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# Absolute molecular weight determination of hypromellose acetate succinate by size exclusion chromatography: Use of a multi angle laser light scattering detector and a mixed solvent

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ABSTRACT

Molecular weight distribution is an important quality attribute for hypromellose acetate succinate (HPMCAS), a pharmaceutical excipient used in spray-dried dispersions. Our previous study showed that neither relative nor universal calibration method of size exclusion chromatography (SEC) works for HPMCAS polymers. We here report our effort to develop a SEC method using a mass sensitive multi angle laser light scattering detector (MALLS) to determine molecular weight distributions of HPMCAS polymers. A solvent screen study reveals that a mixed solvent (60:40%, v/v 50 mM $NaH_2PO_4$ with 0.1 M $NaNO_3$ buffer: acetonitrile, pH* 8.0) is the best for HPMCAS-LF and MF sub-classes. Use of a mixed solvent creates a challenging condition for the method that uses refractive index detector. Therefore, we thoroughly evaluated the method performance and robustness. The mean weight average molecular weight of a polyethylene oxide standard has a 95% confidence interval of (28,443–28,793) g/mol vs. 28,700 g/mol from the Certificate of Analysis. The relative standard deviations of average molecular weights for all polymers are 3–6%. These results and the Design of Experiments study demonstrate that the method is accurate and robust.


## 1. Introduction

The unique features of hypromellose acetate succinate—chemical composition, molecular weight distribution, and associated physicochemical properties—make it ideal for use in a spray-dried dispersion solubilization technology [1–3]. HPMCAS polymer based spray-dried dispersion can increase oral absorption of many poorly water-soluble drug candidates [3]. As molecular weight distribution is an important quality attribute for the spray-dried dispersion, a suitable analytical method is needed to understand and control molecular weight distributions of HPMCAS polymers.

SEC is a widely used technique for determining molecular weight distribution of polymers [4]. Coupling SEC separation with a molecular weight sensitive detector, such as light scattering detector, enables one to directly measure absolute molecular weight distribution of polymer chains in solution. Low angle laser light scattering (LALLS) was on the market since 1970s [5]. Due to the perception that light scattering measurement was difficult and often prone to contamination from dust in the solvents or from column packing shedding, wide use in industrial laboratories was not achieved. The development of MALLS technique since 1980s [6,7], coupled with revolution in computer technology and in improvement of SEC column packing and manufacturing, makes SEC-MALLS combination a preferred method for determining polymer absolute molecular weight distribution today. The principle of MALLS has been reviewed by Wyatt [8] and its application for determining polymer absolute molecular weight has been demonstrated [9–12]. Many applications were focused on utilizing SEC-MALLS to solve specific scientific problems. Some researchers also evaluated certain aspects of analytical procedure for SEC-MALLS method. For example, Jeng et al. and Anderson et al. evaluated instrument precision and accuracy and light scattering equation selection for SEC-MALLS method [13–15]. Tackx and Bosscher evaluated the effect of random noise levels on the calculated molecular weight distribution [16].

For SEC-MALLS method to have wider used in industrial quality control laboratories, proper analytical procedure has to be developed and validation performed in compliance with GMP (Good Manufacturing Practice) regulatory requirements. There is well-established guidance for developing and validating HPLC methods for pharmaceutical applications [17,18]. However, vast majority of HPLC methods is based on measurement of analyte concentration in test solution. It is molecular weight distribution that SEC-MALLS method determines and this makes SEC-MALLS unique in comparison with other HPLC methodologies. Only relative calibration

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method for SEC is covered in USP (US Pharmacopeia) and EP (European Pharmacopeia) [19,20].

In a previous publication, we have shown that neither relative nor universal calibration method works for molecular weight determination of HPMCAS polymers [21]. An advanced mass sensitive detector such as triple detector or MALLS detector is needed for accurate molecular weight determination of HPMCAS polymers. In this paper, we report our effort to develop and validate a SEC-MALLS method to directly determine absolute molecular weight distributions of HPMCAS polymers.

