations, customs checking and, more importantly, eligibility of end-users for substantial EC refunds on food grade starches in excess of 97 % pure if used for non-food applications. Legislation introduced in July 1986 was designed to alleviate the problem of

starch surpluses in the Community and at the same time encourage the utilization of starch as a raw material/feedstock in the chemical and fermentation industries.

Two polarimetric methods and three enzymic methods have been investigated by a starch working party created in the wake of the aforementioned legislation. One of our main objectives is to develop and/or improve existing methods for starch measurement. This paper discusses these methods, their advantages and drawbacks. Also, some results obtained during the development of the Birmingham enzymic method are presented.

The final officially adopted method(s) should give reliable, clear and indisputable results and should preferably have a standard deviation of less than one percent.

EXPERIMENTAL

Materials

Samples of starches were kindly supplied by a number of major manufacturers in the starch field, including ABR, Avebe, Laing National Ltd., CPC (UK) and Cerestar.

Aqueous enzyme solutions (gifts from NOVO) were used for starch hydrolysis; microbial (Bacillus licheniformis) α-amylase, fungal (Aspergillus niger) glucoamylase and microbial (Bacillus species) pullulanase. Enzymes used for glucose determination were glucose oxidase (Type II from Sigma) and peroxidase (Type I from Sigma).

ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid), anhydrous glucose and all other reagents were of analytical grade.

Reagents

"TRIS" - phosphate buffer. Trishydroxymethylaminomethane (36.3 g) and monosodium phosphate ($NaH_2PO_4.H_2O$) (50 g) were dissolved in water (900 ml). The pH was adjusted to 7.0 with 84 % (v/v) phosphoric acid before making up to 1 litre with water.

Glucose-oxidase/peroxidase reagent (GOD/POD). Glucose oxidase (100 mg), peroxidase (3 mg) and ABTS (50 mg) were dissolved in Tris-phosphate buffer (100 ml).

Methods

Determination of enzymic activity. Enzyme activity is defined as the amount of enzyme which produces 1 μmole of glucose (determined as dextrose equivalent by the dinitrosalicylic acid (DNS) method[1]) in one minute from 1% soluble starch solution at 60°C and pH 5.0.

Gel permeation chromatography. Aliquots of the supernatants of the enzymic hydrolysates of starches were injected into a water-jacketed column (56 x 1.3 cm i.d.) packed with Bio-Gel P2 (-400 mesh) and maintained at 60°C. The eluent was 0.1 M sodium chloride with a flow rate of 0.16 ml/min. The column eluent was continuously monitored using an automated L-cysteine sulphuric acid assay[2]. The system was standardized by injecting a mixture of glucose and malto-oligosaccharides.

Solubilization of the starch granules. DMSO method. Aqueous DMSO (20 %, 20 ml) was added to starch (250 mg weighed accurately), which had been previously washed with 40 % ethanol, whilst mixing vigorously on a vortex mixer. The tube was capped tightly,

mixed vigorously for a futher two minutes, boiled for two minutes and cooled to room temperature. The pH of starch solutions were almost always pH 5.0.

Sodium hydroxide method. Starch (250 mg weighed accurately) previously washed with 40 % ethanol, was dispersed with ethanol (1 ml). NaOH (1M, 10 ml) was slowly added whilst mixing vigorously on a vortex mixer. The solution was further mixed for 1 to 2 minutes, then water (10 ml) was added. The pH of the starch solution was adjusted to pH 5.0 with glacial acetic acid, and the solution made up to 25 ml in a volumetric flask. This method of solubilization was incorporated into the Birmingham method. Solutions should be used promptly since retrograding of starch may occur at pH 5.

Water method. Starch (250 mg weighed precisely) which had been previously washed with 40 % ethanol was boiled in water (20 ml) for 5 minutes, cooled down rapidly to room temperature, its pH adjusted with acetic acid to the desired value and diluted to 25 ml with sodium acetate buffer (0.1M of the desired pH) or water.

Hydrolysis of the starch samples. Two-step addition of enzymes. Starch (250 mg) was dispersed in water (20 ml), brought to the boil, then α-amylase (600 units, 25 ml) was added and the solution was boiled for a further two minutes. The solution was cooled to 60°C over 6 minutes. After three minutes at 60°C, glucoamylase (440 units, 24.42 ml) and pullulanase (28 units, 46.67 ml) were added simultaneously. Aliquots (100 ml) of the hydrolysates were taken at specified times for DNS assay[1] and total sugar content determination by the L-cysteine sulphuric acid assay[2]. Stirring was carried out throughout the whole solubilization and hydrolysis processes.

Three-step addition of enzymes. Solubilization and hydrolysis by α-amylase was carried out as described above. The solution was then cooled to 60°C over 6 minutes. Pullulanase (28 units, 46.67 ml) was added and after three minutes glucoamylase (440 units, 24.42 ml) was added. Aliquots (100 µl) of the hydrolysate were then analysed as described above.

Simultaneous addition of enzymes. Similar solubilization technique, heating/cooling conditions and time intervals were carried out as described above but without the addition of α-amylase or the pullulanase. Instead all three enzymes were added simultaneously after three minutes at 60°C. Aliquots of the hydrolysate were then analysed as described above.

Birmingham method of starch hydrolysis. Prepared starch solutions containing 250 mg of starch (by sodium hydroxide method) were heated to 60°C. α-Amylase (15 to 17.3 units, 0.625 to 0.720 ml), glucoamylase (625 units, 34.69 ml) and pullulanase (35 units, 58.33 ml) were then added simultaneously. The hydrolyses was carried out at 60°C for 45 minutes with continuous stirring. The solutions were then cooled to room temperature and made up to 25 ml in a volumetric flask. They were then analyzed by gel permeation chromatography for the degree of hydrolysis or analysed for their glucose content by manual or automated glucose oxidase/peroxidase (GOD/POD) methods. The current protocol of the Birmingham method utilizes a manual GOD/POD method as described below.

Determination of glucose. Manual GOD/POD method. GOD/POD reagent (2 ml) and distilled water (2 ml) were added to starch hydrolysates (1 ml) containing 10 to 40 μg of glucose. The reaction mixtures were left in the dark at ambient temperature for exactly 30 minutes and their absorbance was measured at 560 nm using water (3 ml) and the GOD/POD reagent (2 ml) as a blank. Standard glucose solutions containing 10 to 50 μg of glucose were similarly treated to produce a calibration curve.

