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# Amylose content in starches: Toward optimal definition and validating experimental methods

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ABSTRACT

A new analytical method to define and quantify the amylose content in starches is developed using two-dimensional (2D) macromolecular size/branch chain-length distributions obtained by multidimensional size-exclusion chromatography (SEC, also known as GPC) and enzymatic debranching. This method permits clear separation of amylose (low molecular weights, a small number of long-chain branches), amylopectin (high molecular weights, a large number of short-chain branches), long-chain-branched amylopectin and intermediate components. The results are applied to rice starch, normal maize starch, and two "high-amylose" starches (Gelose 50 and Gelose 80) and used to validate four "single-quantity" techniques for measuring amylose content: iodine colorimetry, concanavalin A precipitation, and 1D SEC debranched (or chain-length) and branched distributions. Quantitatively accurate amylose contents can be obtained with the first three single-quantity methods for starch samples with clear separation of the amylose and amylopectin populations, but the 1D SEC branched distribution seriously overestimates the values compared to the other techniques. For high-amylose starches, the definition of amylose content must be taken with caution: it is impossible to separate the different macromolecular populations unambiguously because of the higher abundance of hybrid species. The 2D structural method serves as a reference to identify amylose content and validate single-quantity analytical procedures.



## 1. Introduction

Starch comprises mainly two types of glucose polymers, amylose and amylopectin. It is generally accepted that amylose is an α-(1→4)-linked glucose polymer of moderate molecular weight (~$10^6$) and with a few long-chain branches linked together by α-(1→6) glycosidic bonds, while amylopectin is of much higher molecular weight (~$10^8$) with a vast number of short-chain branches. The ratios of these two polymers, given as amylose content, have been reported to correlate strongly with various properties of starch (Jane et al., 1999; Sasaki, Yasui, & Matsuki, 2000; Varavinit, Shobsngob, Varanyanond, Chinachoti, & Naivikul, 2002; Zeng, Morris, Batey, & Wrigley, 1997), being a statistically significant factor in cooking and processing quality, texture of the products, and digestibility or nutritional values. Starches with higher amylose have been shown to have lower digestibility or better nutritional value (Li, Jiang, Campbell, Blanco, & Jane, 2008; Witt, Gidley, & Gilbert, 2010; Zhu, Liu, Wilson, Gu, & Shi, 2011). Analyzing the amylose content is thus frequently used as a tool in predicting the properties of starch.

As summarized in Table 1 and by the review of Fitzgerald et al. (2009), many techniques are used to determine the amylose content of starch. These include iodine colorimetry (Juliano et al., 1981; Mahmood, Turner, & Stoddard, 2007; Morrison & Laignelet, 1983; Perez & Juliano, 1978; Zhu, Jackson, Wehling, & Geera, 2008), size exclusion chromatography (SEC, also known as gel-permeation chromatography, GPC) of both debranched and fully branched substrate (Fitzgerald et al., 2009; You & Lim, 2000; Zhu et al., 2008) and concanavalin A precipitation (Gibson, Solah, & McCleary, 1997; Yun & Matheson, 1990). However, the results among different techniques used to analyze the amylose content of a single starch sample can vary noticeably (Duan, Donner, Liu, Smith, & Ravenelle, 2012; Fitzgerald et al., 2009; Juliano et al., 1981; Zhu et al., 2008). For qualitatively different techniques (e.g. SEC on debranched starch, which directly measures chain-length distributions, *vs.* iodine colorimetry, which measures the spectrum of polyiodide in the single helical complex with starch), this is probably because each technique measures a different property that is then converted to a purported amylose content (Gray-Weale & Gilbert, 2009). Variations are also observed in iodine colorimetry, the most commonly used technique to analyze amylose content, within a single laboratory or among different laboratories (Fitzgerald et al., 2009; Juliano et al., 1981; Mahmood et al., 2007;

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**Table 1**
Comparison of methods to analyze amylose content in starch.

