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Reliable measurements of the size distributions of starch molecules in solution: Current dilemmas and recommendations

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ABSTRACT

Characterisation of the distribution functions describing size-related parameters of individual whole starch molecules in solution is important for establishing biosynthesis–processing–structure–property relations, for improvements in human and animal nutrition, and for industrial applications of starch. Current techniques for obtaining these distributions suffer from a number of problems in dissolution procedures, size separation, detection, data processing and data reporting. The need is for techniques that have been shown to give an unbiased sampling of the distribution of the starch in its original state, and which are reproducible (i.e., the same results can be obtained with a given sample in a different laboratory and different experimental set-up). The problems with current methodologies are summarized here by an IUPAC-sponsored international group of specialists in the field. Steps to resolving these problems are also discussed.

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1. Introduction

The work reported here is the first outcome from an International Union of Pure and Applied Chemistry (IUPAC) Working Party on *Critically evaluated techniques for size-separation characterisation of starch*.

There are two types of biopolymers in starch: amylose (an α(1→4)-linked glucan with a small number of long-chain branches, typically reported to have molar mass $\sim 10^5$–10^6 g mol^{-1}), and amylopectin (a hyperbranched α(1→4), α(1→6)-linked glucan with 5–6% α-(1,6) linkages (Buleon, Colonna, Planchot, & Ball, 1998). Amylopectin has a very high molar mass (perhaps 10^7–10^9 g mol^{-1}) with a large number of short-chain branches and large size (tens to hundreds of nanometers in the dissolved state). The distributions of both size and molar mass are broad (Aberle, Burchard, Vorwerg, & Radosta, 1994; Bello-Perez, Colonna, Roger, & Paredes-Lopez, 1998; Bello-Perez, Roger, Colonna, & Paredes-Lopez, 1998; Hanselmann, Burchard, Ehrat, & Widmer, 1996; Hoang et al., 2008; Rolland-Sabate, Amani, Dufour, Guilois, & Colonna, 2003; Yoo & Jane, 2002).

Utilisation of starches for improvements in human and animal nutrition, and industrial applications, require a knowledge and understanding of the relationships between starch biosynthesis, processing, structure and properties. Much progress has been made in relating the genes/enzymes responsible for synthesis of starch with the quantifiable structural composition of starch, such as amylose content and amylopectin branch length distributions, and properties such as aggregation, crystallisation and amylase sensitivity that reflect order at a greater length scale. These starch features underlie important end-use applications. In contrast, there is little knowledge of the biosynthesis factors responsible for determining polymer size, and limited understanding of how molecular size (distribution) affects end-use properties. This is due in part to the lack of standardised reproducible methods for quantifying molecular size. Characterising the molecular size distribution of starch polymers is difficult for a number of reasons, including difficulties in solubilising and subsequently size-separating the

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substrate without degradation, and the broad size distribution of native starch.

The common techniques for size separation of whole starch molecules are size-exclusion chromatography (SEC, sometimes also termed gel permeation chromatography, GPC, and high performance size-exclusion chromatography, HPSEC), field-flow fractionation (FFF) and analytical ultracentrifugation (AUC). Hydrodynamic chromatography (HDC) has also been used (Dias, Fernandes, Mota, Teixeira, & Yelshin, 2008; Klavons, Dintzis, & Millard, 1997). Each of these can be carried out with a range of subtechniques: for example, temperature-gradient FFF, asymmetric-flow FFF, sedimentation FFF, etc. Separation is accompanied by various types of detection (singly or as multiple detection), such as differential refractive index (DRI), multiple-angle laser light scattering (MALLS), in-line viscometry, quasi-elastic light scattering, fluorescence and in-line osmometry (Lehmann, Kohler, & Albrecht, 1996) (this last not being commercially available).

Different detectors provide information about different types of distributions of molecules (number, weight,...). It is essential to recall that for a branched molecule such as starch, there is no unique relation between the size of a molecule and its molar mass: a sample which is perfectly uniform in size may contain a range of molar masses. Thus the distributions from different detectors provide complementary, rather than confirmatory, information. This is very different from the situation for linear polymers of uniform geometry, where size and molar mass are uniquely related, and thus (for example) weight and number distributions are trivially interconverted by multiplying or dividing by molar mass M.

