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Development and evaluation of an integrated method for the measurement of total dietary fibre

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Keywords
total dietary fibre; integrated TDF method; resistant starch; 1,5-pentanediol; diethylene glycol; non-digestible oligosaccharides; integrated procedure; CODEX-compliant.

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Abstract

An integrated total dietary fibre (TDF) method, consistent with the recently accepted CODEX definition of dietary fibre, has been developed. The CODEX Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) has been deliberating for the past 8 years on a definition for dietary fibre that correctly reflects the current consensus thinking on what should be included in this definition. As this definition was evolving, it became evident to us that neither of the currently available methods for TDF (AOAC Official Methods 985.29 and 991.43), nor a combination of these and other methods, could meet these requirements. Consequently, we developed an integrated TDF procedure, based on the principals of AOAC Official Methods 2002.02, 991.43 and 2001.03, that is compliant with the new CODEX definition. This procedure quantitates high- and low-molecular weight dietary fibres as defined, giving an accurate estimate of resistant starch and non-digestible oligosaccharides also referred to as low-molecular weight soluble dietary fibre. In this paper, the method is discussed, modifications to the method to improve simplicity and reproducibility are described, and the results of the first rounds of interlaboratory evaluation are reported.

Introduction

At the 30th session of the Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), the Committee agreed on the following definition for dietary fibre:

> Dietary fibre is carbohydrate polymers[1] with ten or more monomeric units,[2] which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

- Edible carbohydrate polymers naturally occurring in the food as consumed,
- Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,
- Synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

The Committee also agreed on the establishment of an Electronic Working Group (eWG) led by the Delegation of

[1]When derived from a plant origin, dietary fiber may include fractions of lignin and/or other compounds when associated with polysaccharides in the plant cell walls and if these compounds are quantified by the AOAC gravimetric analytical method for dietary fibre analysis: Fractions of lignin and the other compounds (proteic fractions, phenolic compounds, waxes, saponins, phytates, cutin, phytosterols, etc.) intimately 'associated' with plant polysaccharides in the AOAC 991.43 method. These substances are included in the definition of fibre insofar as they are actually associated with the poly- or oligosaccharidic fraction of fibre. However when extracted or even re-introduced in to a food containing non digestible polysaccharides, then they cannot be defined as dietary fibre. When combined with polysaccharides, these associated substances may provide additional beneficial effects.

[2]Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities.

France, open to all Codex members. The specific role of this eWG was to (a) review and update, as appropriate, the list of methods available for dietary fibre analysis, taking into account the new provisions in the draft definition of dietary fibre that would require the selection of methods of analysis, and possible information of new available methods; (b) consider how the results from different methods specific to different types of dietary fibre could be combined together to arrive at the total dietary fibre (TDF) content in a food; (c) evaluate the performance of methods in measuring different types of dietary fibre; (d) make recommendations for methods of analysis for dietary fibre in different food matrices; (e) consider the footnote in the accepted definition that relates to oligosaccharides of degree of polymerization (DP) of 3–9, and to prepare a recommendation as to its revision with regard to the methods of analysis, if necessary.

In their draft document (ALINORM, 2009), the eWG noted that the Official AOAC methods (AOAC 2002) are widely accepted globally for general labeling of nutrient content in foods as well as for health and nutrition claims. The AOAC methods are designed to be accurate, cost effective, and reproducible in various analytical environments on which industry relies. They are the most studied and validated methods available for the quantification of food components. Their use in routine analysis presents no insurmountable difficulty. These methods have passed the rigor of scientific substantiation to achieve the status of reference methods. The eWG also noted that no one AOAC-validated method can measure all non-digestible carbohydrates in foods. AOAC 991.43 (Lee *et al*., 1992) is one of the most widely used 'total' dietary fibre methods. Both this method and AOAC 985.29 (Prosky *et al*., 1985) will measure insoluble polysaccharides and soluble high molecular weight components, i.e. those that are precipitated by alcohol. However, neither fully measures the resistant starch (RS) fraction, nor do they recover the non-digestible oligosaccharide components included in the definition of dietary fibre (Figure 1). They quantify only part of the total RS, inulin, polydextrose (Craig *et al*., 2000), fructo-oligosaccharides (FOS) and resistant maltodextrin (RMD), all of which have relevant physiological functions. Furthermore, some oligosaccharides are not measured at all. The eWG also noted that due to the complexity of the molecular structure of fibers, additional AOAC methods were subsequently developed to validate labelling declarations and claims by measuring specific dietary fibre components in foods that have been shown to exert physiological benefit. Maintaining these methods (e.g. AOAC 999.03, McCleary *et al*., 2002, for fructans) has a number of advantages. By focussing on one component the method is more specific, resulting in higher specificity and accuracy needed to detect fibre present in food products. Equally important, these component-specific methods facilitate routine, cost-effective analysis.

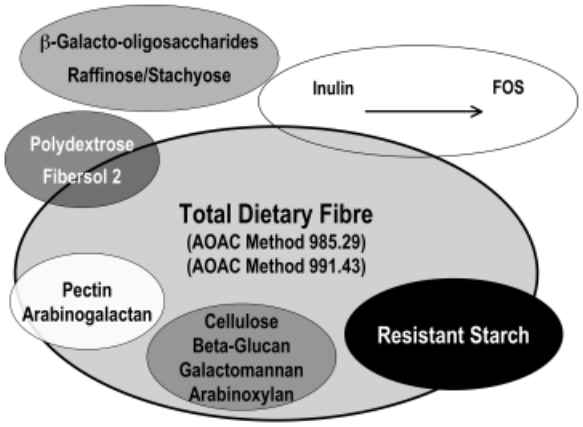

Figure 1 Schematic representation of dietary fibre components measured, and not measured, by AOAC Official Methods 985.29 and 991.43. Also depicted are the problems of partial measurement of RS, Polydextrose® and resistant maltodextrins by current AOAC total dietary fibre methods. Most of the LMWSDF (galactooligosaccharides, fructooligosaccharides, etc) are not measured. The currently described integrated total dietary fibre procedure measures all components shown, with no double counting.