Automated GOD/POD method (YSI (Yellow Springs Instruments) glucose analyser)[3]. Starch hydrolysates were diluted quantitatively with water to 100 ml. Filtered aliquots (250 μl) were injected into the glucose analyser previously calibrated with glucose standard (200 mg/100 ml). One minute after injection a read out of the concentration was given.

Glucose test strips. Glucose contents were in some cases obtained using glucose test strips (Reflolux^{R} IIM, Boehringer). This was achieved by placing a drop of the analyte on the surface of a water resistant plastic dispersion film which had, by the manufacturers, been impregnated with glucose oxidase, peroxidase and the chromogen which is a mixture of o-toluidine and 3-amino-9-(aminopropyl)-carbazole. Excess was wiped off after a minute and after a further minute the reaction colour was measured by an in-built reflectance photometer. Amount of glucose detected was displayed and expressed as mmol/L. The instrument was calibrated by the use of a bar-code film strip.

Determination of the starch content in the residue after enzymic hydrolysis. After enzymic hydrolysis, the hydrolysates were cooled down to room temperature and immediately centrifuged. The residues were washed with water (10 ml) using a vortex mixer. The suspensions were centrifuged and the supernatants discarded. This treatment was carried out twice more. The final residues were hydrolysed with 2M trifluoroacetic acid (5 ml) at 100°C for four hours. The trifluoroacetic acid was evaporated off using a rotary evaporator and the residues were then washed with water which was subsequently evaporated off. This treatment was carried out twice more. The final residues were redissolved in water, left to stand for four hours and assayed for their glucose content using the manual GOD/POD method.

OTHER METHODS EVALUATED:

1. Polarimetric Methods

Modified Ewers Method[4]

The Ewers method is based upon mild, controlled acidic hydrolysis of a starch sample to soluble oligosaccharides. The solution is then clarified and filtered prior to its optical rotation being measured using a polarimeter. It is the method currently specified by the E.C. for the measurement of high grade starches (Supplement to the Special Edition of the Official Journal of the European Community, L123/72). Despite being a notoriously difficult technique to perform reproducibly it was adopted by the E.C. in 1972 as a temporary method for starch purity analysis Over 20 years later the E.C. had to change its policy and consider an alternative technique.

The lack of accuracy of the Ewers procedure has been the subject of much debate in

the starch industry. However, we have shown that accuracy and reproducibility of the method can be increased greatly as a result of stricter standardisation within the procedure. The original rules for the method laid down in the E.C. journal were very nonspecific, containing ambiguous phrasing which was open to misinterpretation. If however one follows a strict procedure for Ewers analysis then individual operator inaccuracy can be bought down to <0.5%. The next step is to get these stringent processes written into the rulings of the E.C. journal. We are now at a stage where individual operator results are becoming consistent, but if the Ewers procedure is to continue as the standard E.C. procedure for starch purity analysis then this improvement must be shown in inter-operator consistency.

Optical activity is not specific for starch, therefore any optically active molecules in the sample can exhibit optical rotation. In addition acid hydrolysis is non-specific and therefore other naturally occurring non-starch polysaccharides (e.g. cellulose and hemicellulose) produce optically active products.

Errors in Ewers "apparent" starch purity values have been shown to originate from a number of sources during sample preparation. Various parameters such as rates of heating and cooling, stirring times and speeds and amount of clarifier used have an effect on the oligosaccharide composition of the acid hydrolysate, and hence have an effect on the optical rotation value of the sample (and therefore on the apparent starch purity value[5-9]). Sample preparation is therefore one area where a number of sources of error can be identified.

One possible method to continually monitor sample preparation reproducibility in the Ewers procedure would be the incorporation of a centrally supplied high purity standard starch which could be analysed parallel to each Ewers sample. This would allow the user to assess the consistency of each analysis with respect to all previous Ewers analyses carried out with that standard. Therefore an inaccurate result (which does inevitably occur occasionally) could be identified and discounted. This is a method which Ewers operators in our own laboratory have developed and used successfully.

A second area which may bring about inaccuracies in the apparent starch content of a sample is pre-treatment. Many commercially utilised starches (particularly when modified or in slurried form) are treated with additives or preservatives, and some of these have been shown to either react with the reagents used in the Ewers analysis (e.g. hydrochloric acid) or to exhibit optical activity themselves. Both of these could give an apparent Ewers analysed starch content different from the actual starch content of the sample.

Starch purity values obtained appear to be affected by the amylose:amylopectin ratio[10] of the sample. Starch purity values decrease with increasing amylose content. This result is due to incomplete solubilisation of starches with higher amylose contents and more extensive hydrolysis of waxy starches. While the method gives differing specific optical rotations for starches from different plants it is unable to distinguish mutant starches from the "same" plant origin (e.g. high amylose and waxy maize starches).

In addition, uncertainties exist over the specific optical rotation values employed, bearing in mind that the original starches used by Ewers to obtain these values were of a

lesser purity than their present day commercial counterparts. Also methods for the determination of non-starch components (ash, protein, fat, moisture and fibre) to obtain the "true" starch value were then most probably inaccurate or non-existent. In most cases, fibre content was not analysed and fat content was underestimated[11] (balance between 100% and the sum content of non-starch components is assumed to equal starch purity).

Further improvements in Ewers accuracy are likely to be realised with the redefinition of the optical rotation values from more specific (non-empirical) methods.

In conclusion, the Ewers procedure, despite its limitation, has the potential to become a good, basic analytical technique, once the guidelines have been clearly defined. The accuracy could be further enhanced by the introduction of an internal standard as a continuous assessment of experimental validity. Whilst the Ewers procedure will not be suitable for all cases (for which techniques of higher specificity and reproducibility are available), it has the potential to grow into a fast, if basic, starch purity determination method.

Calcium Chloride Method[12]

The other polarimetric method investigated is the calcium chloride method. This method is based on the solubilization of starch in a 33% calcium chloride solution at an acidic pH by the addition of acetic acid.