In principle, such a battery of techniques can provide powerful tools for obtaining data which are sensitive to the complex structure of starch, and hence can be used to investigate the relationships between biosynthesis, processing, structure, and properties such as digestibility, mechanical properties, rheology, gelatinisation/retrogradation and biodegradation. However, reliable application of these methods is bedevilled by many problems. Recognizing these, a number of researchers in the field over the last few years have independently devised various protocols, typically involving the use of solvents (eluents) such as methylsulfinylmethane (dimethylsulfoxide, DMSO) with varying amounts of water, with or without other additives such as dimethylacetamide and LiBr, various dissolution regimens, and various flow and separation techniques. However, there is a lack of comparison between the data obtained from different methods and set-ups. This paper examines perceived problems with current methods, and makes recommendations for procedures and reporting of data. Subsequent output from this international group will report experimental studies aimed at overcoming the current problems.

In the present project, only one-dimensional separation techniques are considered (those with a single separation mode); the various multi-dimensional techniques under active development (e.g., Edam, Meunier, Mes, Van Damme, & Schoenmakers, 2008; Pasch, Adler, Knecht, Rittig, & Lange, 2006; Pasch, Adler, Rittig, & Becker, 2005) are not considered at this stage. Size characterisation by methods which do not employ physical separation (such as light scattering on an unseparated sample) are also not considered here. In addressing these problems, the work of the present IUPAC project is complementary to, but not overlapping with, that of an IUPAC project entitled "Terminology and measurement techniques of starch components".

2. Dilemmas

It is convenient to divide size-separation characterisation into the following steps: dissolution, separation, detection, data processing and data reporting.

2.1. Dissolution

The *objective* in dissolution is to find procedures which dissolve (as individual molecules) all of the starch in a sample (which might be, e.g., a whole cereal grain or a processed food) without further degradation, and so that there are no aggregates or other entities that will result in incorrect apparent size distributions. Moreover, the solvent, with or without addition of other material, such as an additional solvent or hydrogen-bond disruptor, must be suitable for use in appropriate size-separation equipment: it must be suitable for use with appropriate detectors (this includes both physical compatibility with seals, etc., as well as providing sufficient physical property contrast when the sample is dissolved, e.g., large specific refractive index increment or molar absorptivity).

Sample preparation, particularly for obtaining native starch from a source such as a cereal grain, may degrade the structure. For example, it is well known that milling can break starch chains. However, some disruption of plant tissue is necessary, because plant cell walls can hinder both solvent access to the starch and egress of dissolved starch molecules into the bulk solvent. Moreover, amylopectin molecules are so large that they are very susceptible to shear degradation, even under minimal shear conditions such as gentle agitation (Han & Lim, 2004). Means of minimizing degradation from sample preparation, and detecting if it has occurred, are needed.