## 2. Experimental

### 2.1. Chemicals and solvents

Polyethylene oxide standard materials (abbreviated as PEOX 20 K and PEOX 30 K) were purchased from Polymer Laboratories, Inc. Amherst, US. The standard reference material of polyethylene oxide (NIST SRM1923) was purchased from National Institute of Standards and Technology, Gaithersburg, MD. Hypromellose (abbreviated as HPMC, CAS 9004-65-3) and hypromellose acetate succinate (CAS 71138-97-1, in three sub-classes of various ratio of acetyl vs. succinoyl derivatization – LF, MF, HF) polymers were purchased from Shin Etsu Chemical Co. Ltd., Tokyo, Japan. Acetonitrile (HPLC grade) and sodium hydroxide (analytical reagent grade) were purchased from J. T. Baker, Phillipsburg, NJ. Water was purified through a MILLIPORE MilliQ system and filtered through a 0.22 μm Millpak filter. The following chemicals were used for the solvent preparation, instrument calibration and column performance check: sodium nitrate (analytical reagent grade), sodium dihydrogenphosphate monohydrate (analytical reagent grade), anhydrous ethylene glycol (99.8%), toluene (99.8%, anhydrous), and anhydrous NaCl (99.999%); all were purchased from Aldrich, St. Louis, MO.

### 2.2. Preparation of normalization, standard, and sample solutions

The normalization solution of PEOX20 K at 5 mg/mL, the standard solution of PEOX30 K at 2 mg/mL, and the sample solution of HPMC or HPMCAS at 2 mg/mL were prepared by adding a weighed amount of polymer into a vial and dissolving it with a measured volume of mobile phase. All solutions were allowed to dissolve at room temperature in the capped vial for 24 h with gentle shaking occasionally. Only the normalization solution was filtered into a HPLC vial through a syringe filter (Whatman Anotop 25, 0.02 μm, 25 mm). The standard solution was directly transferred into a HPLC vial for analysis. The sample solution was transferred into a 15 mL plastic centrifuge tube and centrifuged. Then, a portion of the unperturbed supernatant of the sample solution was transferred into a HPLC vial for analysis. Because light scattering is very sensitive to sample contamination by dirt and dust, all glassware was thoroughly cleaned before use.

### 2.3. Chromatographic condition and run sequence

The SEC-MALLS instrument set-up included a HP1100 HPLC system from Agilent Technologies, Inc. Palo Alto, CA; a DAWN DSP 18 angle laser light scattering detector and a OPTILAB refractive index detector, both from Wyatt Technologies, Inc. Santa Barbara, CA. The analytical size exclusion column (TSK-GEL® GMPWXL, 300 × 7.8 mm, catalog number 08025) was purchased from Toso-Haas, Montgomeryville, PA. The OPTILAB was operated at 35 °C. Both the DAWN and the analytical SEC column were at room temperature (24 ± 5 °C). The mobile phase was a mixture of acetonitrile and the aqueous buffer of 50 mM $NaH_2PO_4$ with 0.1 M $NaNO_3$ (40:60, v/v). The mobile phase was pH adjusted to 8.0 and filtered through a 0.2 μm nylon membrane filter. The flow rate was 0.5 mL/min with in-line degassing. The injection volume was 100 μL and the analysis time was 35 min.

The MALLS data were collected and processed by Wyatt ASTRA software. A suitable analytical procedure was developed that took into consideration regulatory compliance and the well-established practices of HPLC analysis for pharmaceutical use. A representative chromatographic run sequence is given below: B, N1, S1, S2, T1, T2, T3, T4, S2. . . . ., where, B represents blank injection of mobile phase, N1 represents normalization solution; S1 and S2 represent standard solutions one and two, respectively; T1, T2, T3, and T4 represent test sample solutions. No more than 10 sample solution injections were made consecutively before the standard solution was injected again. For every run sequence, duplicate solutions were prepared for the standard and the test samples. Duplicate injections were made for each solution except S1, which was set to three to six injections.

### 2.4. Calibration and normalization

Both the OPTILAB and the DAWN were calibrated periodically according to the manufacturer's recommended procedures and frequency. A 100 μL injection of a 5 mg/mL polyethylene oxide standard (PEOX20 K) was employed for normalizing all angle light scattering detectors relative to 90° detector for each run sequence. Use of this mono-dispersed polymer standard also enabled the volume delay (for our instrument, it was 0.133 mL) between the OPTILAB and the DAWN to be determined, permitting proper alignment of the light scattering signals to the refractive index signal. This is necessary for the calculation of the weight-averaged molecular weight ($M_w$) for each data slice.