The eWG concluded (draft document) that the NSP method does not accurately quantify total dietary fiber. It is inappropriate as a routine technique given its inability to support the now agreed upon Codex definition of dietary fiber. Methods measuring NSP alone (Englyst & Hudson, 1996) give lower estimates than methods for TDF in foods containing RS, resistant oligosaccharides and/or lignin. The eWG did not recommend the inclusion of methods where there is yet no publication about protocol and relevant validation data.

The eWG also noted that the definition encompasses a range of different types of carbohydrate polymers that are recovered to varying extents by different analytical methods. This creates potential problems of double accounting when a carbohydrate fraction is partially or completely measured by more than one method. Examples of this are high molecular weight inulin, which in addition to being measured specifically by enzymatic-chemical fructan methods are also partially recovered in the residue of enzymatic-gravimetric methods (Quemener *et al*., 1994; Quemener *et al*., 1997). The enzymatic-gravimetric methods AOAC 991.43 and 985.29 also recover some, but not all, RS (McCleary & Rossiter, 2004), which can create a double

accounting problem if this data is then combined with that obtained by a separate specific determination of RS. There is also the potential for obtaining a lower than expected value if there is under recovery of a specific fraction by particular methods. The high degree of specificity associated with most direct chemical methods generally means that the problems of combining results from different methods are diminished.

The eWG noted (draft document) that the lack of a validated procedure to combine AOAC methods to determine total fibre content has repeatedly raised concerns during the lengthy process to finalize the definition of dietary fibre. It also noted that in response to this gap in methodology, a new integrated method of analysis of TDF has been developed by McCleary (2007), which measures TDF (including RS), non-digestible oligosaccharides and available carbohydrates. This new integrated method is based principally on existing official AOAC methods 2002.02 and 991.43 and AOAC method 2001.03 (Gordon & Okuma, 2002). A process similar to that described in AOAC Official Method 2001.03 allows for the measurement of non-digestible oligosaccharides in the range of DP 3 to approximately DP 10.

The eWG concluded that 'this new integrated method provides a path forward for analysing the full range of dietary fibres included in the scope of the Codex definition, in a manner that better reflects overall the fiber that is physiologically relevant. This method is in the stage of collaborative study analysis and is likely to achieve AOAC approval'. In addition, the eWG suggested to the committee to consider the inclusion of the new method of analysis for total dietary fiber (McCleary, 2007), once AOAC validation has been completed.

In this paper, the integrated procedure for the measurement of TDF is described, results for the first round of the interlaboratory evaluation are presented and some potential improvements to the method are described.

Materials and methods

Materials

D/L-maleic acid (cat. no. M-0375), bovine serum albumin (cat. no. A-2153), dimethyl sulphoxide (cat. no. D-8779) and sodium azide (cat. no. S-8032) were from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland). Acetic acid (glacial) GR, sodium hydroxide and calcium chloride ($CaCl_2.2H_2O$) were from Merck (Darmstradt, Germany). Partially degraded chicory inulin (Raftilose P-95®) was a kind gift from Raffinerie Tirlemontoise S.A. (Tienen, Belgium). Polydextrose® (Litesse®) was from Danisco (Copenhagen, Denmark) and Fibersol 2® and DE-25 corn syrup (Pinedex®) were from Matsutani Chemical Company, Hyogo, Japan. Regular Maize Starch (Lot 60401; RMS), High Amylose Maize Starch (Lot 60107; HAMS) was from Penford Australasia, Lane Cove, NSW, Australia. Hylon VII® (Ref. 98GH8401), Novelose 330® (Ref. AH17529) and Novelose 240® (Ref. 96LF10063) were from National Starch and Chemical Company, Bridgewater, CT, USA. Native potato starch was from Avebe, Foxhol, the Netherlands. ActiStar® (enzyme-modified tapioca/cassava starch; US Patent 6,043,229) was from Cerestar, Vilvoorde, Belgium. Potato amylose (cat. no. A-9262) and ACS Soluble starch (cat. no. S-9765) were from Sigma Chemical Company (St. Louis, MO, USA). Amyloglucosidase (AMG) (cat. no. E-AMGDF), thermostable α-amylase (cat. no. E-BLAAM), protease (cat. no. E-BSPRT), barley β-glucan (medium viscosity; cat. no. P-BGBM), citrus pectin (cat. no. P-CITPN), wheat arabinoxylan (cat. no. P-WAXM), D-fructose/D-glucose assay kit (cat. no. K-FRUGL), α-Amylase assay kit (Ceralpha; cat. no. K-CERA), total starch assay kit (cat. no. K-TSTA), TDF assay kit (cat. no. K-TSTA), D-sorbitol/xylose assay kit (cat. no. K-SORB) and resistant starch assay kit (cat. no. K-RSTAR) were obtained from Megazyme International Ireland Limited (Bray, Ireland). Amberlite FPA53 (OH^-) and Amberlite 200 C (H^+) are available from Megazyme (G-AMBOH or G-AMBH, respectively), and in bulk from Rohm and Haas France S.A.S (Paris, France).

Methods

The integrated TDF method is based on a method developed by McCleary (2007) (Figure 2) and is modelled on AOAC Method 2002.02 for measurement of RS. The enzymes used are essentially devoid of activity on dietary fiber components and low molecular weight soluble dietary fibre (LMWSDF). The AMG and pancreatic α-amylase enzyme preparations are devoid of activity on pectin and FOS and have negligible activity on β-glucan. Activity on RMD and Polydextrose® is consistent with reported information on partial hydrolysis of the oligosaccharides by α-amylase and AMG (Craig *et al*., 2000; Gordon & Okuma, 2002). The protease used is devoid of α-amylase (an essential requirement in this assay format). The procedure as described here measures high molecular weight dietary fibre (HMWDF) that includes insoluble dietary fibre (IDF) and high molecular weight soluble dietary fibre (HMWSDF), and LMWSDF. An accurate measurement of RS is obtained. The method can be simply adapted to separately measure IDF and HMWSDF.