The *solvent* and *dissolution conditions* need to satisfy the objectives above. Under ambient conditions, starch is relatively insoluble in water and in many common solvents. While starch can be dissolved in water with elevated pressure and/or temperature, and/or with high pH and/or with various pre-treatments (Bello-Perez et al., 1998; Bultosa, Hamaker, & BeMiller, 2008; Fishman & Hoagland, 1994; Hanselmann, Ehrat, & Widmer, 1995; Lee, Nilsson, Nilsson, & Wahlund, 2003; Modig, Nilsson, & Wahlund, 2006; Roger, Baud, & Colonna, 2001; Rolland-Sabate et al., 2003; Rolland-Sabate, Colonna, Mendez-Montealvo, & Planchot, 2007), these conditions may result in degradation (e.g., Bello-Perez et al., 1998; Jackson, Chotoowen, Waniska, & Rooney, 1988; Kim, Huber, & Higley, 2006; You & Lim, 2000). DMSO is commonly (e.g., Bello-Perez et al., 1998; Chamberlain & Rao, 1999; Chuang & Sydor, 1987; Dona et al., 2007; Han & Lim, 2004; Hoang et al., 2008; Jackson, 1991; Millard, Dintzis, Willett, & Klavons, 1997; Shon, Lim, & Yoo, 2005; Song & Jane, 2000; Tongdang, Bligh, Jumel, & Harding, 1999; Yokoyama, Renner-Nantz, & Shoemaker, 1998; Zhong, Yokoyama, Wang, & Shoemaker, 2006), and N,N-dimethylacetamide (DMAc) is less commonly (e.g., Striegel & Timpa, 1995), used to dissolve starch; this also removes some of the lipids and proteins (Lim, Kasemsuwan, & Jane, 1994; Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009). Sometimes dissolution in DMSO is followed by addition of water (because this can simplify subsequent size separation) or some variant thereof (e.g., Song & Jane, 2000). Lithium salts or other hydrogen-bond disrupters are often added to aid dissolution and to prevent adsorption of starch onto SEC columns (e.g., Chuang & Sydor, 1987; Fishman & Hoagland, 1994). It is essential (a) to have a means of determining if all the starch in the sample has been dissolved, and (b) to ensure the dissolution process has not caused significant degradation. It is noted that size distributions will differ for a given sample in different solvents, but these differences are expected to be relatively slight (essentially corresponding to what would be obtained by some simple transformation of the abscissa, arising from different sizes of a molecule dissolved in different solvents), and under ideal conditions there should be no differences in the essential features of the distributions.

After dissolution (and any subsequent addition of another solvent), checks must be carried out to ensure that neither aggregation nor retrogradation occur; retrogradation is the partial

reassociation (often assumed to be helical formation followed by recrystallisation) of a starch molecule with itself or with other starch molecules, and may result in precipitation. These effects result in a sample that contains a component whose structure is significantly different from that of a fully dissolved polymer.

Other components. A native starch source, such as a cereal grain, contains components other than starch: e.g., proteins, lipids, non-starch polysaccharides (including cellulosic material). The dissolution process may result in the presence of some of these dissolved or suspended in the starch solution. Removal of insoluble components by filtration or centrifugation may lead to shear degradation of the dissolved starch, and/or to co-precipitation of some starch with the insoluble components. Procedures for the removal of soluble non-starch components (e.g., degradation of protein with protease, and removal of lipids with methanol extraction) must be such as not to cause degradation of the starch, or biased loss of dissolved starch (e.g., loss of low molar mass starch in an extraction procedure).

Because of the complex procedures typically used to prepare a solution of individual starch molecules for size separation, there can easily be a *dependence on the operator*.

2.2. Separation

The *objective* in the separation stage is to provide optimal means of separating by size (good resolution with minimal band broadening and optimal signal-to-noise ratio), without loss of material during the separation and detection process.

The separation parameter. The parameter by which separation occurs is termed hydrodynamic volume, V_h. It is often unappreciated that the definition of this quantity (Jones et al., 2009) depends on the separation technique. Thus with SEC, it is usually assumed that the universal calibration assumption is valid (Grubisic, Rempp, & Benoit, 1996), in which case V_h is proportional to the product of the weight-average intrinsic viscosity and number-average molar mass (Kostanski, Keller, & Hamielec, 2004). With field-flow fractionation, there is evidence that separation is by centre-of-mass diffusion (Giddings, Yang, & Myers, 1976; van Bruijnsvoort, Wahlund, Nilsson, & Kok, 2001), and V_h is then the hard-sphere size related to this diffusion coefficient by the Stokes–Einstein equation. With AUC, it is the sedimentation coefficient. A dilemma is that there is no rigorous demonstration that these assumptions about separation parameter are applicable to starch, and indeed there is some evidence that this might not be the case under some circumstances (Sun, Chance, Graessley, & Lohse, 2004; Yu & Rollings, 1987). It is also sometimes not appreciated that the hydrodynamic volume, and corresponding hydrodynamic radius, is related to, but not the same as, the radius of gyration, and the relationship between these two quantities depends on the nature and structure of the polymer, and perhaps also on size. The ratio of these two parameters can however give useful information on the solution conformation of the macromolecule of interest (Burchard, 2003; Morris et al., 2008; Rolland-Sabaté, Mendez-Montealvo, Colonna, & Planchot, 2008).