While this method has reportedly more acceptable reproducibility (albeit, not yet of the required standard deviation) it appears to give systematically low starch purity values.[13] The latter was attributed to the possible inappropriateness of the specific optical rotation factor (203°) used for all starches. Confirmation of the suitability of this value for all types of starches under the present conditions for starch solubilization in acidic calcium chloride solution is therefore desirable. Different specific optical rotation values have been reported using slight variations in conditions of analysis[14]. As with the Ewers method uncertainty exists in the correction applied for non-starch constituents to obtain the "true" starch value.

Due to their convenience and the ease with which they are performed, polarimetric methods are preferred by the industrial and customs sectors. Of these, the calcium chloride method is currently favoured due to its superior reproducibility. This is attributed to smaller error during sample preparation since there is no apparent hydrolysis during the starch solubilization process (pH 1.8 and boiling for 30 minutes)[13]. This is not in accord with Dimlers[14] statement that some hydrolysis of starch occurs under these conditions. Studies in our laboratory verify the possible occurrence to a small extent of hydrolysis of different starches under the current prescribed conditions of the method. If hydrolysis does occur, further improvement of reproducibility would be realized by stricter standardization.

While polarimetric methods may be improved to give the repeatability and reproducibility required to qualify as an official method, they suffer from the major drawback of non-specificity. However, the industrial and customs sectors lack confidence in current enzymic methods. This results from apparently low starch purity data and the great variability of starch purity values[13]. The latter is due to inexperience in handling enzymes and micro-biomethods.

2. Enzymic Methods[14]

All enzymic methods being considered for adoption are based on the total and specific hydrolysis of starch to glucose by starch-degrading enzymes[15,16] and the subsequent analysis of the glucose produced. The first enzymic method to be considered was the Swiss enzyme method[17], then the AFNOR (Association Francaise de Normalisation)[18] and most recently, the Birmingham method (see later).

The Swiss method, the official method for the determination of starch in soya-based products, when applied to the determination of high purity starches gives low purity values and unacceptable repeatability and reproducibility data. This could be due to the inability of the (amount of) amyloglucosidase to hydrolyse all the branch points in high purity starches in the length of time prescribed in the method. Thus, the degree of hydrolysis of the starch could possibly be affected by the differences in the amylopectin:amylose ratio between starches from different origins.

The initial disappointment incurred with this method did not stop the further improvement and development of enzymic methods due to the recognition of the significance of such highly specific methods for legislation purposes. Consequently, AFNOR improved the Swiss method by increasing the enzyme to starch ratio. Also, the method for the determination of the released glucose makes use of the glucose oxidase/peroxidase method rather than the hexokinase/glucose-6-phosphate dehydrogenase method[19]. In all other respects, the Swiss and the AFNOR methods are similar.

Although this method showed great improvement over the Swiss method, gel permeation chromatography studies in our lab on the hydrolysates after the enzymic degradation of a wide range of starches still reveal up to 2.5% of the starch existing as oligosaccharides. Furthermore, one of the objections to the Swiss enzymic method, the gelatinization of starch using sodium hydroxide at 60°C for 30 minutes, was not addressed by the AFNOR method. It was thought that such treatment would lead to the possible partial hydrolysis of the starch polymer and, more importantly, to the formation of saccharinic acids and thus to the underestimation of starch.

In the hope of further improving the performance of specific enzymic methods by overcoming the probable and actual problems of the two aforementioned methods, our laboratory introduced further modifications, the most important of which is the utilization of a multi-enzyme regime, consisting of α-amylase (E.C. 3.2.1.1), glucoamylase (E.C.3.2.1.2) and a debranching enzyme, pullulanase (E.C. 3.2.1.41). Incomplete hydrolysis was observed when pullulanase was not present in the enzyme cocktail being applied to branched (amylopectin) starches. Sodium hydroxide solubilization of the starch is carried out at room temperature and the time required for the elimination of soluble oligosaccharides has been shortened considerably.

RESULTS AND DISCUSSION

In the development of an enzymic method, three critical points were considered in order to ensure reliability. These are the total solubilization of the starch granules for

maximum enzyme susceptibility, the total and specific hydrolysis of the starch to glucose, and the accurate and reliable determination of the glucose produced.

Solubilization of the Starch Granules

In the earlier stages of this study, four methods were tried for the solubilization of starch granules, namely: heating the starch in water to boiling point, using 20 % dimethylsulphoxide (DMSO), using 1M sodium hydroxide (NaOH), and using α-amylase. Heating in water, while satisfactory for most starches, was not applicable to high amylose starches. Visual observation showed the presence of ungelatinized starch granules even after prolonged (up to 45 minutes) boiling of the starch suspension. While both 20 % DMSO and 1M NaOH appear to solubilize all types of starches, gel permeation studies of the enzymic hydrolysates of waxy maize, regular maize and high amylose maize starches revealed that, in general, sodium hydroxide solubilization provided the most efficient technique for starch solubilization (Tables 1 and 2) based on the amount of oligosaccharides with DP>15. The apparently low hydrolytic capability of the enzyme(s) in DMSO could be attributed to deactivation in organic solvent, a conformation of either or both the amylose and amylopectin components in 20 % DMSO which could be less susceptible to enzyme attack, and/or a different conformation of the enzyme(s) which could alter their mechanism of action. Also the use of dimethylsulphoxide could result in the oxidation of some of the glucose units. The effect of solubilizing agent was more pronounced in the case of waxy maize which is nearly 100 % amylopectin. Also of great interest are the differences in the oligosaccharide composition of starch hydrolysates from α-amylase action (Table 3); water and sodium hydroxide solubilization give comparable hydrolysate composition after 45 minutes whilst DMSO solubilization does not (Figure 1), in particular those of DP_3 and DP_5. Further studies are required in order to provide a reasonable explanation. It is interesting to note that it has been shown that the conformation of starch molecules can be altered from helices to random coils by varying DMSO/water solvent ratios[20,21].

