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Evaluation of Candidate International Standards for Meningococcal Serogroup W and Y Polysaccharides

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****Full details of MenW/MenY IS Working Group in Annex 1***

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **8 October 2018** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

Until the introduction of International Standards for MenC, MenA and MenX polysaccharides, the standardization of measurement of polysaccharide content within these vaccines was problematic. This was due to the variety of methods and standards employed by different manufacturers and control laboratories for use in the physicochemical assays to test polysaccharide or conjugate vaccines. For determining the MenW and MenY polysaccharide content in vaccines, these problems remain.

For this study, two candidate International Standards for meningococcal capsular group W (MenW) and Y (MenY) polysaccharides were assessed for their suitability as quantitative standards in various physicochemical assays. The intention is that these standards will be used to standardize the quantification of the respective polysaccharide content in meningococcal polysaccharide (conjugate) vaccines and their intermediate components. Twelve laboratories from eleven different countries participated in the collaborative study of candidate International Standard MenW and MenY polysaccharide preparations (coded 16/152 and 16/206, respectively).

Our proposals, on the basis of quantitative (qNMR) data from this study, are 1) candidate standard for MenW polysaccharide (16/152) to be assigned a content of 0.925 ± 0.138 mg MenW polysaccharide per ampoule (expanded uncertainty with coverage factor k = 2.36, corresponding to a 95% level of confidence) and 2) candidate standard for MenY polysaccharide (16/206) be assigned a content of 0.950 ± 0.122 mg MenY polysaccharide per ampoule (expanded uncertainty with coverage factor k = 2.36, corresponding to a 95% level of confidence).

In accelerated thermal degradation studies a decrease in molecular size of the polysaccharide was observed after storage of the lyophilized material at 37°C and 56°C. The candidate material stored at lower temperatures, -70°C, -20°C or 20°C, was considered stable. The amount of polysaccharide per ampoule remained consistent under all conditions over a 12-month period. Real time stability and accelerated thermal degradation studies are on-going.

Introduction

Meningococcal polysaccharide conjugate vaccines have been in use for over 20 years, prior to this, plain polysaccharide vaccines had been available. The first meningococcal conjugate vaccine only contained one polysaccharide serogroup, MenC, but since then MenA, MenW and MenY have been added to produce tetravalent formulations, with MenX conjugates also in development.

Invasive meningococcal disease is a burden worldwide causing mortality and morbidity, particularly in infants. With the incidence of MenC disease controlled in many countries through routine vaccination, remaining disease is largely caused by other serogroups including MenB, MenW and MenY. In recent years there has been an increasing focus on

disease caused by MenW isolates, particularly notable in older age groups. In Europe an exponential increase in cases of MenW disease started in 2009 [1], which led to the introduction of the MenACWY conjugate vaccine in a number of countries. Historically, disease caused by serogroup A was the main problem for the countries of the African meningitis belt; this was until the introduction of the MenA conjugate vaccine. Since then epidemic meningitis in the region is predominately caused by MenW, MenX, MenC and *Streptococcus pneumoniae* albeit in varying proportions from year-to-year [2,3,4]. In recent years, outbreaks of MenW disease have increased globally, in particular in South American countries [5,6]. Although MenY features as less of a global disease burden, epidemiology is closely monitored in Europe [7] and MenY remains the third leading cause of meningococcal disease in the United States of America, after MenB and MenC [8]. The epidemiology of the different serogroups across different geographic regions is notable and the patterns are ever-changing, thus prevention through vaccination against as many groups as possible remains the most effective way to control the disease.

With several manufacturers producing polysaccharide or polysaccharide conjugate vaccines there has been a continued need to harmonize the measurement of polysaccharide content within these products. The measurement of polysaccharide content and, in the case of the conjugate vaccines, the amount of polysaccharide bound to the carrier protein, are the accepted correlates of potency. Correctly measuring both total and free polysaccharide content is imperative as part of the quality control process both by manufacturers and National Control Laboratories (NCLs). Both upstream components of vaccine production, such as bulk (conjugated) polysaccharide and final filled vaccine samples are tested routinely as part of in-process and quality control.