Size not molar mass. It is essential to recall that size separation methods, by definition, separate on size, not on molar mass. Thus it is impossible to obtain a true molar mass distribution by size separation, except when there is a one-to-one correspondence between the two quantities. This is only true for linear chains and some regular comb polymers, and is certainly not the case for branched polymers such as starch. Thus in general, size separation does not give a true molar mass distribution for starch (there may be some exceptions to this general statement, e.g., Konkolewicz, Gray-Weale, & Gilbert, 2007; Konkolewicz, Taylor, Castignolles, Gray-Weale, & Gilbert, 2007). Using a MALLS and a DRI detector, one can determine the value of the weight-average molar mass as functions of hydrodynamic volume, $\bar{M}_w(V_h)$ (note that some versions of commercial software packages do not implement this data processing correctly for branched polymers (Gaborieau, Gilbert, Gray-Weale, Hernandez, & Castignolles, 2007)). One can then express the number and 'SEC' weight distributions as functions of hydrodynamic volume, $N(V_h)$ and $w(\log V_h)$, as $N(M_w(V_h))$ and $w(\log M_w(V_h))$; however, the abscissa is a weight-average, not a true, molar mass; a problem is that some software packages sometimes give such a plot without stating explicitly that the abscissa is not a true molar mass.

It is possible to determine the hydrodynamic volume as a function of elution time (or elution volume) using a combination of in-line viscometry and osmometry detectors, but a problem is that the latter are not available commercially. Calibration of SEC with monodisperse linear standards of known molar masses and known Mark–Houwink parameters for that solvent and temperature can be used to relate elution times and hydrodynamic volume (i.e., obtaining a calibration curve), but using this relies on the assumption of universal calibration (i.e., that separation is only by V_h and does not depend on any other property of the polymer). Moreover, such standards are unavailable for the high hydrodynamic volumes corresponding to amylopectin, which is a problem for SEC. For FFF and HDC, calibration for large hydrodynamic volumes is much easier, because very monodisperse polymer beads (e.g., polystyrene standards) are readily available, and provided they are used in an eluent which does not swell polystyrene and at a temperature significantly below that of the glass transition in polystyrene (100 °C) these behave like ideal hard spheres (non-ideal behavior may otherwise be observed (Brewer & Striegel, 2009)). However, it is essential to ensure that these are colloidally stable in the eluent, to avoid artifacts from coagulation of the standard. For AUC, the sedimentation coefficient is a hydrodynamic parameter that depends on both the molar mass *and* conformation; this requires no calibration, and therefore "true" molar masses can be estimated from the sedimentation coefficient at infinite dilution ($S^0_{20,w}$) and the concentration dependence of sedimentation (k_s) (Harding, 2005; Rowe, 1977), although this may require prior knowledge of the hydration (mass of solvent per unit mass of macromolecule). Sedimentation coefficients are almost always converted to "standard conditions", 20 °C in water; this simple conversion takes into account the density and viscosity of the experimental solvent. Sedimentation coefficient distributions may also be converted to a molar mass distribution using a Mark–Houwink relationship, should one be available.

Non-starch components in the sample which have not been removed in the dissolution procedure will pose problems if these elute in a region which overlaps that of the starch in the sample. Contamination (e.g., by small amount of lipids or phospholipids that can cause starch molecules to form helical complexes and entangle with one another) or blockages can also result in incorrect data. Furthermore, some solvents (e.g., DMSO with lithium salts) corrode parts of the set-up such as seals and membranes, which not only results in diminution of performance but can also lead to artifacts.