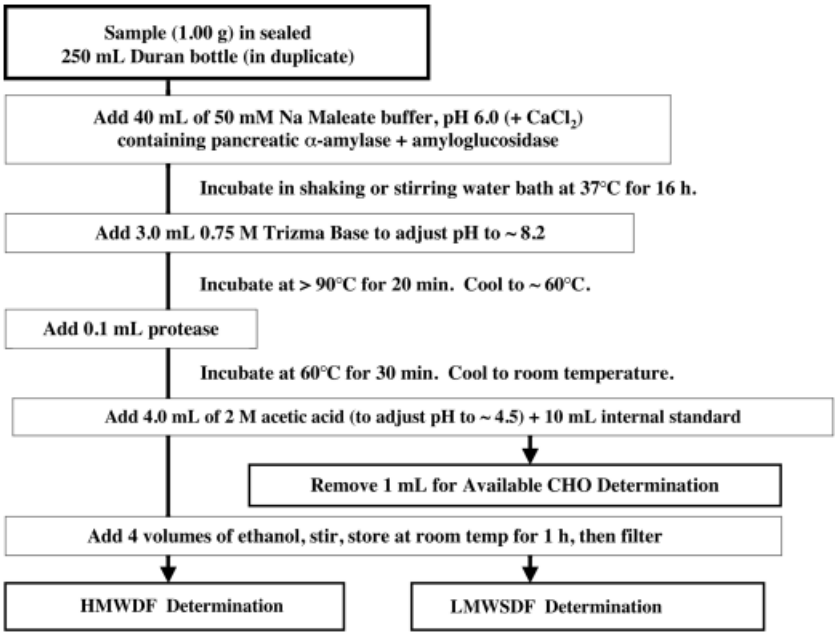

Figure 2 Schematic representation of the integrated TDF assay procedure, also showing where samples can be removed for determination of available carbohydrates (not part of this study).

Sample treatment and analysis of HMWDF

Food samples should be analysed 'as eaten'. Wet or moist samples should be freeze-dried and milled to pass a 1-mm screen; pasta should be cooked, freeze-dried and milled; bread samples should be freeze-dried and milled; etc. Incubate duplicate 1.000 g quantities of the sample being analysed in 250 mL Duran® bottles in a shaking incubation bath set at 150 rev/min in orbital mode, with pancreatic α-amylase and AMG for exactly 16 h at 37 °C. Alternatively, a 2mag Mixdrive 15 submersible magnetic stirrer apparatus as described later, can be used. During this time, non-RS is solubilized and hydrolysed to D-glucose by the combined action of the two enzymes. Adjust the pH to approximately 8.2 with Trizma base and incubate the reaction solutions at > 95 °C to inactivate α-amylase and AMG and to denature protein. (In preliminary work, it was found that if the protein in not denatured by cooking, it is not hydrolysed by protease and thus is a major proportion of residue weight, leading to large errors when it is subtracted from the residue weight to determine HMWDF.) Digest denatured protein with protease at 60 °C and adjust the pH of the reaction mixture to approximately 4.5 with acetic acid. Add internal standard (1 mL of sorbitol or glycerol at 100 mg mL^{-1}; or preferably, diethylene glycol; see later), followed by four volumes of ethanol with mixing to precipitate HMWSDF (including RS that is solubilized, but not depolymerized, in the > 95 °C incubation step). Filter the suspension and wash the residue sequentially with 76% ethanol, 95% ethanol and acetone; dry and weigh. Analyse one duplicate for protein and the other for ash (incubation at 525 °C). HMWDF is the weight of the filtered and dried residue less the weight of the protein and ash.

Analysis of LMWSDF

Concentrate the aqueous ethanol filtrate plus washings, desalt, re-concentrate by rotary evaporation (at 60 °C) and analyse by high-performance liquid chromatography (HPLC) to determine LMWSDF. The method used is modelled on AOAC Method 2001.03 (Gordon & Okuma, 2002). In that method, the low molecular weight RMDs (LMWRMD) that are soluble in 78% v/v ethanol are recovered and analysed by HPLC. In the current method, the same principle is used to measure all of the LMWSDF likely to be in the food product or to have been added. The aqueous ethanol filtrate is concentrated by rotary evaporation, desalted through ion exchange resins [25 g Amberlite FPA53 (OH^-) and 25 g Amberlite 200 C (H^+) or equivalent (Rohm and Haas France S.A.S.], concentrated and analysed by HPLC. This can be done using a Waters Sugar-Pak® (Waters Corporation, Milford, MS, USA) 6.5 × 30 cm (part no. WAT085188) column, with D-sorbitol-1,5-pentanediol

or diethylene glycol as the internal standard. Alternatively, this can be performed by gel permeation chromatography according to AOAC Method 2001.03 [using two TSK-GEL® G2500 PWXL, 7.8 mm × 30 cm (Tosoh Corp., Tokyo, Japan) gel permeation columns in series with a TSK® guard column PWXL 6.0 mm id × 4 cm (Tosoh Corp.)] with glycerol as internal standard (Gordon & Okuma, 2002). (Since glycerol is present in many food products and also in many of the analytical enzymes used, this is not an ideal internal standard.)

Results and discussion

A range of starches, milled grain and some food samples were assayed for RS using the procedure described here as well as with AOAC Method 2002.02 (RS), and the results are shown in McCleary (2007; table 1). Clearly, there is a good agreement in values for all samples except for native potato starch, ActiStar® and green bananas. Native potato starch and ActiStar® completely dissolve in the $> 95\,^{\circ}C$ incubation step (as does most of the banana starch), and subsequent precipitation by ethanol is apparently not complete. While the RS values for these two samples are underestimated by the current method, it should be noted that with AOAC Method 985.29 or 991.43 the TDF (and thus RS) value is essentially zero. From ileostomy studies, native potato starch has been shown to contain high levels of RS. However, this is a very fragile starch, with the granule structure being readily destroyed by heat or physical treatment. Thus it is unlikely that native potato starch will ever be used as a source of RS in food products. Actistar®, which is prepared by partial hydrolysis of tapioca starch with α-amylase and isoamylase, is also very susceptible to heat treatment. Consequently, ActiStar® can only be used in foods designed for consumption without cooking.