| Methods | Measurement | Possible interference |
|---|---|---|
| Iodine colorimetry (blue value) – apparent amylose (Juliano et al., 1981; Mahmood et al., 2007; Morrison & Laignelet, 1983; Perez & Juliano, 1978; Zhu et al., 2008) | Blue color (wavelength at 590–720 nm) of amylose–iodine complex without prior defatting of starch | • Interference by the color developed from amylopectin–iodine complex overestimating amylose content (Juliano et al., 1981; Morrison & Laignelet, 1983; Perez & Juliano, 1978)<br>• Different standard curves depending on the inclusion of amylopectin in the standards (Juliano et al., 1981) and the sources of amylose and amylopectin (Fitzgerald et al., 2009; Perez & Juliano, 1978; Zhu et al., 2008)<br>• Unreliable standard curve due to residual *n*-butanol and amylopectin in commercial amylose (Vilaplana & Gilbert, 2011; Zhu et al., 2008)<br>• Inconsistent absorbance depending on pH (Juliano et al., 1981; Perez & Juliano, 1978), time (Morrison & Laignelet, 1983), temperature (Morrison & Laignelet, 1983), wavelength(s) (Juliano et al., 1981; Mahmood et al., 2007; Zhu et al., 2008), dissolution technique (Mahmood et al., 2007; Morrison & Laignelet, 1983), sample concentration and $I_2$/KI concentration (Morrison & Laignelet, 1983) |
| Iodine colorimetry (blue value) – total or absolute amylose (Juliano et al., 1981; Mahmood et al., 2007; Morrison & Laignelet, 1983; Perez & Juliano, 1978) | Blue color (wavelength at 590–720 nm) of amylose–iodine complex with prior defatting of starch | • Same as those in "iodine colorimetry – apparent amylose"<br>• Incomplete removal of lipids, especially internal lipids (Morrison & Laignelet, 1983; Schoch, 1942)<br>• Residual solvent, such as methanol or ethanol, used to defat starch preventing the complex formation with iodine |
| Iodine potentiometry – apparent amylose (Bates, French, & Rundle, 1943; Duan et al., 2012; Jane et al., 1999; Kasemsuwan, Jane, Schnable, Stinard, & Robertson, 1995) | Increase in voltage because of unbound iodine ions after the defatted starch is completely complexed with iodine | • Incomplete removal of lipids, especially internal lipids (Morrison & Laignelet, 1983; Schoch, 1942)<br>• Residual solvent, such as methanol or ethanol, used to defat starch preventing the complex formation with iodine<br>• Different iodine binding resulted from different $I_2$/KI concentrations (Bates et al., 1943) |
| Iodine potentiometry – real or absolute amylose (Jane et al., 1999; Kasemsuwan et al., 1995) | Increase in voltage because of unbound iodine ions after the defatted starch is completely complexed with iodine corrected with the binding of iodine by purified amylopectin/intermediate component | • Same as those in "iodine potentiometry – apparent amylose"<br>• Residual amylose in the purified amylopectin used to correct the amylose content<br>• Molecular degradation of amylopectin and intermediate components by chemical fractionation process |
| 1D whole-molecule (fully branched) size distribution of starch by size exclusion chromatography (You & Lim, 2000; Zhu et al., 2008) | AUC of the smaller molecules among the two populations of starch molecules | • Different $dn/dc$ of amylose and amylopectin due to their different confirmations in solution (Tizzotti, Sweedman, Tang, Schaeffer, & Gilbert, 2011)<br>• Artifacts caused by incomplete dissolution and low SEC recovery (You & Lim, 2000)<br>• Intermediate component eluding at the same hydrodynamic size as amylose (Vilaplana & Gilbert, 2010a,b)<br>• Shear scission of amylopectin by SEC increasing the peak area of amylose peak (Cave et al., 2009) |
| 1D branch chain-length distribution of starch by size exclusion chromatography (Fitzgerald et al., 2009) | AUC of the branches with DP > ~100 | • Long branches of amylopectin or intermediate component eluding at the same hydrodynamic size as amylose branches (Vilaplana & Gilbert, 2010a,b) |
| Concanavalin A precipitation followed by enzymatic or chemical hydrolysis (Gibson et al., 1997; Yun & Matheson, 1990) | Precipitation of highly branched molecules using concanavalin A and amylose was determined from the supernatant by enzymatic or chemical analysis | • Co-precipitation of amylose underestimating the actual amylose content (Gibson et al., 1997; Yun & Matheson, 1990)<br>• Incomplete removal of lipids, especially internal lipids (Morrison & Laignelet, 1983; Schoch, 1942) |
| Differential scanning calorimetry (Mestres, Matencio, Pons, Yajid, & Fliedel, 1996; Sievert & Holm, 1993; Zhu et al., 2008) | Endotherm from the dissociation of starch–lipid complex | • Inconsistent endotherm/exotherm of amylose-lipid complex due to heating and/or cooling conditions (Mestres et al., 1996), botanical sources (Sievert & Holm, 1993; Zhu et al., 2008), and presence of retrograded amylose (Sievert & Holm, 1993) and non-starch components (lipid, protein, etc.) (Zhu et al., 2008)<br>• Unreliable amylose standard due to residual *n*-butanol and amylopectin in commercial amylose (Vilaplana & Gilbert, 2011; Zhu et al., 2008) |
| Amylose fractionation using *n*-butanol followed by β-amylolysis (Banks & Greenwood, 1967) | Maltose from β-amylolysis of fractionated amylose analyzed as reducing sugar | • Incomplete fractionation and the presence of residual amylopectin after *n*-butanol fractionation (Vilaplana & Gilbert, 2011; Zhu et al., 2008)<br>• Incomplete β-amylolysis of amylose as it contains few branching points that can hinder β-amylolysis |

Morrison & Laignelet, 1983; Perez & Juliano, 1978; Zhu et al., 2008); such single-technique variability arises from technical problems such as different standards, pH of buffer, standing time, temperature, wavelength(s), dissolution technique, sample concentration, and $I_2$/KI concentration being used for the measurement. Moreover, most of these techniques do not take into consideration the presence of hybrid components (highly branched glucose polymers with molecular weight similar to amylose, or with very long branches of amylopectin; Vilaplana & Gilbert, 2010b). Although these species of glucose polymers are minor components in most native starches, their amounts are substantial in some mutant starches, such as high-amylose maize starch (Jane et al., 1999; Klucinec & Thompson, 1998; Li et al., 2008), and may greatly affect the measurement of amylose content. In addition, two definitions for apparent and absolute amylose contents are found in the literature (Table 1), which creates confusion when discussing the relationship between amylose content and starch properties.

The origin of this question is that there is no rigorous definition of just how a molecule can be classified unambiguously as amylopectin or amylose. One has *characterization* definitions (e.g. defining amylose content as the result obtained by iodine colorimetry) or *structural* definitions (e.g. the relative amounts of

big molecule/small branches and small molecule/long branches), but neither type is satisfactory or unambiguous, and each is case-dependent.

A solution to this problem has been put forward (Gray-Weale & Gilbert, 2009; Vilaplana & Gilbert, 2010b) through another way of defining amylose and amylopectin; this is *both* a structural and characterization definition and is not affected by the presence of complexing lipids. One starts by obtaining (using methods summarized below) the experimental two-dimensional (2D) distribution function giving, as the first dimension, the weight (or number) of molecules as a function of the total size of the molecule (usually the hydrodynamic volume $V_h$, which is the separation parameter in SEC) and, as the second dimension, the weight (or number) of individual branches in the macromolecules as a function of their (branch) degree of polymerization (DP) $X$ (actual examples of these are given later in this paper in Fig. 2). If amylopectin and amylose are clearly separated, as large-size molecules with small branches *vs.* small molecules with long branches respectively, these would be manifest as two clearly separated "mountains" in such a 2D distribution, and a well-defined line can be drawn separating the two. The amylose-to-amylopectin ratio would be then obtained from the areas under these separate "mountains". If on the other hand there is no such clear separation in a particular sample, then alternative dividing lines could be drawn depending on taste, but in general it is only the 2D function that contains useful information in such cases, not fractions in an arbitrary division.