The use of α-amylase for solubilization of starch granules has been shown to be promising, although high amylose starches still need prior sodium hydroxide gelatinization before being susceptible to amylolytic attack[22]. Also, α-amylase solubilization at the conditions prescribed by Karkalas[22] (30 minutes at 85°C) is time consuming. Furthermore, a lesser amount of α-amylase than that used by Karkalas[22] at 60°C for three minutes leads to almost maximum hydrolysis(Figure 2). Also, the α-amylase preparations used contain calcium ions which destabilise amyloglucosidase, the most important enzyme in the complete hydrolysis of starch. Thus, it would appear that a lesser amount of enzyme is sufficient to solubilize starch, bearing in mind that very low molecular weight oligosaccharides are less susceptible to glucoamylase attack[16].

Comparative studies were carried out on the efficiency of simultaneous, 2-step and 3-step addition of a three enzyme regime to hydrolyse starch. Figure 3 shows that waxy maize was hydrolysed to the greatest extent when enzymes were added simultaneously, whilst a two-step addition appeared to work best on the high amylose maize starch. As mentioned earlier high amylose maize was not sufficiently gelatinized in water and therefore the

Table 1

Composition of Glucoamylase Hydrolysates of Starches Solubilized in Different Media

Starch sample	Solubilizing media	Incubation time (min)	Composition of starch hydrolysates							
			DP_1	DP_2	DP_3	DP_4	DP_5	DP_6	DP_{7-15}	$DP_{>15}$
Regular maize	Water	1	38.5	0.7	0.6	0.2	0.2	0.3	5.6	53.8
		45	82.8	0.2	tr[1]	0.1	0.3	0.4	6.1	10.0
	DMSO,20%	1	43.7	(8.3)[2]		2.8	1.5	0.8	8.7	34.2
		45	73.6	1.5	tr	0.6	0.8	1.0	6.5	16.0
	NaOH	1	46.2	(20.7)		2.6	1.8	1.2	7.5	20.0
		45	80.0	2.5	tr	0.4	1.1	1.0	4.0	11.0
Waxy maize	Water	1	31.1	1.1	0.8	0.6	0.5	0.3	18.9	46.7
		45	82.8	0.2	tr	0.1	0.3	0.4	6.1	10.0
	DMSO,20%	1	15.4	tr	0.3	0.5	0.7	0.3	12.1	70.7
		45	62.8	tr	tr	0.3	0.6	0.9	4.4	31.0
	NaOH	1	57.8	4.4	0.4	0.5	0.8	0.2	4.4	31.5
		45	86.3	(tr)		0.4	0.6	0.6	8.6	3.5
High amylose (70%) maize	DMSO,20%	1	18.9	(0.2)		0.5	0.6	0.3	4.5	75.0
		45	88.0	0.7	tr	0.2	0.5	0.6	3.3	6.7
	NaOH	1	30.0	(30.0)		2.1	1.7	1.9	16.4	17.7
		45	91.0	0.6	tr	0.2	0.7	0.8	2.6	4.1

[1] Trace = less than 0.1%.

[2] Figures in bracket elute between DP_2 and DP_3.

DP = degree of polymerization.

Table 2 **Effect of Solubilization Technique on the Extent of Hydrolysis of Starches**

Starch sample	Enzyme system	Solubilization media	Composition of starch hydrolysates (%)		
			DP_1	DP_{2-15}	$DP_{>15}$
Waxy maize	1[1]	Water	99.8	0.0	0.2
		DMSO,20%	91.9	7.7	0.4
		NaOH	98.9	0.9	0.2
	2[2]	Water	99.1	0.7	0.2
		DMSO,20%	94.1	0.9	5.0
		NaOH	98.0	1.8	0.2
High amylose	1	DMSO,20%	99.0	0.6	0.4
		NaOH	98.8	1.0	0.2
	2	DMSO,20%	98.3	0.6	1.1
		NaOH	99.6	0.1	0.3

[1] System 1 = 120 units α-amylase; 440 units glucoamylase; 28 units pullulanase.
[2] System 2 = 440 units glucoamylase; 28 units pullulanase.

Table 3

Composition of α-Amylase Hydrolysates of Starches Solubilized in Different Media

Starch sample	Solubilizing media	Incubation time (min)	Composition of starch hydrolysates							
			DP_1	DP_2	DP_3	DP_4	DP_5	DP_6	DP_{7-15}	$DP_{>15}$
Regular maize	Water	45	6.0	13.0	17.0	7.0	36.0	2.0	15.0	4.0
	DMSO,20%	45	6.0	13.0	10.0	6.0	41.0	2.0	17.0	5.0
	NaOH	45	6.0	14.0	17.0	6.0	34.0	2.0	17.0	5.0
Waxy maize	Water	45	6.0	15.2	14.0	9.0	28.0	4.0	18.8	5.0
	DMSO,20%	45	6.0	11.2	3.2	8.5	43.7	3.8	18.6	5.0
	NaOH	45	6.5	16.4	18.7	9.0	27.8	4.5	13.5	3.6
High amylose (70%) maize	DMSO,20%	45	12.0	17.0	11.0	12.0	38.0	2.0	7.0	1.0
	NaOH	45	13.0	14.0	25.0	13.0	27.0	1.0	6.5	0.5

DP = degree of polymerization.