MenW and MenY polysaccharides are both composed of repeating disaccharide units of either sialic acid and galactose (Gal) for MenW →6-O-α-D-Gal*p*-(1→4)-α-D-NeuAc-2→ or sialic acid and glucose (Glc) for MenY →6-O-α-D-Glc*p*-(1→4)-α-D-NeuAc-2→. The degree of acetylation of sialic acid is variable at C7 and/or C9 [9]. Determination of polysaccharide content is primarily by physico-chemical assays using polysaccharide, sialic acid, glucose or galactose standards. Commonly used assays include the Resorcinol assay for the measurement of sialic acid content [10] and High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) [11].

Unitage assignment and uncertainty of World Health Organization (WHO) International Standards that are defined in Système International d'Unités (SI units) should be derived from and traceable to a physicochemical method, rather than a bioassay. The first polysaccharide International Standards for *Haemophilus influenzae* type b (Hib) and meningococcal serogroup C polysaccharides were assigned in SI units, using secondary physicochemical methods [12,13,14]. A primary method is one “whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of SI units, and whose results are, therefore, accepted without reference to a standard of the quantity being measured” (Bureau International des Poids et Mesures [15,16]. Since the MenC and Hib polysaccharides were assigned unitage using secondary methods, qNMR has been proposed as a suitable primary method for the quantitation of organic compounds, whereby the unitage is derived from the traceability of the qNMR standard to the SI unit. [17]. Of crucial importance is that the internal standards used have defined purity and uncertainty and are SI-traceable. Such standards can be termed primary certified reference materials (CRMs) and the production of which is a growing field [17,18]. MenA, MenX and Vi (for typhoid) polysaccharide International Standards were assigned unitage using qNMR. [19,20]. Furthermore, the MenW and MenY polysaccharides have been extensively characterized by ^{1}H-NMR [21,22]. The proposal to assign unitage based in qNMR to

candidate polysaccharide standards for MenW and MenY for their intended use as primary calibrators of secondary in-house standards in physical chemical assays was endorsed by the Expert Committee on Biological Standardization (ECBS) in 2015 [23].

We also report on the stability of the candidate standards. Stability was assessed by studies in real-time, accelerated thermal degradation and of the reconstituted material.

Participants

The MenW and MenY IS Working Group was formed to complete the collaborative study and comprised twelve laboratories from eleven countries (Annex 1). A code number was assigned to each laboratory, in no particular order and does not correspond to the order given in Annex I. These organizations included eight NCLs, two vaccine manufacturers, one Pharmacopoeial laboratory and one university. Seven laboratories performed qNMR spectroscopy, six laboratories performed HPAEC-PAD, six laboratories performed the Resorcinol assay and one laboratory each performed the HPLC, Anthrone and Nephelometry assays.

Candidate Materials and Preparation of Candidate International Standards

Lyophilized purified bulk polysaccharide material was received from GSK Vaccines S.r.l. to be used for evaluation of suitability as International Standards for use in quantitative assays. The polysaccharides were stored at -70°C until required. Data from the Certificates of Analysis provided by the manufacturer are shown in Table 1. All results were compliant with the in-house specifications set by the manufacturer. For this study the repeating unit of the polysaccharides was calculated to have a functional weight of 514.465 g/mol based on a fully *O*-acetylated calcium salt of the materials. Following accurate determination of the *O*-acetylation content of each standard, the functional weights were adjusted (see Annex 2).

Based on previous studies of candidate materials for other meningococcal polysaccharide serogroups, MenC, Men A, and MenX, trial fills of the material were performed with reconstituted polysaccharides in water. Stable plugs of material were formed after lyophilization and the decision made to proceed to definitive fill with the same formulation.

For preparation of the candidate International Standards, the lyophilized polysaccharides were added to MilliQ (deionized 18.2 M Ohm) water and reconstituted by stirring for at least 4 hours at room temperature before transferring to 4°C overnight. The polysaccharides were filled the following day by the Centre for Biological Reference Materials (CBRM) in 2016.

The bulk material was stirred at 4°C during the filling process and was dispensed in 1.0 ml volumes into 5 ml DIN glass ampoules (Adelphi tubes, Haywards Heath, UK) on a Bausch and Strobel AFV 2090 ampoule filling/sealing machine. The ampoules were then partially stoppered and freeze dried.