## 3. Results and discussions

### 3.1. Solvent screen & use of a mixed solvent: a counter-intuitive but necessary choice

HPMC and three grades of HPMCAS polymers were dissolved in several solvents and their solution behaviors were studied by SEC-MALLS. In the screen experiments, a Waters StyraGel 5E column at 24 °C was used for THF (tetrahydrofuran) solvent. A Polymer Laboratories PLGel Mixed-B LS column at 70 °C was used for both DMAC (dimethylacetamide) and DMSO (dimethyl sulfoxide) solvents. For all these three solvents, the flow rate was set at 1 mL/min and the rest chromatographic parameters were the same as specified in Section 2. For all aqueous buffer and mixed solvents, the chromatographic parameters specified in Section 2 were used. The solutions were prepared at a concentration of 2–5 mg/mL and were clear by visual inspection, except for some floating fibrous materials that were centrifuged down.

Interestingly, the HPMCAS polymers appeared to "dissolve" in solvents of very different properties, for example, in non-polar solvent such as THF, in polar solvents such as DMAC, DMF, and DMSO, and in various aqueous buffer organic solvent mixtures. This is somewhat expected since the HPMCAS polymers are substituted with both hydrophobic and hydrophilic functional groups. However, MALLS showed the subtle differences in solution behaviors of these polymers.

In THF, the light scattering signals started to register near the total exclusion volume and continued tailing after the total elution volume. In contrast, the differential refractive index signal was very weak from the total exclusion volume to the total elution volume, indicating very low concentration of "species" were eluted from the column. THF is not a good solvent for HPMCAS polymers as the polymer chains were not molecularly dispersed in the solvent. As

**Table 1**
Summary of solvent screen results.

| Solvent system | $M_w$ (g/mol) | $M_w/M_n$ | <Rg>$z$ (nm) |
|---|---|---|---|
| | | | *HPMC* |
| DMAC + 0.75% LiCl | 22,090 | 1.54 | 18.8 |
| 8.66 mM $KH_2PO_4$, 30.3 mM $Na_2HPO_4$, pH 7.5 | 67,900 | 2.22 | 50.7 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0 | 21,960 | 1.78 | 21.0 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0 + 0.1% ethylene glycol | 25,410 | 2.08 | 89.9 |
| 80:20% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: Methanol | 25,380 | 1.79 | 18.7 |
| 65:35% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: ACN | 25,440 | 1.67 | 18.6 |
| 55:45% (v/v) 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0: ACN | 21,260 | 1.63 | 14.5 |
| | | | *HPMCAS – LF* |
| DMAC + 0.75% LiCl | 794,420 | 1.79 | 28.3 |
| 8.66 mM $KH_2PO_4$, 30.3 mM $Na_2HPO_4$, pH 7.5 | 271,700 | 2.04 | 55.2 |
| 50 mM $NaH_2PO_4$, 0.3 M NaCl, pH 8.0 | 176,000 | 4.30 | 45.6 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0 | 154,600 | 3.45 | 47.4 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0 + 0.1% ethylene glycol | 131,800 | 3.87 | 45.2 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 8.0 | 158,900 | 3.79 | 47.2 |
| 80:20% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: Methanol | 137,100 | 3.96 | 35.8 |
| 65:35% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: ACN | 135,700 | 4.09 | 46.2 |
| 55:45% (v/v) 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0: ACN | 125,175 | 4.19 | 46.1 |
| | | | *HPMCAS – MF* |
| DMAC + 0.75% LiCl | 169,100 | 2.41 | 26.2 |
| 8.66 mM $KH_2PO_4$, 30.3 mM $Na_2HPO_4$, pH 7.5 | 257,800 | 2.00 | 50.5 |
| 50 mM $NaH_2PO_4$, 0.3 M NaCl, pH 8.0 | 390,500 | 3.71 | 52.5 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0 | 200,400 | 3.42 | 48.5 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0 + 0.1% ethylene glycol | 171,900 | 4.48 | 45.9 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 8.0 | 233,800 | 3.85 | 51.1 |
| 80:20% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: Methanol | 266,000 | 3.13 | 43.4 |
| 65:35% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: ACN | 143,700 | 4.15 | 45.1 |
| 55:45% (v/v) 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0: ACN | 133,820 | 4.41 | 39.9 |
| | | | *HPMCAS – HF* |
| DMAC + 0.75% LiCl | 156,650 | 3.54 | 41.9 |
| 8.66 mM $KH_2PO_4$, 30.3 mM $Na_2HPO_4$, pH 7.5 | 841,900 | 2.30 | 58.7 |
| 50 mM $NaH_2PO_4$, 0.3 M NaCl, pH 8.0 | 1,430,000 | 2.46 | 45.8 |
| 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 8.0 | 916,800 | 3.24 | 58.6 |
| 80:20% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: Methanol | 757,700 | 2.26 | 50.8 |
| 65:35% (v/v) 50 mM $NaH_2PO_4$, 0.1 M NaCl, pH 7.0: ACN | 365,800 | 3.32 | 44.8 |
| 55:45% (v/v) 50 mM $NaH_2PO_4$, 0.1 M $NaNO_3$, pH 7.0: ACN | 249,125 | 4.36 | 47.9 |