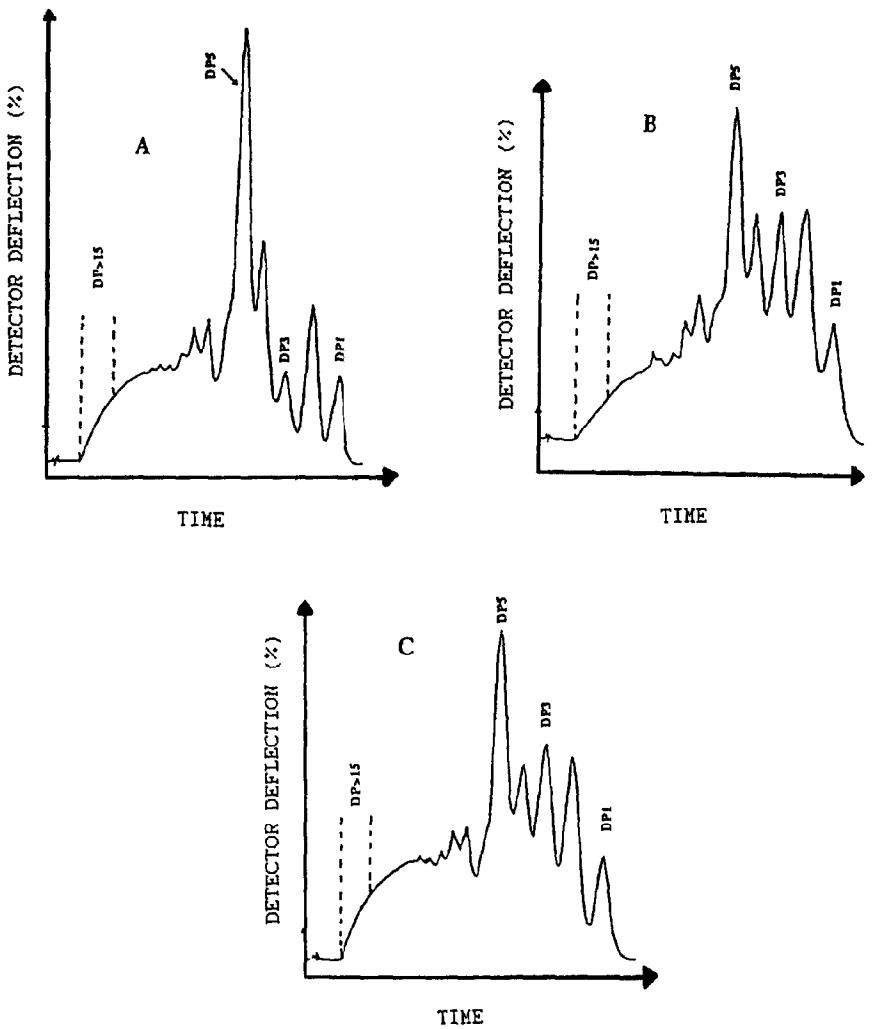


Figure 1. Gel permeation profile of α-amylase hydrolysates of waxy maize starch solubilized in (A) 20% DMSO, (B) sodium hydroxide and (C) water.

starch granules were not very susceptible to hydrolysis. However, it would appear that the use of α-amylase helped solubilise the starch and made available more non-reducing groups for glucoamylase attack. Surprisingly, a three-step addition gave slightly lower efficiency in hydrolysing high amylose starch. Alternatively this could be due to the addition of pullulanase to a still insufficiently gelatinized starch granule which may have inhibited further α-amylase activity resulting in lesser amounts of non-reducing ends. Such enzyme inhibition effects could also occur with the simultaneous addition of all three enzymes on ungelatinized starch granules, although these differences may be small enough to be comparable to experimental error.

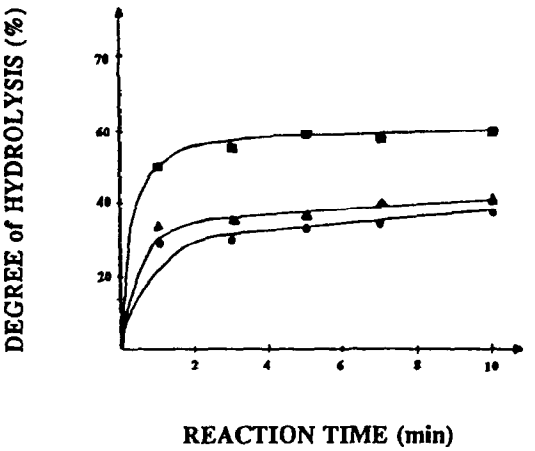
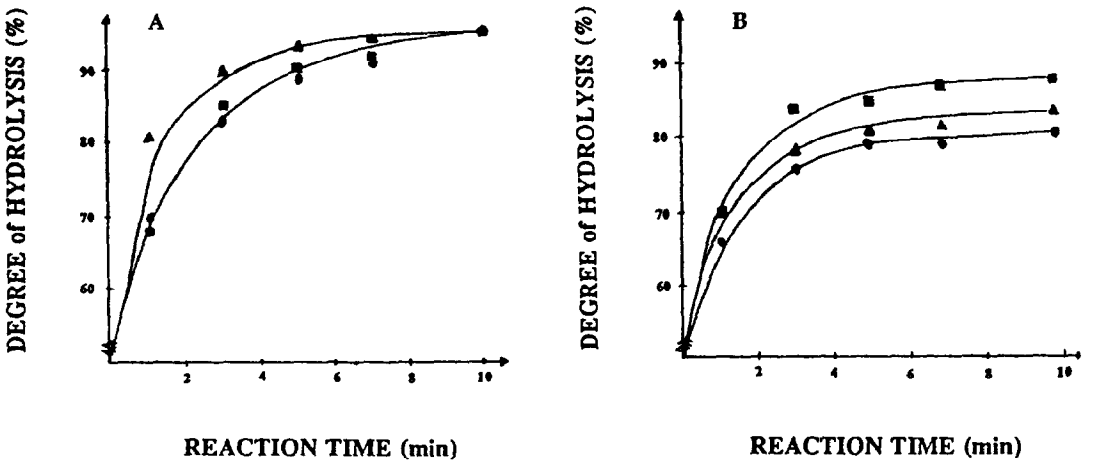


Figure 2. Kinetic curve of 120 units (50 µl 120L Termamyl) α-amylase per 250 mg of various starches solubilized in water; soluble starch (■), regular starch (▲) and waxy maize starch (●).



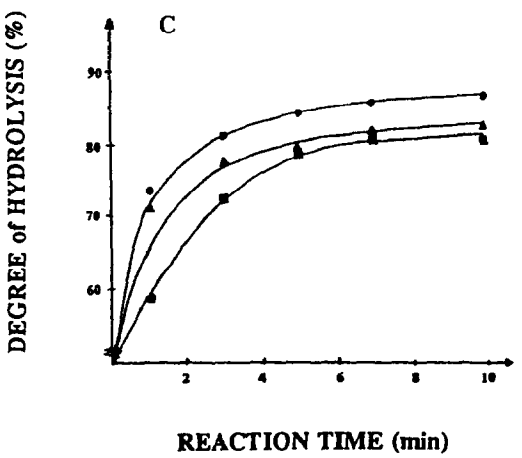


Figure 3. Kinetic curves of a three-enzyme regime added simultaneously (■), 2-step (●), and 3-step (▲) to (A) regular maize, (B) waxy maize and (C) high amylose (70%) maize starches solubilized in water.