In the current procedure, incubation at $> 95\,^{\circ}C$ is incorporated into the method to ensure denaturation of protein and subsequent hydrolysis of the bulk of this by protease. In our experience, there is little hydrolysis of protein in non-cooked materials by subtilisin A (protease) at pH 8 if the cooking step is deleted. This means that the final residues for many samples have a very high protein content, leading to large errors when this protein weight is subtracted from residue weight to obtain HMWDF. Similar results were obtained when other proteases were used, including pepsin at pH 2. In this latter case, problems are also experienced in the acid hydrolysis of certain oligosaccharides, particularly FOS.

TDF values for samples traditionally used to check the efficacy and purity of enzymes used in dietary fibre analysis (e.g. β-glucan, larch arabinogalactan, wheat arabinoxylan, pectin and casein) were essentially the same with the integrated TDF method (McCleary, 2007) and AOAC Method 991.43. However, for high-amylose maize starch (HAMS), a value of 46.5% was obtained with the integrated TDF method compared with 29.3% with AOAC Method 991.43. The much higher DF value for HAMS reflects a true measure of the RS content of this sample. With wheat starch, a TDF value of ~0.8% was obtained, compared with a value of 0.1% by AOAC Method 991.43, demonstrating that even wheat starch contains a small percentage of RS.

The TDF values for a range of RS containing samples have previously been determined with AOAC Method 991.43 and the integrated TDF method and are shown in McCleary (2007; table 3). In general, the TDF values determined with the new method were much higher than those obtained with AOAC Method 991.43. Just two samples, Novelose 240 and Novelose 330, showed similar values with the two methods. Both samples are retrograded. The integrated TDF procedure for HMWDF gives a more accurate measure of DF in samples containing RS.

The procedure described in this paper for the measurement of LMWSDF is based on AOAC Method 2001.03 for measurement of LMWRMD. Since in the current procedure, the samples are subjected to incubation with pancreatic α-amylase plus AMG for 16 h, followed by heat treatment and incubation with protease, it was important to demonstrate that there is no degradation of the LMWSDF during this process. HPLC traces for Neosugars® (FOS) dissolved in water and analysed, compared with the same material subjected to the full enzymatic incubation sequence are shown in Figure 3. The traces are essentially identical, showing that no degradation has occurred. A glycerol peak is evident in the material subjected to enzymatic treatment, which was due to the presence of glycerol as a stabilizer in the AMG and protease enzyme preparations. Raftilose® (FOS) also showed no enzymatic degradation. With Fibersol 2® and Polydextrose®, there is some degradation, however this is consistent with information supplied by the manufacturers. Thus, analysis of the aqueous ethanolic filtrate from the DF incubations will give a true measure of the LMWSDF in the original sample.

In AOAC Method 2001.03, glycerol is used as the internal standard. However, many enzyme preparations used in TDF assay procedures contain glycerol as a stabilizer. Consequently, a range of other sugars and sugar alcohols have been evaluated as a potential replacement for glycerol. Of these, D-sorbitol had the best chromatographic properties on the Waters Sugar Pac® column, but was not suitable for