This 2D distribution function is obtained experimentally by carrying out the separation in the first dimension by macromolecular size (e.g. $V_h$) using preparative SEC, collecting the resulting fractions at elution times, debranching these samples enzymatically in the conventional way using isoamylase, and then finding the chain-length distribution of the resulting linear glucans as the second dimension (Vilaplana & Gilbert, 2010b, 2011). The size of these linear glucans is related to the corresponding DP by the Mark–Houwink relationship. Technical details of this procedure, which is a demanding one to implement, have been given elsewhere (Vilaplana & Gilbert, 2010b, 2011).

It is emphasized that SEC separates by molecular size ($V_h$); thus it separates neither by branching structure alone (amylose having a small but significant number of long branches, and amylopectin an enormous number of short ones) nor by molecular weight (amylose having relatively low molecular weight, and amylopectin very high molecular weight): two polymer molecules can have the same $V_h$ (and thus co-elute in 1D SEC) but very different branching structures and molecular weights. The new 2D method however separates by both size and by branching structure.

In the present paper, this 2D method is applied to a test set of starch samples: rice, normal maize and two "high-amylose" starches. The information obtained in the full 2D distributions is compared to the results of measurements using four conventional methods for finding "amylose content": iodine colorimetry, concanavalin A precipitation, area under the curves (AUC) of 1D macromolecular size distributions (obtained from SEC of whole, i.e. fully branched, starch molecules), and AUC of 1D branch chain-length distributions (obtained from SEC of debranched starch molecules). The results of these measurements, each of which yields a single quantity, are compared to the much richer information obtained from the 2D distributions. In cases where there is found to be two well-separated amylopectin/amylose peaks, the results of these single-quantity measurement should be close to the relative areas under these 2D peaks. In more complex situations without clear separation of distinct regions (as found here with the high-amylose starches), it is possible to see just what quantity the methods measure, i.e. which arbitrary line dividing the 2D distributions into two regions produces relative amounts close to those of a given single-quantity method. In the latter case, the single-quantity values can be compared to the well-defined values from the 2D method. This then enables one to both test the validity of the methods for cases of clear separation, and to infer just what is measured in intermediate cases.

## 2. Materials and methods

### 2.1. Materials

Rice flour (MRQ74) was kindly donated by Malaysian Agricultural Research & Development Institute (Kuala Lumpur, Malaysia). Normal maize, Gelose 50, and Gelose 80 starches were obtained from Penford Australia Ltd. (Lane Cove, NSW, Australia). The amylose/amylopectin kit (K-AMYL), which employs concanavalin A, was obtained from Megazyme International Ltd. (Co. Wicklow, Ireland). Filtered dimethyl sulfoxide (DMSO, ACS grade, Merck & Co, Inc., Kilsyth, VIC, Australia) with 0.5% (w/w) LiBr (ReagentPlus, Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) was employed in sample dissolution and as eluent for separation by SEC.

### 2.2. Iodine colorimetry

The amylose content of starch or rice flour (100 mg) was analyzed using standardized iodine colorimetry in triplicate following ISO Method 6647-2-2011 (International Standardization Organization, 2011). Five rice flour samples obtained from the International Rice Research Institute (IRRI, Los Baños, Philippines) with agreed amylose contents of 0.00, 4.19, 11.32, 17.19 and 23.52%, were used as the standards to generate the calibration graph. The amylose contents of the rice flour standards were determined by inter-laboratory studies (chaired by Dr Melissa Fitzgerald, IRRI) from the AUC of amylose branches in the 1D SEC debranched distribution. The absorbance of the starch-iodine mixture was obtained at 620 and 720 nm. Using rice flours of known amylose contents as a set of standards eliminates the need to correct the resulting amylose content from the non-starch components in rice flour sample, assuming that the amounts of these components are the same in all rice flours. However, for starches which have been isolated from the non-starch components in the grains, such as normal maize, Gelose 50, and Gelose 80 starches, a correction factor is needed. The correction factor of 85% was used in the present study as it is the average dry starch content in rice flour. Only the results from the absorbance at 720 nm are used as comparison with other methods. Additional data are given in the Supporting Information.

### 2.3. Concanavalin A

The amylose content of starch or rice flour was determined in duplicate using the Megazyme amylose/amylopectin kit following the procedure provided by the manufacturer.

### 2.4. Whole-molecule size distributions and branch chain-length distributions from SEC

One-dimensional (1D) size distributions of (i) the whole starch macromolecule and (ii) chain-length distributions of the starch branches after debranching with isoamylase, both obtained from SEC measurements, were used to estimate amylose content. The extraction, dissolution, and debranching procedures were those reported previously (Syahariza, Li, & Hasjim, 2010; Tran et al., 2011). SEC experiments were performed on an Agilent 1100 series system (PSS, Mainz, Germany) equipped with a triple detection set-up consisting of multiple-angle laser light scattering (MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York), refractive index detector (RID; Shimadzu RID-10A, Shimadzu Corp., Japan) and a viscometric detector (ETA-2010, PSS).