The ampoules were dried in the CS100 freeze drier (Serail, Le Coudray Saint Germer, France) using a 4-day cycle (FD0076 V03). Filled ampoules were loaded onto shelves pre-cooled to 4°C and then frozen to -50°C over a period of 2 hours. This temperature was held for 2 hours before a vacuum was pulled to 100 µbar. The shelf temperature was then raised to -20°C and was maintained for 30 hours for primary drying. The temperature was then ramped to 25°C and held for another 30 hours at a vacuum of 30 µbar for secondary drying. Once lyophilization was complete the freeze drier chamber was back-filled with nitrogen and the

stoppers fully inserted before removing them from the freeze drier. The ampoules were then flame-sealed.

Assessments of coefficient of variation (CV) of fill, headspace oxygen concentration, residual moisture, bacterial and mould/yeast colony counts were performed; the results of which are given in Table 2. The material is not infectious. Three thousand ampoules each of 16/152 (MenW) and 16/206 (MenY) are offered to WHO. NIBSC will act as the custodians of the materials. Both materials are stored at -20°C at the CBRM, NIBSC.

Study design

The study comprised two parts. The aim of Part 1 was to assign unitage to the candidate MenW and MenY polysaccharide standards by qNMR and to measure the degree of *O*-acetylation of both polysaccharides. Unitage was measured in milligrams of polysaccharide per ampoule by participants using their own method for qNMR. Participants were requested to measure the polysaccharide content of four ampoules of each polysaccharide (two ampoules tested on two different days). Participants using other methods were also asked to assign unitage to the candidate standards although these data were used for comparison purposes only. The candidate MenY standard was labelled as CS581 Standard 1 and the candidate MenW standard was labelled CS581 Standard 2. The identity of the MenW and MenY candidate standards was not revealed to the participants at the time of the study but could be determined by appropriate methods.

The aim of Part 2 of the study was to assess the fitness for purpose of each candidate standard by comparing the use of in-house standards and the candidate International Standards in the different methods. Participants were asked to measure the MenW and MenY polysaccharide concentration of three liquid samples, labelled A, B and C, using candidate standards and their in-house standards. The candidate standards, labelled appropriately as CS581 Candidate IS MenW and CS581 Candidate IS MenY, were supplied lyophilized alongside the test samples and assigned an arbitrary unitage of 1 mg/ml. The identity of each test sample was unknown to the participants at the time of the study. Sample A was a MenW bulk conjugate, Sample B was a MenA polysaccharide and Sample C was a MenY bulk conjugate sample. Participants were supplied with four microtubes of each sample and asked to test each one using the relevant standard in four separate assays. The polysaccharide concentration was reported in µg polysaccharide/ml.

Methods

Quantitative ^{1}H-NMR

Quantitative ^{1}H-NMR (qNMR) relies on different hydrogen-1 nuclei resonating at specific frequencies when a magnetic field is applied. Integration of the integrals is calculated for each resonance for the sample and standard. Peak intensity, after normalization for the number of protons for the relevant nuclei, is proportional to the concentration of the sample. Sample concentration can therefore be calculated from the known quantity and purity of the internal standard.

Different internal standards (TSP-d_4, maleic acid, caffeine and ascorbic acid and DSS-d_6) as well as instruments (magnetic field from 400 to 700 MHz) were used across the different NMR laboratories, the details of which are presented in Table 3. Where appropriate, data was amended to account for the purity of the standard used.

The laboratories used a number of different methods to perform qNMR, using different internal standards, listed above and different peaks to integrate. Participants 1, 6, 4 and 2 all

used sodium deuterium oxide to determine the % of *O*-acetylation. Participant 2 used the ratio of N-acetyl (N-Ac) to free acetate to determine the % of *O*-acetylation, as well as H3 of NeuNAc to determine the total amount of polysaccharide. Participants 1 and 6 used the ratio of Gal/Glc-H1 to free acetate to determine the % of *O*-acetylation of the polysaccharides [22], both laboratories used Gal/Glc-H1 to determine the total amount polysaccharide in the ampoules. Participant 4 used the ratio of N-Ac to acetate to determine the percentage of *O*-acetylation and the combination of H3 of NeuNAc and a range of peaks between 3.4 to 4.1 ppm, which encompasses 13 protons, to determine the absolute mass of polysaccharide. Participant 5 used H3 of NeuNAc to determine the absolute amount of polysaccharide and the ratio of H7 and H9 of H7(OAc) and H9(OAc) to H3 of NeuNAc to calculate the % of *O*-acetylation. Finally, Laboratory 7 used a method to remove possibly overlapping signals from H1 to determine the absolute mass of polysaccharide, while they used H7, H9 and H9' compared to H1 of Gal/Glc to calculate the % of *O*-acetylation.