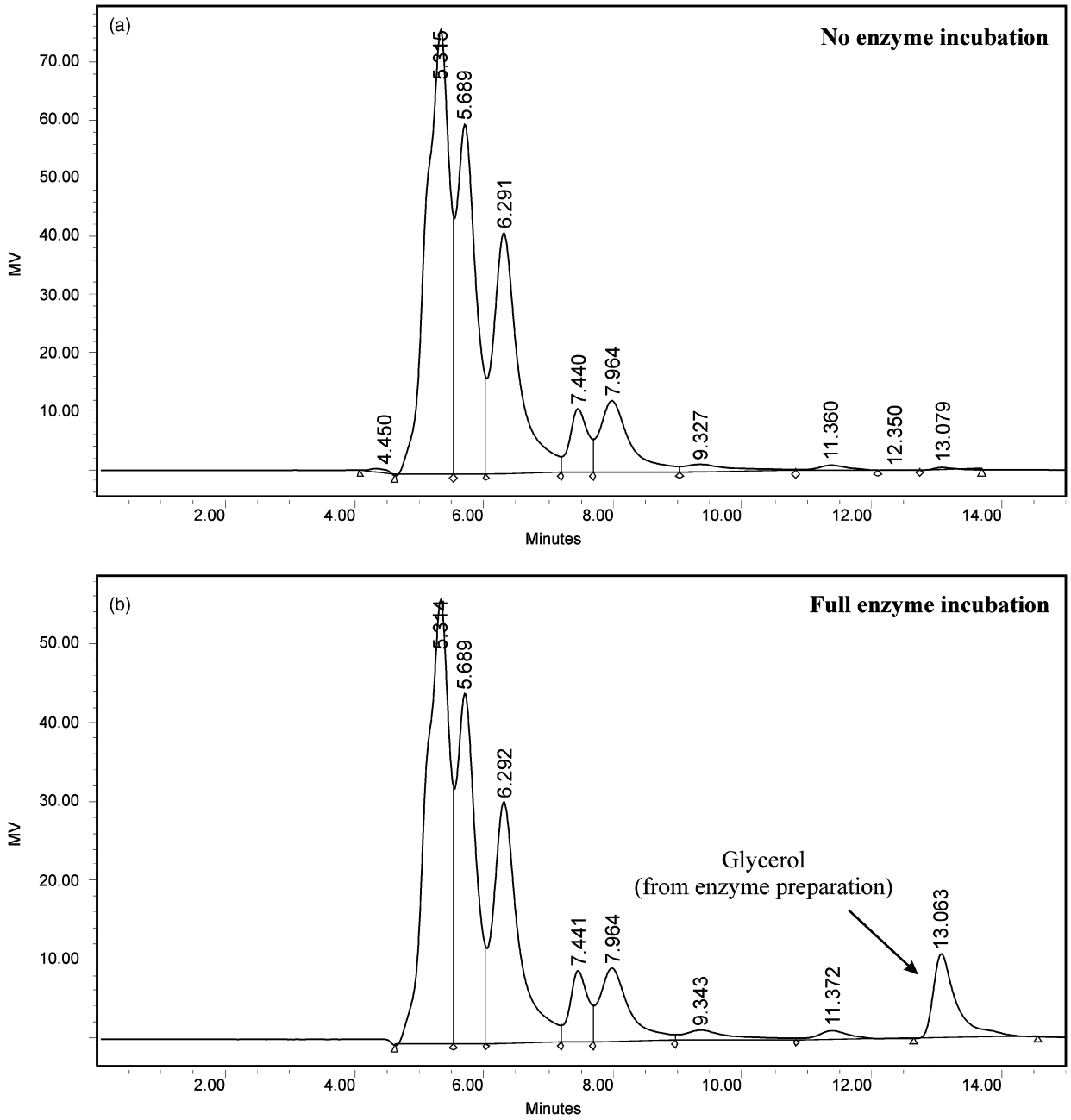

Figure 3 HPLC trace for Raftilose® dissolved in water and analysed directly, compared with Raftilose® recovered as NDO after running through the current integrated TDF procedure. Column: Waters Sugar-Pak® (6.5 × 300 mm, part no. WAT085188) column. Solvent: distilled water containing EDTA (50 mg L^{-1}); flow rate: 0.5 mL min^{-1}; temperature 90 °C; with D-sorbitol, 1,5-pentanediol or diethylene glycol as the internal standard.

the gel permeation columns as it elutes at the same point as D-glucose. Glycerol is recommended (Gordon & Okuma, 2002) as the internal standard for this column arrangement. However, this is not ideal as glycerol is used widely in food products and also is present in many of the enzyme preparations used in dietary fibre analysis.

Interlaboratory evaluation

The integrated method as described, for the determination of TDF, as defined by the CODEX Alimentarius, was the subject of an AOAC International interlaboratory evaluation and has been accepted as AOAC Official First Action

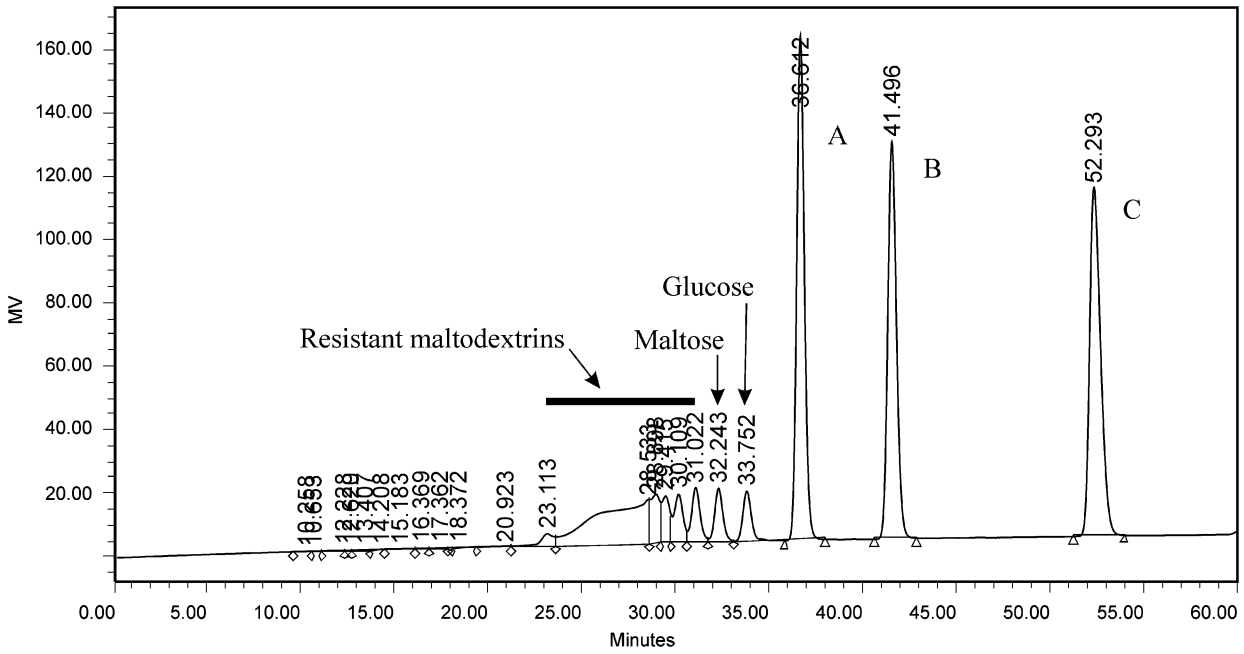

Figure 4 HPLC of DE-25 corn syrup plus glycerol, 1,2-pentanediol and 1,5-pentanediol on two TSK-GEL® G2500 PWXL, 7.8 mm × 30 cm (Tosoh Corp) gel permeation columns connected in series. (A) glycerol; (B) 1,2-pentanediol; and (C) 1,5-pentanediol. Solvent: distilled water; flow rate 0.5 mL min^{-1}; temperature 80 °C.

Method 2009.01. Eighteen laboratories participated with 16 laboratories returning valid assay data for 16 test portions (eight blind duplicates) consisting of samples with a range of traditional dietary fiber, RS, and non-digestible oligosaccharides. The dietary fiber content of the eight test pairs ranged from 11.57% to 47.83%. Digestion of samples under the conditions of AOACI 2002.02 followed by the isolation and gravimetric procedures of AOACI 985.29 and 991.43 results in quantitation of HMWDF. The filtrate from the quantitation of HMWDF was concentrated, deionized, concentrated again and analyzed by HPLC to determine LMWSDF, i.e. all non digestible oligosaccharides of DP 3 or higher. Total dietary fiber was calculated as the sum of HMWDF and LMWSDF. Repeatability standard deviations (s_r) ranged from 0.41 to 1.43, and reproducibility standard deviations s_R ranged from 1.18 to 5.44. This is comparable to other Official dietary fibre methods.

Alternative internal standards

Since it would be useful for the internal standard for HPLC to be appropriate for both the gel permeation chromatographic format as well as for the ion-exchange system with the Waters Sugar-Pak® column, several potential internal standards were evaluated. Initial studies indicated that 1,2-pentanediol and 1,5-pentanediol were possibilities. The chromatographic patterns of these compounds against D-glucose and glycerol in the gel permeation system, is shown in Figure 4. 1,2-Pentanediol was excluded as a possibility because some is lost when column eluates are concentrated by rotary evaporation. This was not the case with 1,5-pentanediol, where loss on evaporation was negligible (Figure 5a); the compound chromatographs distinctly from sugars, oligosaccharides and glycerol with a good peak shape with no broadening even though it elutes well after the other components (Figure 4), and; there is no loss on evaporation at 60 °C nor adsorption to the desalting resins (Figure 5b). Glycerol is not an ideal internal standard because this compound is widely used as an ingredient in the food industry.