The whole-molecule size distributions were obtained using a GRAM pre-column, and 30 and 3000 analytical columns (PSS) with DMSO/LiBr (0.5%, w/w) as the mobile phase at a flow rate of 0.3 mL $min^{-1}$ and 80 °C. Separation of the debranched samples were in this case with combined GRAM pre-column, 100 and 1000 analytical columns (PSS) with a flow rate of 0.6 mL $min^{-1}$ of DMSO/LiBr (0.5%, w/w) at 80 °C.

### 2.5. 2D structural distributions from multidimensional SEC × SEC

The 2D distributions based on macromolecular size/branch chain-length were obtained by an analytical procedure combining size fractionation by preparative SEC, collection of size-separated fractions, enzymatic debranching of these fractions with isoamylase, and analysis of the branched and debranched fractions by analytical SEC with multiple detection (refractometry, viscometry and light scattering). The final result is the weight distribution of whole starch molecules as a function of hydrodynamic size and of the hydrodynamic size of an individual branch; the latter can be expressed either as the hydrodynamic radius of the branch resulting from debranching, $R_{h,de}$ or equivalently as the corresponding DP, $X$, calculated from the hydrodynamic size using the Mark–Houwink relation. Full details about the experimental procedure are given elsewhere (Vilaplana & Gilbert, 2010b, 2011).

The amylose content in starches can be structurally defined from the results of the 2D size/branch chain-length distributions. This new analytical method implements the theoretical development of an earlier paper (Gray-Weale & Gilbert, 2009) as follows. From the definition of the SEC weight distribution $w(\log V_h)$, the mass of polymer in a logarithmic increment $d \log V_h$ is proportional to $w(\log V_h)\, d \log V_h$ (within an arbitrary normalization constant), or equivalently, if expressed in terms of hydrodynamic radius $R_h (V_h = 4/3\pi R_h^3)$, to $w(\log R_h)\, d \log R_h$. Similarly, for the 2D distributions, the mass of polymer in a 2D increment $d \log R_{h,br}$, $d \log R_{h,de}$ is $w(\log R_{h,br}, R_{h,de})\, d \log R_{h,br}$, $d \log R_{h,de}$. Thus:

$$\text{mass in a given 2D region} = \iint_{\text{region}} w(\log R_{h,br}, R_{h,de}) d \log R_{h,br}\, d \log R_{h,de} \quad (1)$$

The integration range for the amylose region is chosen by inspection of the 2D plots, as illustrated in Section 3. A line is chosen which separates the amylose and amylopectin regions, then the 2D numerical integration performed to give the total mass of polymer in each region by a double application of Simpson's rule (a standard numerical integration method) in each dimension. The intervals were chosen here to be evenly spaced in each of $\log R_{h,br}$ and $\log R_{h,de}$, and to be sufficiently small in each dimension to give an accuracy of ~1%, which was typically 50–100 intervals.

## 3. Results and discussion

### 3.1. Overview of results

Prior to discussing the results from each technique in detail, we present an overview of the collected data. Table 2 presents the results for defatted starch using iodine colorimetry, concanavalin A, 1D whole-molecule size distribution (fully-branched SEC), 1D branch chain-length distribution (debranched SEC) and the new 2D method.

The ratios of each single-quantity method to those obtained from the new 2D method are displayed graphically in Fig. 1, where this comparison includes the ratios both for the cases where there is a clear single dividing line between the amylose and amylopectin "mountains" (rice and normal maize starches), and where alternate choices of this dividing line can be made, i.e. for the Gelose 80 and Gelose 50 starches.

The amylose contents are in the order rice starch < normal maize starch < Gelose 50 starch < Gelose 80 starch for all methods, but the actual values are sometimes significantly different for different methods. Iodine colorimetry, concanavalin A, 1D debranched SEC and the new 2D method give similar results for rice flour/starch and normal maize starch, but those for 1D fully branched SEC are evidently different (>20%) from those obtained by other four methods in agreement. For Gelose 50 and Gelose 80 starches, as will be seen in detail later, the 2D data reveal that there are two alternative ways of reasonably defining an amylose region; as stated, these high-amylose starches are expected to be problematic for all the single-quantity methods. The results for concanavalin A and iodine colorimetry are similar with both of those regions from the 2D method for Gelose 50 starch, and the value from 1D debranched is in moderate accord with one of the two values from the 2D method, while the value from 1D fully branched SEC is noticeably different from the two 2D values. For Gelose 80 starch, the value from iodine colorimetry is close to the 2D result from one defining region, and the value from concanavalin A is close to both 2D values, while 1D debranched and 1D fully branched SEC results are evidently different from the two 2D values and from each other.

The results from each method are now considered in detail.

### 3.2. The new method using 2D macromolecular size/branch chain-length distributions

Fig. 2 shows the 2D distributions for the four starch samples as contour plots. The various one-dimensional SEC distributions which are processed to yield these 2D distributions are given in the Supplementary Information. These contributing one-dimensional SEC distributions are those from preparative SEC, and the SEC debranched distributions for each of the fractions as indicated; they are similar to those we have given elsewhere (Vilaplana & Gilbert, 2010a,b, 2011). The debranched distributions in the Supplementary Information clearly indicate the amylose and amylopectin fractions for maize and rice starches, where these regions are well separated.

Distinct and well-resolved surfaces can be observed for the amylose and amylopectin populations in rice and normal maize starches. On the other hand, a more complex 2D behavior is observed for the high-amylose starches, related to the presence of amylopectin, amylose and hybrid macromolecular populations (intermediate components (Takeda, Shitaozono, & Hizukuri, 1990; Wang, White, Pollak, & Jane, 1993; Whistler & Doane, 1961) and amylopectin with long-chain branches) in these samples, with no clear separation between the different macromolecular populations in the surface plots. The 2D plots indicate effectively baseline resolution in cases where there are clearly separate regions (Fig. 2 for normal maize, rice and some regions of both Gelose 50 and Gelose 80).