In the case of waxy maize, the starch granules were fully dispersed in water and were very accessible to enzyme attack. Prior extensive action of α-amylase would result in the production of a great amount of low molecular weight oligosaccharides which are broken down more slowly by glucoamylase than polysaccharides[16]. Not surprisingly, simultaneous addition of enzymes worked more efficiently for waxy maize. However, three-step addition of enzymes appeared to hydrolyse waxy maize better than two-step addition. It is possible that glucoamylase did not work efficiently on β-limit-dextrins with branch points near the non-reducing ends compared with smaller molecular sized linear glucose oligomers produced from prior pullulanase hydrolysis. Also, pullulanase hydrolyses (1->6)-glucosidic linkages with at least two glucose units in the side chains[16]. However, three-step addition worked more efficiently for regular maize than simultaneous or two-step addition of enzymes, although all three gave the same degree of hydrolysis by the tenth minute. The simultaneous and two-step addition of enzymes gave the same kinetic curve for regular maize. From the above results the observed differences, although small, suggest that simultaneous enzyme addition is applicable to starches of differing amylose:amylopectin ratio provided the starch granules are sufficiently gelatinized. It also provides better handling and manageability. Hence, simultaneous addition of enzymes was adopted in the final developed method.

Hydrolysis of Starch

Under the prescribed conditions of the Swiss[17] and the AFNOR[18] methods the enzyme glucoamylase does not completely hydrolyse the starch sample. The unhydrolysed oligosaccharide sections were attributed to the branched portion of starch which is not easily hydrolysed by glucoamylase. Addition of α-amylase does not solve this problem since it only acts on (1->4)-D-glucosidic linkages. However, it could minimize the problem because it can produce more non-reducing terminals after the branched portions for glucoamylase attack. To obtain total hydrolysis more efficiently the addition of a debranching enzyme together with α-amylase and glucoamylase was deemed necessary. This principle has been applied to the greater production of glucose from starch in the industrial setting[23]. After screening several enzyme regimes of varying amounts of the three enzymes, the enzyme regime consisting of 17.3 units α-amylase, 625 units glucoamylase and 35 units pullulanase was tried on a wide range of starches. Pullulanase was chosen as the debranching enzyme only because of its availability; isoamylase could be applicable.

Gel permeation chromatography studies on the hydrolysates obtained by the aforementioned enzyme regime revealed that all types of starch are almost totally hydrolysed to glucose (>99.5 %), as shown in Figure 4, except for potato starches which are 98.5 to 99.5 % hydrolysed (Table 4). The latter was attributed to the presence of phosphate groups linked to carbon six of some of the glucose units consisting the potato starch of which their glucosidic linkages are not attacked by any of the enzymes used. The presence of a brown residue after the enzyme hydrolysis of a rye starch suggests that this particular starch is not of very high purity. The larger than 1.5 % high molecular weight portion in its starch hydrolysate could very well be soluble hemicellulose. Other non-starch polysaccharides give a positive response to the L-cysteine sulphuric acid used for the detection of the eluted components from gel permeation chromatography. If the high

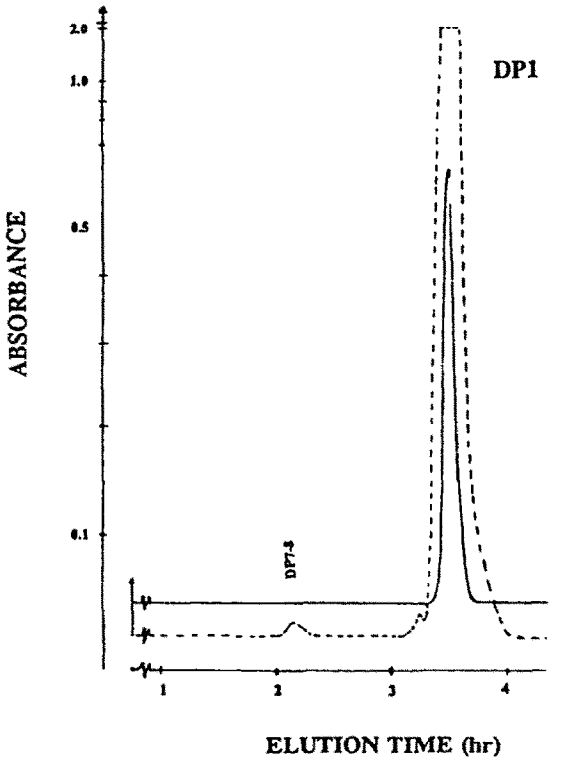


Figure 4. A representative gel permeation chromatogram of a starch hydrolysed by the Birmingham multi-enzyme regime showing greater than 99.5% degree of hydrolysis. [(----) = 10 times the injection load of (——)].

molecular weight portion is due to non-starch polysaccharides, the efficiency of hydrolysis of rye starch to glucose is underestimated. This could also be true for wheat B (less pure than wheat A) starches and for the pea fibre.

While pullulan could be hydrolysed to glucose by an enzyme regime containing pullulanase and glucoamylase, pullulan is too expensive to use as a "diluent" of commercial starches to increase apparent starch purity.

About 0.5 % of most of the starches is not completely hydrolysed to glucose. Studies are at hand to investigate this "unhydrolysed" portion. However, further increase in the amount of the pullulanase results in an apparently lower conversion rate of starch to glucose. This could suggest enzyme inhibition due to the molecular size of the pullulanase.