Recently, Dr. Okuma, Matsutani Chemical Company, recommended (K. Okuma personal communication) the use of either diethylene glycol or triethylene glycol as an internal standard. We have confirmed that both of these compounds are interesting and neither compound is lost on evaporation during concentration, or by adsorption to resins. In fact, of the three compounds under consideration, 1,5-pentanediol, triethylene glycol and diethylene glycol, the latter has been selected as best since it separates best from the compounds of interest in both chromatography systems evaluated (Figures 6a and b and 7a and b).

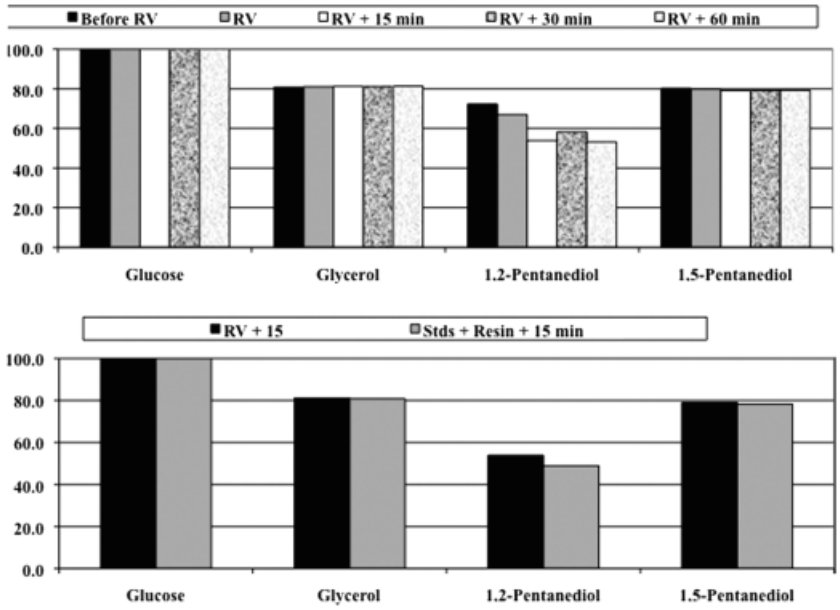

Figure 5 (a) Recovery of glycerol, 1,2-pentanediol and 1,5-pentanediol relative to D-glucose on rotary evaporation of 200 mL of solution containing 100 mg of each component at 60 °C. The graph shows original solution mix (a); concentration to near dryness (b); further evaporation for 15 min (c); 30 min (d); and 60 min (e). All re-dissolved to a final volume of 10 mL. (b) Recovery of glycerol, 1,2-pentanediol and 1,5-pentanediol relative to D-glucose in original solutions (a) and following desalting of solutions through ion exchange resins and rotary evaporation at 60 °C to dryness and further evaporation for 15 min (b).

Manual desalting of samples in preparation for liquid chromatography is time consuming. An in-line desalting system has been evaluated and appears to be suitable for this application (J.W. DeVries, personal communication). However, cost per sample of the disposable desalting cartridges may be prohibitive.

Alternative incubation conditions

A shaking water bath is used (McCleary, 2007) for the incubation of sample with α-amylase plus AMG. In AOAC Method 2002.02 (RS) shaking was preferred over stirring as it gave values more in line with ileostomy results. If samples were stirred in glass tubes using a magnetic stirrer the RS granules were ground between the stirrer bar and the glass base of the tube resulting in significant solubilization, and subsequent underestimation of the RS content of some samples. Since sample stirring could have advantages over suspension using a shaking water bath (e.g. cost of equipment, number of samples that can be handled at one time), the effect of shaking and stirring of samples during the incubation step with enzymes was evaluated. The three incubation arrangements were:

1. shaking of reaction solution in a 250 mL Duran® bottle in rotary motion (150 rpm),
2. stirring using a suspended stirrer with no contact between the stirrer bar and the bottom of the bottle (Figure 8), and
3. conventional magnetic stirring with the stirrer bar added to the reaction container.

In the latter two cases, stirring was achieved with a 2mag Mixdrive 15 submersible magnetic stirrer (http://www.2mag.de/english/stirrer/multiple/stirrer_multiple_04_mixdrive6_15.html) set at 170 rpm and using a 7 × 30 mm stirrer bar. All incubations were performed at 37 °C. A comparison of results obtained with the three mixing systems is shown in Figures 9a, b and c and in Table 1. With all samples studied, the suspended stirrer arrangement gave the same results as for the shaking bottle arrangement. When incubations were performed in bottles with conventional stirring with a magnetic stirrer (not suspended) results obtained were the same for all samples studied except for native potato starch. Native potato starch is known to be a very fragile starch and thus is unlikely ever to be used as a source of RS in processed foods. On the basis of these results, and for the sake of convenience and simplicity, the