The sources of uncertainty in such 2D plots have been considered in detail elsewhere (Vilaplana & Gilbert, 2010a,b, 2011). There are three such sources.

The first is SEC band broadening: a perfectly monodisperse analyte sample will be eluted over a greater or lesser range of elution volume. This is especially problematic with the first step in the 2D method, preparative SEC. In the absence of monodisperse standards over the large size range covered by starch, this effect cannot yet be quantified, but conditions were chosen to minimize this problem as much as possible (Vilaplana & Gilbert, 2011). However, the 2D plots all show clear separation between peaks, and this separation is close to baseline in rice and normal maize starches. Thus, despite the quantitative uncertainty, this good separation implies that using these 2D data to calculate amylose fraction will be subject only to negligible errors from band broadening.

**Table 2**
Amylose content (%, dry starch basis) determined using iodine colorimetry, concanavalin A precipitation, 1D whole-molecule size distribution, 1D branch chain-length distribution, and the new 2D method.

| Sample | Iodine colorimetry[a] | Concanavalin A (Megazyme kit)[a] | SEC: 1D whole-molecule (fully branched) size distributions | SEC: 1D branch chain-length distributions ("debranched" SEC) | SEC: 2D method (macromolecular size/branch chain-length distributions) |
|---|---|---|---|---|---|
| Rice flour/starch | 22.8 ± 0.1 | 31.8 ± 0.3 | 40.1 | 29.9 | 28.1 |
| Normal maize | 30.2 ± 0.3 | 28.7 ± 0.6 | 43.6 | 30.2 | 34.1 |
| Gelose 50 | 48.2 ± 1.3 | 46.1 ± 1.1 | 77.0 | 64.0 | 50.7[b]<br>54.7[c] |
| Gelose 80 | 64.5 ± 5.4 | 58.9 ± 1.2 | 93.3 | 83.7 | 53.1[b]<br>63.1[c] |

[a] Mean ± standard deviation. The iodine colorimetric results following ISO 6647-2-2011 (International Standardization Organization, 2011) were obtained from absorbance at 720 nm and correction factor of 85% was used for normal maize, Gelose 50, and Gelose 80 starches.
[b] Line 1 in Fig. 2.
[c] Line 2 in Fig. 2.

Shear scission, the second problem, cannot be avoided in any current SEC set-up for amylopectin, and hence it is impossible to analyze the amylopectin component of starch which has been completely molecularly dissolved without degradation (Cave, Seabrook, Gidley, & Gilbert, 2009). However, shear scission during SEC analysis of the branched starch molecules is expected to affect only the amylopectin component, through selective cleavage near the center of the molecule (Basedow & Ebert, 1977; Cave et al., 2009), and therefore not to affect the branch chain-length distributions of amylopectin that are later obtained in the second SEC analysis after debranching (Liu, Halley, & Gilbert, 2010). As also discussed below, the effect of shear scission on the second-stage SEC analysis, that on the debranched samples, is expected to be negligible, because these are well below the size where shear scission becomes significant.

The third problem is the lack of size standards for the SEC system necessary to analyze the size of starch molecules (pullulan standards being the best currently available for this purpose). This puts an upper bound of $R_h \sim 50$ nm on the quantitative size axis. Extrapolation can be used for larger sizes, but the hydrodynamic volumes so obtained are very sensitive to small fluctuations in the SEC (Vilaplana & Gilbert, 2010a). The size, as radius of gyration $R_g$, can be accurately obtained for these larger sizes using multiple-angle laser light scattering (MALLS), and fortunately there is good evidence (Vilaplana & Gilbert, 2010a) that $R_g$ and $R_h$ are

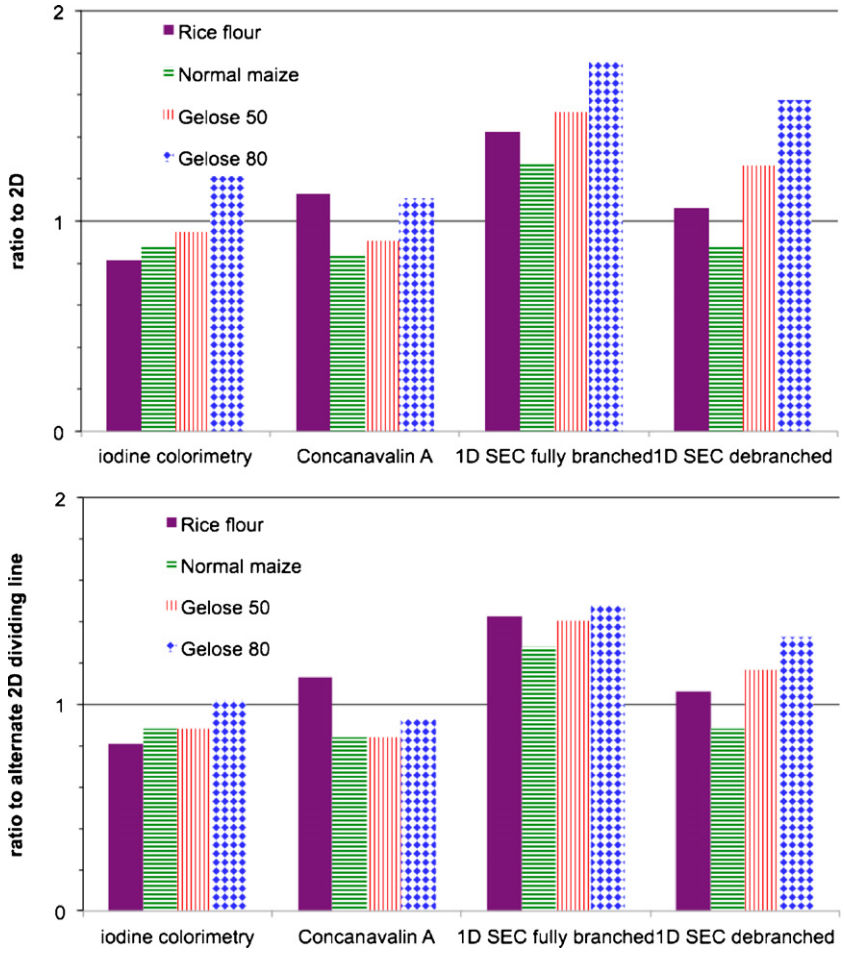


**Fig. 1.** Data of Table 2, as ratios of single-quantity measurements of amylose content to the same quantity obtained by integrating the 2D distributions, which contain much more information. The ratios are presented for the two alternative choices of dividing lines of the amylose and amylopectin peaks, as in Gelose 50 and Gelose 80 starches.