Shear degradation during separation and detection is very likely to be significant with a giant molecule such as amylopectin. One way of detecting this in SEC is by varying the flow rate and seeing if this results in a change in the apparent size distribution. If, for example, halving the flowrate does not result in a change of all or part of an apparent size distribution, then this (part of the) distribution is probably free of artifacts from shear scission (Cave, Seabrook, Gidley, & Gilbert, 2009). If, on the other hand, part of an apparent size distribution were to change to larger sizes when flowrate increases, this may be due to so-called slalom chromatography occurring (Liu, Radke, & Pasch, 2005). This is also applicable

for sedimentation velocity in AUC, in which case the rotor speed (gravitational force) can be varied; however, the very large size of amylopectin means that experiments need to be performed at low speeds. In flow FFF, the polymers are only exposed to gentle tangential shear forces, with the possible exception of higher shear rates at the inlet valve. One way of detecting this in flow FFF is by varying the sample injection flow rate. Although FFF involves much lower shear than does SEC, shear scission may occur, at least in thermal FFF (Janca & Strnad, 2004).

Band broadening (whereby a sample which is perfectly monodisperse in size elutes over a range of elution volumes) is inevitable in any size separation technique. While modern columns (SEC) or flow designs (FFF) may reduce this effect, it is always present. Band broadening with the best available set-ups might result in only minor effects on averages such as $\bar{M}_w$. However, the effect can be very important if one is trying to obtain more sensitive information, such as testing if the distribution in the sample follows a form suggested by theory. An example of this in SEC is testing to see if a sample has a single-exponential number distribution $N(V_h)$: band-broadening causes a predicted straight line produced in a $\ln N$ plot to become curved (van Berkel, Russell, & Gilbert, 2005). While some SEC software packages claim to take band broadening into account, in fact the result is only a partial compensation for the effect. In AUC, diffusion causes boundary broadening, which for a monodisperse polymer may allow the estimation of the diffusion coefficient and hence the molar mass from the Svedberg equation; recent developments (Brown & Schuck, 2006) have gone a long way to extending this to polydisperse and/or non-ideal systems.

Aggregation of materials during the course of the method may occur, giving false apparent distributions.

Exclusion limits. It is well known that the larger molecules of amylopectin lie outside the exclusion limit of current SEC columns. While column development is ongoing, a single column or combination is unlikely to be optimal for all needs, e.g., good resolution of both amylose and amylopectin fractions while giving accurate distribution for each. It is possible that packed column or open tubular HDC may be of assistance.

Sample recovery. Unless quantitative recovery of sample following a separation technique is demonstrated, there will be uncertainties as to whether sample losses are size-specific, thereby resulting in a biased size distribution profile. The very small quantities often used for size separation pose a problem for the sensitive and quantitative detection of separated samples that is required to establish efficient recovery.

2.3. Detection

The *objective* is to detect the amount of starch present in a given elution slice, with the signal dependent only on a chosen property of the starch (e.g., distribution of the number or weight of chains) in that slice.

The types of detectors commercially available for SEC and FFF are DRI, UV absorption, in-line viscometry, MALLS (or other types of light scattering), fluorescence and QELS (quasi-elastic light scattering, also known as PCS, photon correlation spectroscopy, and also as DLS, dynamic light scattering); for AUC, DRI, UV and fluorescence are commercially available. It is essential to be aware of what each detector measures, and under what approximations a distribution may be obtained. For example, in-line viscometry in SEC alone gives the number distribution (Gray-Weale & Gilbert, 2009), but only if the universal calibration assumption holds. Again, if the starch sample is labelled with a fluorophore on the reducing end, then fluorescence detection gives the number distribution, but only if labelling is quantitative, or at least independent of size of the sample (Hanashiro & Takeda, 1998; O'Shea et al., 1998). If labelling with a chromophore is performed randomly, the UV detection will give a weight distribution with a good signal-to-noise ratio. However, this will be the case only if the labelling is truly random, a hypothesis for a complex branched polymer such as starch which can only be tested by comparing the weight distribution obtained by another method – which defeats the purpose of labelling. Information from QELS (and also from static light scattering) depends on the scattering angle, and especially for starch, false inferences can be drawn if there is no access to angular-dependent data, because both local motions and translational diffusion are probed (Galinsky & Burchard, 1997). There are also limits to detection techniques that pose problems for the derivation of quantitative size distribution data, e.g., viscometry or light scattering are useful for detecting small amounts of very large components but these may not be accurately quantified by DRI. Most commercial QELS detectors are single-angle detectors. Furthermore, current on-line QELS systems are not well adapted for very large molecules such as amylopectin (such molecules diffuse very slowly, and thus the dwell time in the measurement cell is not long enough to obtain reliable data). While viscometry and light scattering can detect small amounts of very large components, good signal-to-noise can be a problem. Signal-to-noise problems can arise when the value of dn/dc in a particular solvent is small (n is the refractive index and c the polymer concentration). Small dn/dc values are not a problem in water as a solvent, but starch in this medium is prone to degradation; small dn/dc values are a significant problem in DMSO. Further, viscometric detectors work through pressure differences, and therefore provide a technical challenge in systems such as asymmetric-flow field-flow fractionation which involve pressure variations.