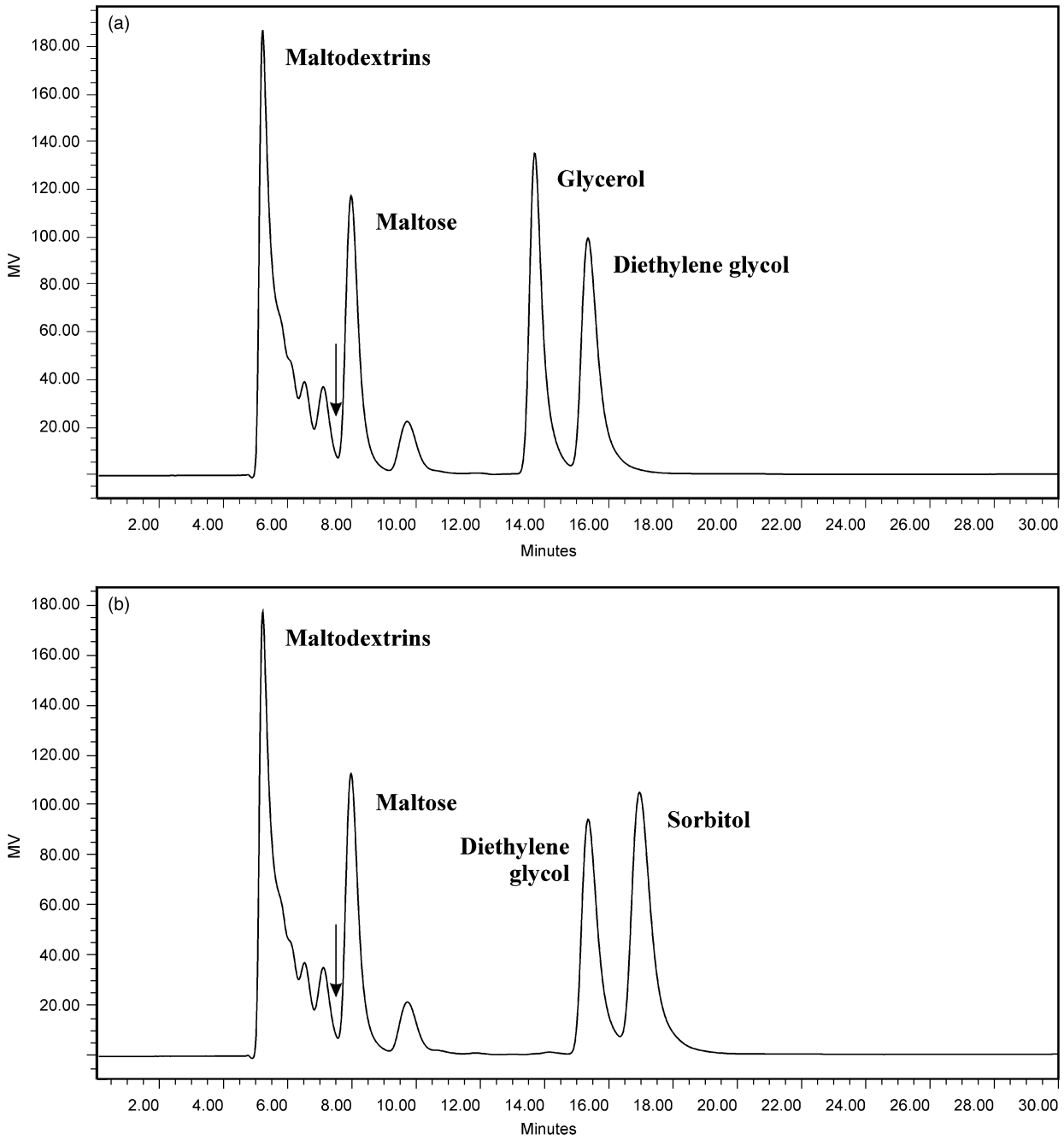

Figure 6 Chromatography of a mixture of maltodextrins, maltose, diethylene glycol plus either (a) D-sorbitol, or (b) glycerol on a Waters Sugar-Pak® (6.5 × 300 mm, part no. WAT085188) column. Solvent: distilled water containing EDTA (50 mg L^{-1}); flow rate: 0.5 mL min^{-1}; temperature 90 °C; with D-sorbitol, 1,5-pentanediol or diethylene glycol as the internal standard. The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).

use of conventional magnetic stirring at 37 °C for 16 h is recommended for the incubations.

Conclusions

A method has been developed and validated through an interlaboratory study, for the determination of TDF (CODEX compliant) in cereal and food products. The method (integrated TDF) involves separate determination of HMWDF and LMWSDF and summation of determined values. Several steps in the procedure were re-assessed and improvements or modifications developed. Incubations can be performed, as recommended, in a shaking water bath, but equivalent results