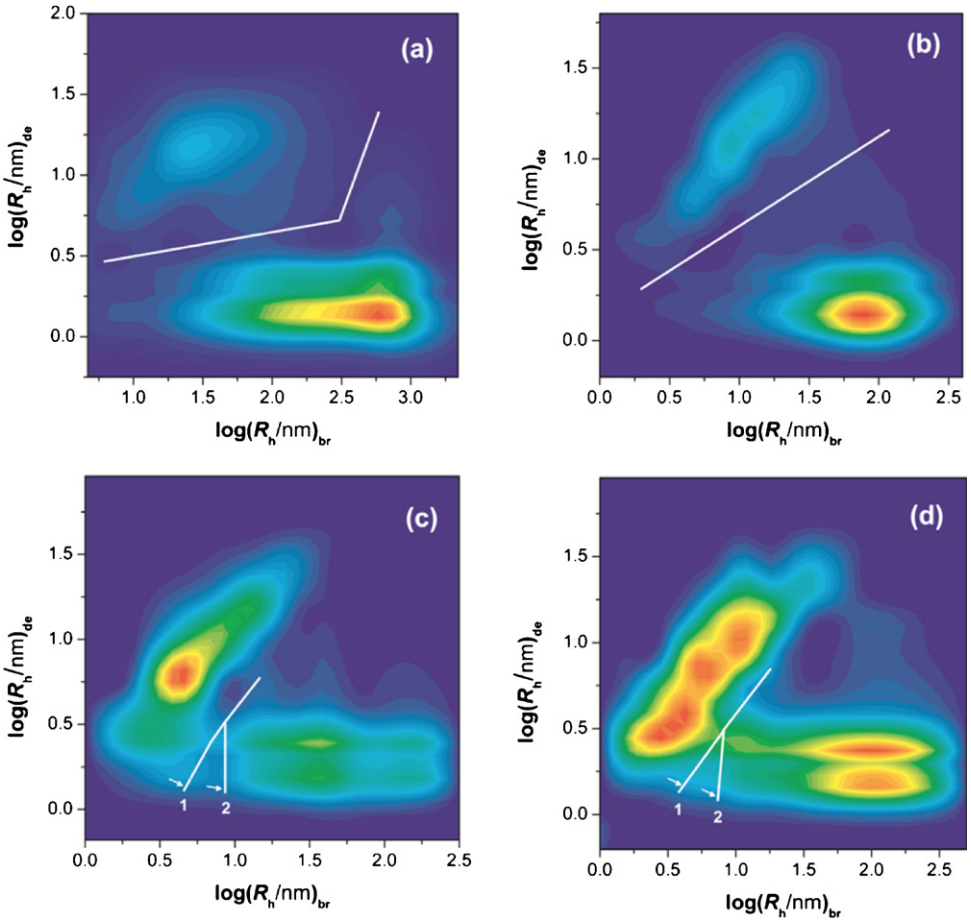


**Fig. 2.** Two-dimensional macromolecular size/branch chain-length distributions for the different starch samples: (a) rice; (b) normal maize; (c) Gelose 50; (d) Gelose 80.

quantitatively similar in the present solvent system (DMSO with 0.5% (w/w) LiBr) and temperature (80 °C). MALLS detection was however not used for the present goals, because although the size axis above ~50 nm is inaccurate, this will have no effect on the actual shapes or on the masses obtained by integration as long as the extrapolations are done consistently. Moreover, the extrapolation procedure has little effect on the AUC of the branched distributions.

Hence the effect of these three SEC problems will not be significant for the present purpose of using the 2D distributions to define and measure amylose content.

The reduction of the detailed information provided by the 2D distributions to a single-quantity measurement, the amylose content, requires choice of a line separating the amylose and amylopectin regions (Eq. (1)). It was deemed best to choose this separating line manually, by assigning the separation line based on the relative minimum values of the 2D distributions. This procedure could be automated for straightforward cases where there is a clear separation between the amylose and amylopectin mountains (e.g. for the rice and normal maize starches in Fig. 2a and b, respectively), and a line along the bottom of the valley between the two mountains can be easily assigned from the unique minimum line separating the two mountains in the 2D distributions. While this is unambiguous for rice and normal maize starches, this is not the case with the high-amylose starches where there is no clear separation between the amylopectin and amylose populations, and alternative minimum lines can be identified in the 2D distribution data (Fig. 2c and d, for Gelose 50 and Gelose 80 starches, respectively). Indeed, for these cases, a number of different choices can be made depending on whether the relative minimum is found on either of the two dimensions (macromolecular size or branch chain-length); these choices are shown in Fig. 2 by white lines.

This ambiguity for high-amylose starches is not unexpected: it is merely a reflection of the fact that the conventional definitions of amylose and amylopectin are not really applicable for these systems, and it is the 2D distributions, rather than simple single-value measurements, which best describe these systems.