Insoluble materials were observed to be present in all enzymic hydrolysates of starches studied. The insoluble materials could be attributed to a) the presence of ungelatinized/unsolubilized starch granules, b) the presence of insoluble glucose-protein moieties formed via Schiff's base reaction of the glucose produced with the enzymes/proteins from the starch sample itself and/or with one or more of the starch-degrading enzymes employed, c) the presence of water-insoluble oligosaccharides as reversion products, d) the presence of water-insoluble retrograded oligosaccharides [D.P. <

Table 4 **Degree of Hydrolysis Achieved by the Birmingham Multi-enzyme Regime on Starch Samples**

Reference No.	Starch sample	Composition of hydrolysis		
		G^1	G_2-G_{15}	$G_{>15}$
PUR-10	Regular maize	99.8	0.2	0.0
PUR-38	Regular maize	99.8	0.2	0.0
PUR-2	Waxy maize	99.8	0.2	0.0
PUR-15	Waxy maize	99.7	0.3	0.0
PUR-25	High amylose (50%) maize	99.8	0.2	0.0
PUR-39	High amylose (50%) maize	99.7	0.3	0.0
PUR-26	High amylose (70%) maize	99.9	0.1	0.0
PUR-40	High amylose (70%) maize	99.7	0.3	0.0
PUR-28	Wheat A	99.8	0.2	0.0
PUR-33	Wheat A	99.6	0.4	0.0
PUR-13	Wheat B	99.1	0.6	0.3
PUR-46	Wheat B	98.9	0.7	0.4
PUR-48	Potato	99.3	0.7	0.0
PUR-65	Potato	99.0	1.0	0.0
PUR-37	Rice	99.6	0.4	0.0
PUR-44	Rice	99.9	0.1	0.0
PUR-4	Rye	98.0	0.2	1.8
PUR-5	Tapioca	99.8	0.2	0.0
PUR-12	Sago	99.6	0.4	0.0
PUR-14	Pea fibre	96.8	1.9	1.3

10 do not retrograde] formed during the solubilization and hydrolysis processes, e) the presence of insoluble glucose-containing carbohydrates such as cellulose and hemicellulose which occur naturally in starch granules and f) denatured enzymes/proteins. Occurrence of a, b, c and d to a large degree will result in inaccurately low starch purity values. However, analyses of the starch contents of the residues obtained from the enzymic hydrolysates show less than 0.1 % based on the original dry weight of the sample (Table 5). This is virtually negligible and would not affect starch purity values.

Glucose Measurement After Enzymic Hydrolysis

The present technique used in the Birmingham method is the (manual) enzymic method using glucose oxidase and peroxidase. Repeatability of the data obtained in our laboratory gave a coefficient of variation of less than 1% (Table 6). This method is widely used in the determination of glucose in complex biological samples (e.g. blood) and also in the food industry. Objections to enzymic methods for the determination of the released

Table 5 **Amount of Starch in Residue after Enzyme Hydrolysis**

Reference No.	Starch sample	Starch in Residue (% dry basis)
PUR-10	Regular maize	0.04
PUR-38	Regular maize	0.08
PUR-2	Waxy maize	0.04
PUR-15	Waxy maize	0.09
PUR-25	High amylose (50%) maize	0.09
PUR-39	High amylose (50%) maize	0.06
PUR-26	High amylose (70%) maize	0.05
PUR-40	High amylose (70%) maize	0.08
PUR-28	Wheat A	0.07
PUR-33	Wheat A	0.03
PUR-13	Wheat B	0.08
PUR-46	Wheat B	0.08
PUR-48	Potato	0.05
PUR-65	Potato	0.03
PUR-37	Rice	0.04
PUR-44	Rice	0.05
PUR-4	Rye	0.07
PUR-5	Tapioca	0.00
PUR-12	Sago	0.05
PUR-17	Pea fibre	0.27

glucose are that they are time consuming and laborious and the need for low concentrations of the final glucose analyte renders it susceptible to a number of errors due to handling, especially by inexperienced analysts. Thus, the use of a commercially available biosensor and a test strip based on the same principle as the manual GOD/POD method were considered because of their ease of use. Employment of the automated YSI glucose analyser gave comparable data to those obtained by the manual method while the glucose test strips were very irreproducible (Table 6). It must be borne in mind that the analyst who performed the above repeatability test is experienced in handling biochemical assays.

Comparison of the starch purity values (using the hydrolytic conditions of the Birmingham method) given by the three methods show that the biosensor gave comparable results to that of the manual GOD/POD method. For hydrolysates obtained by the AFNOR method, the YSI glucose analyser gave apparently higher purity values in comparison with the manual method (Table 7). Glucose test strips always gave inaccurately high purity measurements. Studies with a glucose standard show that the values obtained

Table 6 **Repeatability[1] of Glucose Determination Techniques**

Method	Regular maize	Waxy maize	High amylose	Wheat A	Wheat B	Potato	Rice
Manual GOD/POD[2]	0.44	0.38	0.40	0.63	0.60	0.52	0.45
YSI Glucose Analyser	0.31	0.38	0.35	0.48	0.52	0.52	0.43
Glucose Test Strips	9.30	8.20	8.80	9.50	10.00	8.50	9.00

[1] In terms of standard deviation.
[2] Method for glucose measurement incorporated in the Birmingham 2 method as currently written.

Table 7 **Starch Purity Values Obtained by Various Methods**

Starch Sample	Starch purity (% wet basis)			
	Manual GOD/POD		YSI analyser	
	Birmingham 2	AFNOR	Birmingham 2	AFNOR
Maize	87.4	85.4	85.8	85.9
Maize	82.7	83.0	83.6	85.8
Wheat A	84.6	83.8	83.6	85.4
Wheat A	82.4	82.0	82.2	83.6
Potato	82.1	80.4	81.5	82.4
Potato	79.6	79.8	79.6	80.6

were 120 to 140 % of the actual value. This could be due to their primary use in the determination of blood glucose and hence, would be calibrated correspondingly. Calibrating such instruments for food application could improve their accuracy for the determination of starch purity values; however, their degree of precision would still be unacceptable.

In this study, starch hydrolysates were immediately analysed upon hydrolysis. In the case of the AFNOR hydrolysates, the significantly higher starch purity values obtained by the biosensor as compared to the manual method might suggest that when analysed the AFNOR hydrolysates exist in an anomeric equilibrium different from that of the standard, due to either the non-attainment of an anomeric equilibrium or a different ratio of α- to β-D-glucopyranose at their respective anomeric equilibrium (i.e. if anomeric equilibrium is achieved just after hydrolysis). Glucoamylase only produces β-D-glucose from starch and glucose oxidase is specific for β-D-glucose. Differences in anomeric equilibrium between sample and standard are not as significant as when using the manual method. During the thirty minute "standing" time of the manual GOD/POD method, the almost complete isomerization of α–D-glucose to the β-anomer is reportedly achieved with the β-D-glucose consequently being completely converted to gluconic acid by the glucose oxidase[6]. The automated glucose analyser used has a reaction time of only one minute and hence, will not allow for (near) complete conversion of the α-isomer to its β-form and results will depend greatly on the anomeric ratio existing at the time of analysis, although more α-anomer is expected in freshly hydrolysed starch with glucoamylase.