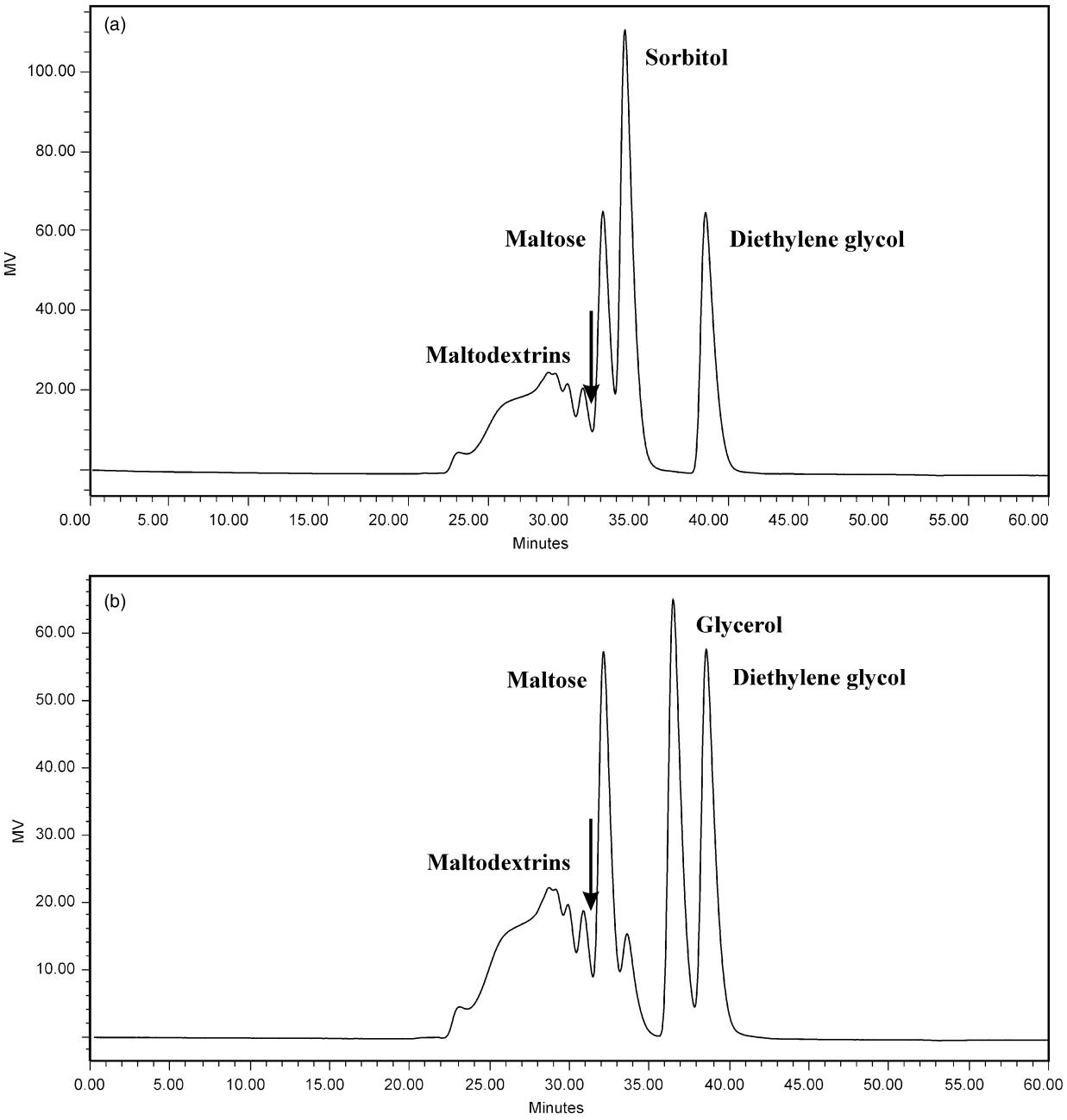

Figure 7 Chromatography of a mixture of maltodextrins, maltose, diethylene glycol plus either (a) D-sorbitol, or (b) glycerol on two TSK gel filtration columns (G2500PWXL) in series. Solvent: distilled water; flow rate 0.5 mL min^{-1}; temperature 80 °C. The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).

are obtained for all samples studied (except native potato starch) when incubations are performed in Duran® glass bottles with agitation by magnetic stirring at a defined speed. An alternative internal standard, diethylene glycol, has been evaluated and is now recommended. This standard performs well with both the Waters Sugar Pak® column and the TSK gel permeation columns. It is considered that other improvements to this basic procedure will be introduced as analysts become familiar with the format. An alternative, in-line procedure for desalting of samples for HPLC has been developed (DeVries, personal communication). A further interlaboratory evaluation is planned in

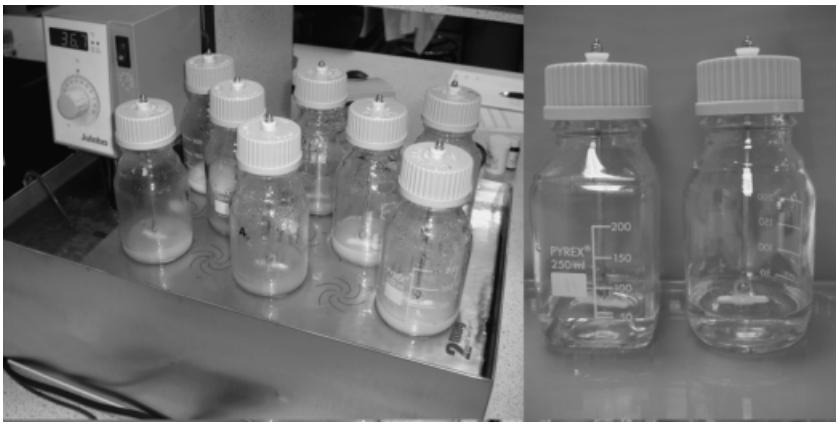

Figure 8 Arrangement for mixing suspensions of resistant starch containing samples using a suspended magnetic stirrer (to avoid grinding of sample between the stirrer bar and the glass bottle).

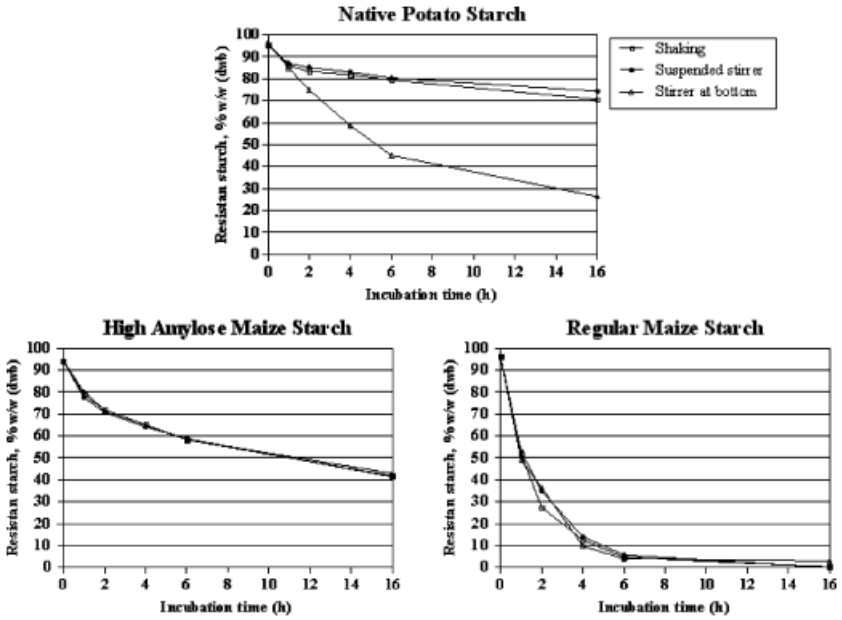

Figure 9 Effect of shaking, suspended stirring and magnetic stirring on the time course of hydrolysis of regular maize starch, high amylose maize starch and native potato starch.

Table 1 The effect of the shaking or stirring conditions on the measured level of resistant starch in a range of samples

	Resistant starch % w/w (dry weight basis)		
Sample	Shaking	Suspended stirring	Conventional magnetic stirring
Regular maize starch	0.3	0.1	0.3
Kidney beans	5.3	5.6	5.7
Green banana	51.0	48.4	46.6
High amylose maize starch	41.7	41.4	42.9
Hylon VII (high amylose maize starch)	52.6	–	49.7
Native potato starch	70.6	74.4	26.3

which IDF, HMWSDF and LMWSDF will be determined separately.

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