Nevertheless, despite this ambiguity, it is clearly useful to use these *complete* 2D descriptions to calculate *approximate* amylose fractions, because this quantity is widely used. However, it is essential to be aware that the value for amylose content cannot be unique.

For this purpose, the numerical integration was performed for two reasonable choices of dividing line, as given in Table 2. For the case of Gelose 50, an amylose content between 50.7% and 54.7% is obtained depending on the position of the different relative minima in the 2D distributions (Lines 1 and 2 in Fig. 2c, respectively); for Gelose 80, the calculated amylose content values range between 53.1% and 61.1% (Lines 1 and 2 in Fig. 2d, respectively). Both dividing lines exclude most, but not all, of the intermediate component, which is small macromolecular sizes (e.g., $1.0 < \log R_{h,br}/\mathrm{nm} < 1.7$) with short branches (e.g., between $0.2 < \log R_{h,de}/\mathrm{nm} < 0.5$), and amylopectin with long branches (e.g., $\log R_{h,de}/\mathrm{nm} > 0.5$) from the AUC of amylose peak.

### 3.3. Iodine colorimetry

Two wavelengths (620 and 720 nm) are recommended for the iodine colorimetry in the ISO 6647-2-2011 (International

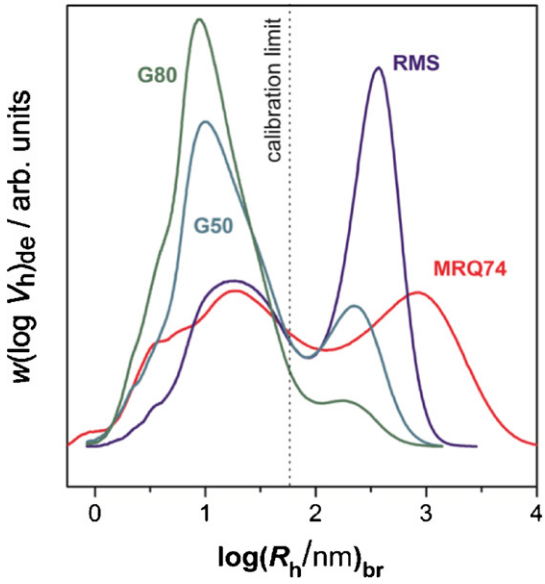


**Fig. 3.** Normalized 1D whole-molecule (fully branched) size distributions from SEC for the different starch samples. Normalization is to the total AUC of the distributions (which are proportional to the total mass). The upper limit of size calibration of the SEC is indicated.

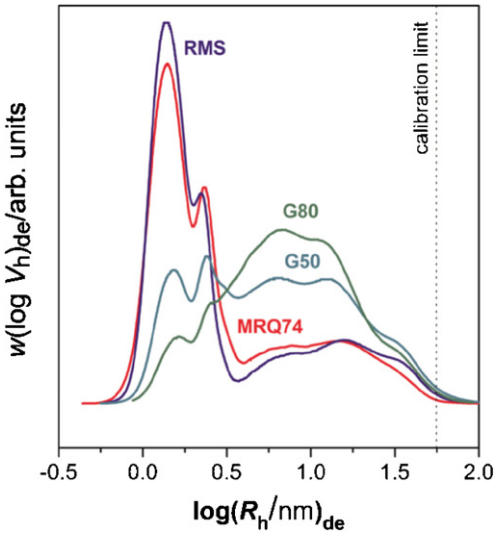


**Fig. 4.** Normalized 1D branch chain-length distributions (debranched size distributions) from SEC for the different starch samples after enzymatic debranching. Normalization is to the total AUC of the distributions (proportional to the total mass). The upper limit of size calibration of the SEC is indicated.

Standardization Organization, 2011). The results from the two wavelengths are summarized in the Supplementary Information. The results from the two wavelengths for the rice flour are similar, but the difference between the two wavelengths becomes larger when the amylose content is higher. Furthermore, the standard deviation from triplicate measurements is also larger when the amylose content of the sample is higher.

Without the correction factor, the results from iodine colorimetry seriously underestimated the amylose content of isolated starches (Supplementary Information) because the calibration graph has corrected the amylose content from the non-starch components in rice flour, which are not present in isolated starch. Hence, only the results from the absorbance at 720 nm and with correction factor of 85% for isolated starches are used for comparison as they are closer to the results from the 2D method than that those at 620 nm and /or without correction factor.

### 3.4. Concanavalin A

Concanavalin A interacts with the non-reducing end of glucan polymers, resulting in associations which can precipitate; the association is least with amylose because there are far fewer reducing ends than in amylopectin (Matheson & Welsh, 1988). Precipitation of amylopectin using concanavalin A allows the determination of amylose in the supernatant by an enzymatic method, the principle of the Megazyme amylose/amylopectin kit. The amylose contents obtained from this method are given in Table 2 and, in an alternate way, in Fig. 1.

### 3.5. 1D whole-molecule size distributions from SEC

SEC data from differential refractive index detection yields the SEC weight distribution $w(\log V_h)$, which is (to within an arbitrary normalization constant) the total weight of polymer in a logarithmic increment of hydrodynamic volume $d \log V_h$, or equivalently the same quantity in terms of the corresponding hydrodynamic radius $R_h$ (because $d \log V_h = d \log 4/3\pi R_h^3 =$ (constant) + (constant)$d \log R_h$). Fig. 3 shows the branched size distributions for the four starch samples considered here. The peaks are assigned to the amylopectin (at $\log(R_h/\text{nm}) > 2$) and amylose populations (at $\log(R_h/\text{nm}) < 2$). The amylose content was calculated from these distributions as the ratio of the AUC of the amylose peak to the total AUC of both amylopectin and amylose peaks; the minimum between the two peaks was taken as the limit of integration to separate the area contributions to the amylopectin and amylose populations (Table 2).