The above results might suggest that the Birmingham hydrolysates exist at a different anomeric equilibrium from that of the AFNOR hydrolysates but are comparable just after hydrolysis to that of the glucose standard. The presence of calcium ions from the α-amylase preparation used might have resulted in the apparently smaller amount of the α-anomer (hence, lower starch purity values) in the Birmingham hydrolysates. It should be noted that calcium ions reportedly shift the anomeric equilibrium of glucose to the β-anomer[24]. Also, α-amylase produces small quantities of α-D-glucose from starch[16].

High pressure liquid chromatography methods are also being considered as an alternative to manual GOD/POD for glucose measurement. They offer short analysis times as well as being unaffected by the anomeric equilibrium of the sample (i.e. using a non-chiral column). Studies on glucose standards using a system similar to one of the HPLC systems described by White et al[25] show very good repeatability. However, the present mobile phase system (80/20 acetonitrile/water with 0.01 % of 1,4-diaminobutane) does not provide a resolved glucose peak from that of the sodium chloride and studies are in hand to further improve the resolution and/or eliminate the salt during sample clean-up and also using an internal standard. Obviously other HPLC systems could be used according to what is available to the analyst.

In any of the above methods for glucose determination the purity of the glucose standard will be crucial to the accuracy of the starch purity results obtained. At present, while enzymic methods for starch purity determination recommend the checking of the purity of the glucose standard to be used by HPLC, it does not guarantee thorough impurity measurement e.g. moisture and inorganic materials (normally less than 0.2 %).

Comparison of Purity Values Obtained by the Ewers & Birmingham Methods.

Table 8 shows that for regular and waxy starches, starch purity values obtained by the Birmingham enzymic method give comparable, if not more believable results, to those obtained by the Ewers polarimetric method. For high amylose starches, higher starch purity values are obtained by the Birmingham method. The low starch purity values for high amylose starches given by the Ewers method are due to the non-solubilization of some starch granules which are filtered off before the determination of the optical activity. Based on gel permeation studies of potato starch hydrolysates, the purity values obtained by the

Table 8

COMPARISON OF STARCH PURITY VALUES OBTAINED BY USE OF THE BIRMINGHAM METHOD WITH THOSE OBTAINED BY THE EWER'S METHOD

	Purity (% dry weight)	
Starch Type	Birmingham 2	Ewer's
Wheat	99.64	92.80
Wheat	99.86	98.50
Maize	92.45	95.50
Maize	93.01	96.90
Maize, waxy	94.72	96.08
Maize, waxy	99.74	96.08
Maize, 50% amylose	96.49	89.30
Maize, 50% amylose	92.49	80.53
Maize, 70% amylose	97.76	76.30
Maize, 70% amylose	97.50	73.83
Potato	96.28	94.80
Potato	96.39	102.20
Rice	97.38	95.23
Rice	99.83	99.57
Rye	91.71	91.20
Sago	96.10	98.50
Tapioca	99.38	101.20

Birmingham method are underestimated by 1-1.5 %; This factor would appear therefore to be required in the calculation of potato starch purity values by this particular enzymic method. The low values obtained for several of the starches are at present unexplained, although in the majority of cases the value obtained for the starch purity as assessed by the Ewers method is of a similarly low level, thus suggesting that the starch purity actually is low, and that an underestimation at the measurement stage has not occured.

CONCLUSIONS

Aside from the advantage of being specific, enzymic methods are not empirical methods. The principle on which they are based is scientifically sound (i.e. percentage of conversion to glucose is known) and they are therefore more reliable than the polarimetric methods currently available. Uncertainties regarding the applicability of the specific optical rotation values used under the currently prescribed conditions of sample preparation need to be eliminated for these methods to become reliable. To obtain true starch values of the reference starches employed to obtain the specific optical rotation values, the starches should be analysed using an enzymic method. Consistently lower purity values obtained by procedures that measure starch directly suggest that the Difference method (still currently the reference method in terms of accuracy by most associations in charge of developing

analytical procedures for standardization and subsequent legislation) underestimates non-starch components, although errors in the other procedures also exist.

The further acceptance of enzymic methods will be realized with more experience by relevant analysts in handling enzymes and semi-micro quantities of materials which inevitably accompany enzymic procedures due to the high sensitivity and high cost of enzymes. The weighing of semi-micro quantities of enzymes could be eliminated with the availability of kits and/or liquid preparation of (pre-mixed or individual) enzymes, allowing easier handling. The use of automated glucose determination equipment (e.g. YSI glucose analyser providing α/β-anomeric equilibrium is shown to be similar to the glucose standard used for calibration) and/or HPLC to minimize sources of error incurred during handling of the hydrolysates for glucose content analysis is highly recommended. These methods use greater representative amounts of the analyte. Consequently, the required repeatability and reproducibility of data from a ring test could then be insured to be obtained for subsequent adoption/legislation.

Ultimately, when all methods being considered have comparably acceptable standard deviation in their repeatability and reproducibility, then choice would probably depend upon their respective reliability, in terms of specificity. After taking the trouble of selecting and developing an accurate and reliable method, mainly because of its economic significance, the EC should not be satisfied with a method that could be easily manipulated.

In conclusion, at present, The Birmingham enzyme method, despite possessing some limitations, appears to provide significant improvements over all other methods being considered. The use of a multi-enzyme regime consisting of α-amylase, glucoamylase and pullulanase affords quantitative hydrolysis of starch to glucose regardless of their amylose:amylopectin ratio, providing specificity. If analyses are performed in duplicate (as our group do as a matter of course) then accuracy is also provided. Solubilization in the Birmingham method is carried out under very mild conditions to ensure non-destruction of the starch molecules and the time required for the elimination of soluble oligosaccharides has been reduced considerably.

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