A single lot of each polymer was used for the screen experiments except for HPMCAS-HF. For HPMCAS-HF, one lot was used for all solvents except DMAC + 0.75% LiCl. The lot used for DMAC + 0.75% LiCl was subsequently analyzed using the optimized solvent (See Section 2.3) and the $M_w$ is 376,775 g/mol. The same lots except this lot of HPMCAS-HF were also used for the performance test of HPMCAS polymers (LF, MF, HF). $M_n$ is the number weight average molecular weight; <Rg>$z$ is $z$-average root mean square radius [9].

a result, the poorly dissolved polymer chains were probably still entangled together to form a small number of aggregates, which gave a weak MALLS signals but not concentrated enough to give a detectable refractive index signal. Notice that light scattering signal is proportional to $M_w$ (weight average molecular weight) $\times$ $C$ (concentration), whereas the refractive index signal is only proportional to $C$. The fact that the light scattering signal continued to tail after the total elution volume is a result of the aggregates being trapped inside the column and then slowly eluting out by the flow pressure.

DMSO is thought as a universal solvent for many polymers and we found that it (with the addition of 0.75% LiCl) indeed is a good solvent for HPMCAS. Unfortunately, values of $dn/dc$ (change of refractive index per change of concentration) for HPMCAS polymers in DMSO are very small, as DMSO has a large refractive index that is very close to that of the cellulose polymers. This results in very weak MALLS signals, as light scattering signal is also proportional to $(dn/dc)^2$.

The results of SEC-MALLS analysis of HPMCAS and HPMC polymers in other solvents are summarized in Table 1. We see that aqueous buffer alone is not a good solvent for HPMCAS polymers, as the values of measured $M_w$ are in general larger than those in mixed solvents. The best solvent for HPMCAS-LF and -MF is a mixed solvent of aqueous buffer and acetonitrile, while that for HPMCAS-HF is DMAC with 0.75% LiCl. For HPMC, we see that either of the following three solvents works equally well: DMAC with 0.75% LiCl, 50 mM $NaH_2PO_4$ + 0.1 M $NaNO_3$ at pH 7, and the mixed solvent of 50 mM + $NaH_2PO_4$ with 0.1 M $NaNO_3$ at pH 7 and acetonitrile (55:45, v/v).

HPMCAS is a cellulose derivative that has four functional groups of diverse properties (methoxyl, hydroxypropyl, acetyl, and succinyl) substituted along its cellulose backbone. It is the four functional groups that introduce subtle interplay of hydrophobic and hydrophilic interaction sites along the polymer chain. From this study and an earlier study using SEC with a triple detector [21], we believe that both acetyl and succinoyl groups may not be evenly substituted along the HPMCAS polymer chains. This non-homogeneity in the substitution pattern would cause the polymer chains to behave like a block co-polymer in terms of solution behavior. While a "homogeneous" polymer chain such as HPMC in a good solvent would have an extended random coil conformation, an unevenly substituted "heterogeneous" HPMCAS polymer chains would behave like block co-polymer. A particular solvent may be good for dissolving one block, whereas it may not be good for dissolving another block. Thus, the "heterogeneous" HPMCAS polymer chains in solution would form a "Shell-Core" type aggregation. For the "heterogeneous" HPMCAS polymers, the best solvent is a mixed solvent of aqueous buffer and acetonitrile.