These fully branched SEC distributions suffer from band-broadening and shear scission; as discussed above, the additional problem of calibration for $R_h > 50$ nm is unimportant for the goal of finding total mass ratios. The data of Fig. 3 were obtained under SEC conditions which minimize but certainly do not eliminate both artifacts (Cave et al., 2009). While a number of methods for correcting for band broadening are available (Baumgarten, Busnel, & Meira, 2002; Busnel, Foucault, Denis, Lee, & Chang, 2001; Castro, van Berkel, Russell, & Gilbert, 2005; Chang, Lee, Lee, Park, & Ko, 1999; Grushka, 1972; Hatada, Kitayama, Ute, & Nishiura, 2004; Konkolewicz, Taylor, Castignolles, Gray-Weale, & Gilbert, 2007; Lee, Chang, Harville, & Mays, 1998; Lee, Lee, et al., 1998; Mader & Schnoell-Bitai, 2005; Schnoell-Bitai, 2005; Schnöll-Bitai, 2005; Vega & Schnoell-Bitai, 2005; Yossen, Vega, & Meira, 2006), all of these require further development to be applicable to starch. Both band broadening and shear scission therefore cause overlap of amylopectin and amylose molecules on elution, even in situations (such as is the case for rice and normal maize starches) where these molecules are clearly separated in the 2D distributions (Fig. 2). This will thus introduce unavoidable error into measurement of the amylose content by 1D whole-molecule size distributions from SEC.

### 3.6. 1D branch-length distributions from SEC

The branch chain-length distributions of the starch samples are presented in Fig. 4. Typical distribution patterns are observed for all starch samples, with a bimodal peak assigned to the short amylopectin branches at $\log(R_h/\text{nm}) < 0.7$ (corresponding approximately to DP < 100) and a broader peak for the longer amylose chains at $\log(R_h/\text{nm}) > 0.7$. The separation between the amylopectin and amylose branch peaks is almost baseline-resolved for the normal starch samples (rice and normal maize starches), but some overlap is apparent for the high-amylose starches. The

amylose content was calculated as the ratio of the AUC of the SEC distributions curves for the larger branches to the total AUC for all branches, being the lower limit of integration the minimum of the distributions between the amylopectin and the amylose peaks (Table 2). The impossibility of clearly separating the long-branch region attributed to amylose and the short-branch region of amylopectin in the distributions of high-amylose starches, where overlap is inherent, introduces ambiguities in the inferred value of the amylose content, as in any single-value measurement.

### 3.7. Comparison of the different experimental methods for determining amylose content

The amylose content values vary considerably with different methods (Table 2 and Fig. 1). This variability arises from the different analytical properties that are measured in each case. There is therefore a need to introduce a structural definition of amylose as the small-sized long-chain branched macromolecular populations present in starch. In this case, the 2D macromolecular size/branch chain-length distributions currently offer the clearest distinction between the different macromolecular populations in starch and it is here used as reference method to compare to the other methods for the amylose content.

The amylose content values from iodine colorimetry are in an agreement with those from the 2D method. In general, the iodine colorimetry method gives slightly lower values than the new 2D method, the difference being the largest (about 19%) for the starch in rice flour among the four samples considered in the present study.

The amylose content results from the concanavalin A test offer a reasonable approximation when compared to the values from the 2D distributions. However, amylose can co-precipitate with amylopectin when treated with concanavalin A, underestimating the amylose content (Gibson et al., 1997; Yun & Matheson, 1990) and this effect is also generally observed here when comparing the results with the 2D method.

The amylose content values from the 1D whole-molecule size distributions are evidently higher than those from the other methods. This is attributed to limitations in the separation of branched starch using SEC, as discussed in detail above.

The amylose contents from the 1D branch chain-length distributions for rice and normal maize correspond well with those obtained from the 2D distributions, but are somewhat higher for the high-amylose starches. These are both cases where no clear separation of the branch chain-length distributions is observed between the amylopectin- and amylose-branch populations, as apparent in the 2D distributions.

## 4. Conclusions

The objective of this paper has been to show that two-dimensional (total size/branch length) distributions of starch provide an unambiguous way of determining amylose content, in situations where these 2D distributions show a clear separation of amylose and amylopectin "mountains". In situations ("high-amylose" starches) where the 2D distributions show that no clear separation is possible, the 2D distribution provides a means of quantitatively determining the relative amount of each such component. The 2D distributions also contain significant information about the underlying biosynthetic processes, a clear direction for future research.

Current measurement methods of amylose content often yield different values for this quantity, even when cross-checked against lab, operator and calibration variability. This is ascribed to the fact that different methods measure different physical quantities.

In the comparison between the results of four single-quantity measurements and those from the 2D distribution where it is possible to make unambiguous separation between amylopectin and amylose (rice and normal maize starches being the examples given here), three of these methods, standardized iodine colorimetry, concanavalin A precipitation and 1D branch chain-length distribution, give acceptable agreement with the 2D results, although there is respectively a moderate under- or over-estimate in each of these cases. In each case, the 1D whole-molecule size distribution significantly overestimates the amylose content, due to band broadening and shear scission, both of which are very difficult to avoid with current technology. As seen in Fig. 1, the errors in the single-quantity measurements (compared to 2D reference results) depend on the nature of starch sample: a constant correction cannot be applied.

Where there are substantial amount of hybrid components (the high-amylose starches Gelose 50 and Gelose 80 being the samples used here), all single-quantity methods give significantly different answers from the 2D distributions, and indeed the 2D distributions show that there is no unique way of separating amylose and amylopectin components in these cases. In these cases where hybrid components with smaller macromolecular sizes are present, it is meaningless to attempt to distinguish which branches are originally attributed to almost-linear amylose and which to branched intermediate components, which biases the outcome of the measured amylose contents.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.11.072.

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