There is also the need to avoid artifacts from non-starch materials: for example, detecting if a protein elutes at a hydrodynamic volume which overlaps that of the starch sample.

2.4. Data processing

The *objective* in data processing is to convert the raw signal(s) from one or more detectors (these signals depending on the particular set-up of the separation and detection system) into one or more distribution functions (e.g., a number or weight distribution), which depend only on the sample and not on the separation/detection system. This is implemented by expressing the data as hydrodynamic volume distributions.

A problem with presenting data in terms of hydrodynamic volume distributions for SEC is that such distributions can only be obtained if the universal calibration assumption is valid, or if it is possible to measure the hydrodynamic volume directly.

Sometimes software packages used in size separation have components which are "black boxes", by which is meant it is impossible to find details of the data processing treatments employed (e.g., in the treatment of MALLS data, what assumptions are made in the use of dn/dc, etc.). There is a need to have software that gives fully exportable data and for which the assumptions in data processing are fully set out.

2.5. Data reporting

The *objective* for data reporting is to present the distribution data along with sufficient information that the experiment is reproducible (i.e., can be performed by a different operator in a different laboratory using a similar experimental set-up but different equipment). There is rarely sufficient information on this given in the literature.

3. Recommendations

3.1. Dissolution

All dissolution procedures should involve a check that all the starch in the sample has actually been dissolved (i.e., individual isolated molecules), which can be implemented by measuring the starch content in the starting material by one of several means. It is also recommended that tests be performed to assess whether significant degradation prior to or during dissolution has occurred, such as seeing if there are changes in apparent distributions with, e.g., milling time or mechanical agitation during dissolution. Checks for aggregation and retrogradation can be implemented by performing replicate experiments, and also performing measurements with different concentrations of starting material in both the dissolution and separation steps. Another way to check for the occurrence of aggregation and/or retrogradation is to obtain $\bar{M}_n$ and $\bar{M}_w$ in different eluents. It is recommended that techniques such as NMR or specific (bio)chemical assays be used to detect the presence of significant amounts of non-starch substances in the starch solution to be used for size separation. The fraction of starch dissolved with the chosen procedure should always be measured and reported with information on parameters such as dn/dc included. Inherent in all of these recommendations is the need to determine the optimal solvent (mixture) and dissolution protocol for fully dissolving starch without degradation.

3.2. Separation

There is a need to establish the nature of the separation parameter (hydrodynamic volume) for starch with the commonest techniques, SEC and FFF. As with dissolution, checks should be carried out for aggregation and/or retrogradation and/or adsorption (e.g., onto a membrane in flow FFF) during the separation step, which can include seeing if there is a change in apparent distribution with concentration. Moreover, information should be published which presents evidence that the equipment is operating in true size-separation mode, i.e., there is no significant interaction with the stationary phase (i.e., the column for SEC; FFF and AUC have no stationary phase).

Columns (for SEC) and membranes, separation method and channels (for FFF) are continually being improved by manufacturers, with improved exclusion limits, improved separation and less band broadening. As stated, band broadening can never completely be eliminated. While methods exist to take this into account (e.g., Konkolewicz et al., 2007, for SEC, and use of monodisperse polymer colloid standards for FFF), even after such deconvolution techniques, band broadening will always cause loss of some information.

To detect the occurrence of shear degradation during separation, it is strongly recommended that data are obtained with a range of flow rates, to see if the apparent size distribution depends on shear.