We proceeded to optimize the mixed solvent for the analysis of both HPMCAS-LF and MF, which is listed in Section 2, under Chromatographic condition. Representative chromatograms of the HPMCAS-MF polymer are shown in Fig. 1. The mixed solvent of the aqueous buffer and acetonitrile is necessary for the dissolution of HPMCAS polymers. However, its use creates a challenging condition for the SEC-MALLS method that uses refractive index detector as a concentration detector, as the refractive index of a mixed solvent is very sensitive to its composition fluctuation. This fluctuation

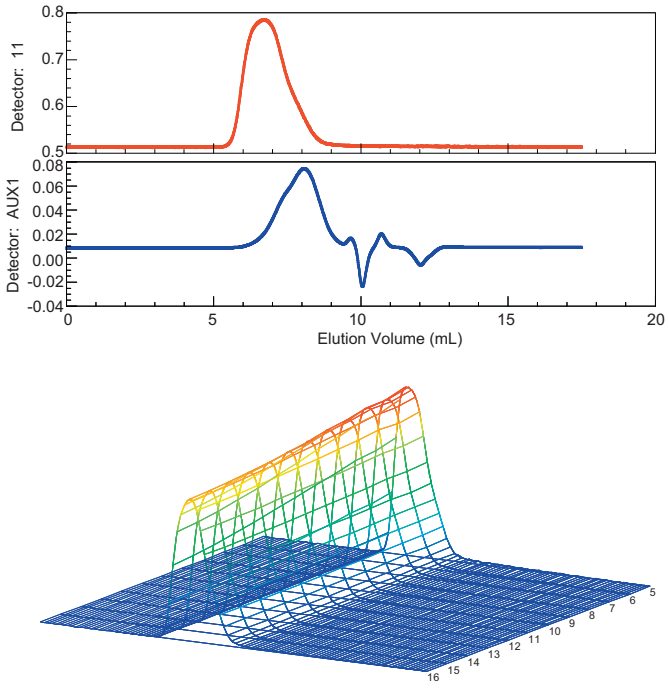


**Fig. 1.** Representative chromatograms of the HPMCAS-MF polymer. The upper trace is the 90° signal from the MALLS detector, the middle trace is the signal from the refractive index detector, and the 3-D plot show the signals from the twelve angles in the MALLS detector.

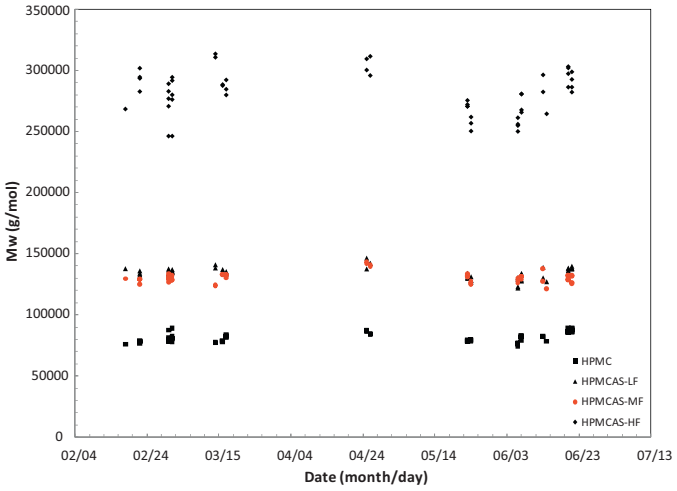


**Fig. 2.** Graphic representations of all experimental data of $M_w$ for the HPMC and HPMCAS polymers used in the method performance evaluation.

would introduce additional measurement error, on top of already vulnerable light scattering detection. Therefore, a comprehensive evaluation of method performance and robustness is needed.

### 3.2. Method validation – evaluation of performance

The purpose of this SEC-MALLS method is to determine the molecular weight distribution, not the analyte concentration, therefore, not all of the performance parameters for HPLC method validation are applicable [17,18]. In the authors' opinion, the minimum relevant parameters for validating a SEC molecular weight determination method should include the following: system suitability, accuracy, precision, and robustness. The use of a well characterized external polymer standard (S1) with multiple injections serves as a system suitability check for instrument precision. The agreement of S1 and a duplicate preparation of the same standard (S2) serves as a check of analyst preparation precision. The measured $M_w$ for S1 is compared to that listed in the Certificate of Analysis of the standard. This serves as an accuracy check, similar to the standard check used for HPLC analysis. In addition, the injection of a mobile phase blank tests for interferences from any other sources.

As long as the signals are within the acceptable ranges of the refractive index and the MALLS detectors, there is no need to validate the linearity range, limit of detection (LOD) and limit of quantification (LOQ), because we measure molecular weight distribution, not the analyst concentration. This is confirmed by an experiment we performed while doing the solvent screen using the last mixed solvent listed in Table 1. In the experiment, we injected four samples of HPMCAS-MF in the concentration range of 0.5–2 mg/mL. The measured peak area from the refractive index signal correlated to the sample concentration linearly with $R^2$ of 0.9999, but the measured $M_w$ remained the same (mean of 133,750 g/mol with 3.0% RSD).

We decided not to use pre-determined $dn/dc$ values in calculating molecular weight distributions; instead, we only used measured light scattering signals, refractive index signal, sample concentration and injection volume for the calculation. Note that all these data were obtained simultaneously from the same single experiment. No requirement of $dn/dc$ value in the calculation is advantageous for the method to be used in daily operation of today's fast-paced pharmaceutical quality control laboratories, as measuring sample $dn/dc$ value requires additional experiment. In addition, the low angle light scattering (Nos. 1–4, 17, and 18) signals were very prone to interference of strait light and were not used in the molecular weight calculation. The Zimm's fitting method was chosen for processing all the data, as Zimm plots have been reported to work equally well in comparison to other fitting methods for middle-sized macromolecules (RMS radius ~20–50 nm) [15].

The measurement accuracy of the SEC-MALLS method was assessed by the PEOX30 K standard, which was used in all run sequences. The statistics for the measured values of $M_w$ are summarized in Table 2. These measurements were made over a period of 9 months. Overall, there were 49 sample preparations and 198 injections. The $M_w$ from the Certificate of Analysis for this lot of PEOX30 K is 28,700 g/mol by static light scattering (without SEC column separation). The measured $M_w$ mean has a 95% confidence interval of (28,443–28,793) g/mol, proving that the method is capable of making accurate measurement of molecular weight distribution.

The measurement precision is better assessed by the percent relative standard deviation (%RSD) calculated from all measurements for each polymer type. The percent relative standard deviation is the most used statistical parameter for analytical method evaluation in validation. In Fig. 2, all experimental data for HPMC, HPMCAS-LF, MF, and HF polymers are shown to assist visual apprehension of variability of the repeated measurements. The complete numerical data set is in the Supplement data set. These measurements were made from different sample preparations, different injections, different mobile phase preparations, and different (three) columns over a period of 5 months. The statistics of these measurements are summarized in Table 2. It can be seen that the %RSD of HPMC measurements (4.9%) is comparable to that for PEOX30 K (4.4%), while the %RSD for both HPMCAS-LF and MF polymers (3.5% and 3.4%, respectively) are lower than that for PEOX30 K. The relatively high %RSD for HPMCAS-HF (6.3%) is likely related to the properties of this type of polymer [21], as the current mixed solvent is not the best solvent for this type of polymer (See discussions in the previous subsection). Nevertheless, the level of %RSD (3–6%) achieved by this method using a mixed solvent is very impressive,

**Table 2**
Descriptive statistics of the measured weight average molecular weights ($M_w$ in g/mol) for PEOX30 K, HPMC, and HPMCAS polymers.