3.3. Detection

The commercial development of new types of detectors is strongly encouraged, particularly ones that can be used to find the hydrodynamic volume as a function of elution time, without needing to make the assumption of universal calibration; one such is in-line osmometry. Obviously, the more types of detectors, the more information about starch structure can be gleaned by size separation, although one-dimensional methods are always limited to single moments of an infinite-dimension distribution (Gray-Weale & Gilbert, 2009). While labelling the reducing end group with a chromophore will give $N(V_h)$ directly, this requires verification that end-group labelling is quantitative, which does not seem to be possible without obtaining $N(V_h)$ independently. However, if universal calibration is valid, then in-line viscometry alone gives $N(V_h)$ directly, so if the same apparent distribution were to be obtained by a separate end-group-labelling measurement on the same sample, this would support the validity of both assumptions.

Recovery should always be measured and reported, and for preference moisture contents also given (as this can affect, for example, the rate of solubilisation in DMSO (Dona et al., 2007)). Within the scope of this goal, this IUPAC project will undertake an investigation of recommended methods to measure recovery and make recommendations on optimal procedures.

3.4. Data processing

It is regarded as a high priority that some errors noted above in the treatment for branched polymers are corrected in commercial size-separation software packages, and that all packages have a readily accessible option to report data in terms of the separation variable V_h. It is emphasized that data that are reported in terms only of elution time or elution volume, without additional calibration information, are irreproducible, because the result depends on the particular instrumentation set-up, and indeed will vary from day to day in the same set-up. Moreover, commercial packages should be such that it is easy to extract the raw signal, and that assumptions made in the data processing method are clearly spelled out. In addition, the output should be available in a clearly defined instrument-independent form, such as $\bar{M}_w(V_h)$, $N(V_h)$ or $w(\log V_h)$, depending on the type of detector.

3.5. Data reporting

Data reporting should contain a complete description of the starting material (including the provenance and pedigree of the plant source, or batch number for processed starch), and the dissolution technique, including a statement of what fraction of starch in the starting material was actually dissolved. Recovery data should always be provided. It is important that software include means of obtaining and reporting hydrodynamic volume distributions. A full description of the separation set-up should be provided, including for example flow rate, material used for the column or membrane (or the proprietary name thereof), and so on. It is also important that data reporting should include full calibration information: not just calibration curves reporting maxima of the standards as a function of elution volume, but for preference also the calibration elugrams themselves (such details can usually be deposited as supplementary information). It is important to state what kind, if any, of column and interdetector band broadening correction was performed and what type of smoothing function, if any, was used on the data. For universal calibration, specifying the values of the band broadening parameters used, and how these were determined, is paramount.

Results must always be reported in a way that depends only on the properties of the sample and which would, in the absence of non-idealities such as band broadening and shear scission, be independent of the set-up: that is, in terms of distributions of hydrodynamic volume: $N(V_h)$, $w(\log V_h)$ and $\bar{M}_w(V_h)$. Sometimes it might be impossible with the particular system used to convert calibration data directly into V_h: for example, if Mark–Houwink parameters for the particular standards, solvent and temperature are unavailable to the group reporting the results; it is then especially important to give calibration curves so that the results can be converted into V_h distributions when the Mark–Houwink parameters do become available, i.e., so that the experiment is reproducible. It is good practice to provide such information at all times. Data on

starch size separation should never be reported as distributions in terms of molar mass, because this is wrong; it is however correct to present data as $N(\bar{M}_w(V_h))$ and $w(\log \bar{M}_w(V_h))$, but $\bar{M}_w(V_h)$ data should also be presented. Commercial software packages should reflect this reality.

A round-robin test will be implemented within this IUPAC project. This will involve three agreed materials available to all participants: a commercial processed starch as available from chemical suppliers, plus two cereal grains, wheat and rice. The tests will include inter-laboratory comparison of dissolution procedures, and of both raw and processed data obtained with different separation modes, set-ups and data-processing packages. This will lead to recommended procedures to enable laboratories throughout the world to obtain reliable size distributions of undegraded whole starch, for subsequent use in establishing the relations between biosynthesis, processing, structure and properties.

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