| | PEOX30 K | HPMC | HPMCAS-LF | HPMCAS-MF | HPMCAS-HF |
|---|---|---|---|---|---|
| Mean | 28,618 | 81,403 | 134,145 | 130,458 | 281,463 |
| Median | 28,300 | 80,165 | 134,400 | 129,850 | 282,550 |
| Minimum | 26,600 | 74,640 | 122,100 | 121,400 | 246,300 |
| Maximum | 32,300 | 89,110 | 146,200 | 143,700 | 313,500 |
| Standard Deviation | 1249 | 3950 | 4696 | 4452 | 17,618 |
| %RSD | 4.4 | 4.9 | 3.5 | 3.4 | 6.3 |

The HPMC lot used for the performance test was different from the one used for solvent screen. For HPMCAS (LF, MF, HF) polymers, the same lots used for solvent screen were used for the performance test.

considering that size exclusion chromatographic separation is in general less robust than the reverse phase liquid chromatographic separation and a more complex and vulnerable multi angle laser light scattering detector was used.

### 3.3. Method validation – evaluation of robustness

Robustness of the method was evaluated using DOE (Design of Experiments). The five variables under investigation were mobile phase pH, acetonitrile content in the mobile phase, buffer ionic strength, flow rate, and sample concentration. Analysis of variance (ANOVA) was used to determine the impact of the five variables to the measured weight average molecular weights. Note that instrument-to-instrument and analyst-to-analyst variations were not included in the study, as they were mainly related to the method transfer. In addition to the five polymers that were used previously for method performance evaluation, we also included a NIST Standard Reference Material for polymer molecular weight determination, SRM1923, in the robustness study, as it was the most accurately measured standard available.

The details of the experimental design and the experimental data are included in the Supplement data set. The analysis is explained below for each polymer type. For SRM 1923 and HPMC, no variables are deemed statistically significant in affecting changes in $M_w$. Although the most statistically significant variables that affect PEOX 30 K are flow rate, acetonitrile percentage, and sample concentration, the magnitude of changes is very small (overall span 2.81–3.13 $\times$ $10^4$ g/mol). The most statistically significant variables that affect $M_w$ measurements of HPMCAS polymers are pH (for all grades) and acetonitrile percentage (only for LF and HF). As pH increases, values of $M_w$ for all grades decrease. As the acetonitrile percentage increases, the value of $M_w$ for HPMCAS-LF decreases and that for HPMCAS-HF increases discernibly. The somewhat bigger impact of acetonitrile content to the measured $M_w$ of HPMCAS-HF polymer is likely related to the properties of this type of polymer [21], as the current mixed solvent is not the best solvent for this type of polymer. In all other cases, the impact of these statistically significant factors to the measured $M_w$ of each polymer (PEOX30 K, HPMCAS-LF, MF, and HF) is practically insignificant as compared to the variations from other sources (sample preparation, injection, column-to-column, mobile phase preparation, etc.). The method is robust within boundaries covered by the evaluated five variables.

## 4. Conclusions

A SEC-MALLS method was developed to directly determine the absolute molecular weights of hypromellose acetate succinate. Because HPMCAS has four functional groups of diverse properties that introduce both hydrophobic and hydrophilic interaction sites along the polymer chain, dissolution of HPMCAS polymer is a no small task. A solvent screen revealed that the best solvent for HPMCAS-LF and -MF is a mixed solvent of aqueous buffer and acetonitrile (60:40%, v/v 50 mM $NaH_2PO_4$ with 0.1 M $NaNO_3$ buffer: acetonitrile, pH* 8.0), while that for HPMCAS-HF is DMAC with 0.75% LiCl. Use of a mixed solvent creates a challenging condition for the SEC-MALLS method that uses refractive index detector as a concentration detector. Therefore, we validated the method with the consideration of uniqueness of SEC (measuring the molecular weight distribution, not the analyte concentration), and used statistical tools to analyze the data. The measured mean $M_w$ of PEOX30 K standard has a 95% confidence interval of (28,443–28,793) g/mol vs. 28,700 g/mol from the Certificate of Analysis. The relative standard deviations (%RSD) for the polyethylene oxide standard, the hypromellose, and the HPMCAS three grades are in the range of 3–6%, which is very impressive, considering that SEC separation is in general less robust than the reverse phase liquid chromatographic separation and a more complex and vulnerable multi angle laser light scattering detector was used. These results and the Design of Experiments study demonstrate that the method is accurate and robust for determining HPMCAS absolute molecular weight distributions.

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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.jpba.2011.07.035.

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