To ensure full solubilization of the material, participants were requested to reconstitute the material 24 hours before use, firstly for 2 hours at room temperature and then at 4°C for the remaining period.

NMR data was reevaluated by a single analyst at NIBSC using the following approaches. Where the material was untreated by NaOD the peak used to determine the mass of the material were H1 of the polysaccharide. Percentage *O*-acetylation was determined using the sum of the peak areas of H7 and H9', this value was divided by the area under the signal for H1. Where the polysaccharides were treated with NaOD, the percentage of *O*-acetylation was determined using the area under the free acetate and H1 signals, the former being divided by the later. It was observed that the data provided, even within participant datasets, varied in quality. During this analysis by a single analyst the upmost care was taken to handle the spectra consistently, when processing the spectra and integrating the peaks in question.

NIBSC performed an extended study on nine and ten ampoules of the MenW and MenY candidate standards, respectively; the data from which was used in part to determine the uncertainty of measurement.

HPAEC-PAD and other HPLC-based methods

The weakly acidic nature of the carbohydrates makes them suitable candidates for separation by anionic exchange at high pH. The carbohydrate sample is first hydrolyzed with strong acid before the resultant monosaccharides are separated by anion exchange chromatography [11]

Six participants performed High Performance Anion Exchange Chromatography with a pulsed amperometric detector, HPEAC-PAD, (Participants 5, 6, 7, 8, 10 and 11). All laboratories used TFA (trifluoroacetic acid) hydrolysis for the depolymerization of MenW or MenY prior to chromatographic analysis. Acid hydrolysis allows for the measurement of the saccharide repeating unit, of partly *O*-acetylated alternating units of NeuNAc and D-Gal (linked with α(2→6) and α(1→4) glycosidic bonds) for MenW or of partly *O*-acetylated alternating units of NeuNAc and D-Glc (linked with α(2→6) and α(1→4) glycosidic bonds) for MenY. Participant 10 submitted data for Part 1 of the study with results from HPAEC-PAD assay using either Glc/Gal standards, as well as MenW and MenY polysaccharide standards. If MenW or MenY purified polysaccharides were used as a standard (Participants 5, 6, 10 and 11) the polysaccharide content can be directly measured. For laboratories which used Gal (for MenW) or Glc (for MenY) as a standard (Participants 7, 8 and 10) a conversion factor is required. All laboratories used CarboPac columns (PA1, PA10 or PA100) and resolved the relevant peaks using NaOH and sodium acetate, with the exception of Participant 10, where only NaOH was used. Further details for each laboratory are listed in Table 4.

Noticeable differences include the standards used and the milder acid treatment performed by two of the laboratories (7 and 8).

Participant 9 used a slightly different HPLC method, making use of the Ludger Tag 2-AA Monosaccharide Release and Labeling Kit [24]. Like other methods, saccharides were hydrolyzed with 2 M TFA. The resultant monosaccharides have a free reducing terminus which is labelled with a fluorescent tag – 2-aminobenzoic acid (2-AA). These labelled monosaccharides are separated by HPLC. Fluorescence peaks for test samples are then compared to a standard curve of monosaccharide concentration versus fluorescence. The standard curve is composed of a mixture of glucosamine hydrochloride, galactosamaine hydrochloride, galactose, mannose, fucose and glucose.

Resorcinol assay

The resorcinol assay is the current European Pharmacopoeial method for the determination of sialic acid content and is therefore a commonly used method for the determination of MenC, MenW and MenY polysaccharide content in meningococcal vaccines [10,25]. It is a colorimetric assay used to measure sialic acid content after acid hydrolysis of the sample. Sialic acid reacts with the resorcinol reagent in the presence of copper sulphate and hydrochloric acid when incubated at >100^{o}C for 15-30 minutes. The resultant blue colour can be quantified by measuring absorption at 564-585 nm and concentration is then determined through comparison to a standard curve. Six laboratories performed the resorcinol assay (Participants 4, 6, 7, 10, 11 and 12) and details are listed in Table 5 All the laboratories except laboratory 12 used a sialic acid standard and therefore require a conversion factor to quantity the polysaccharide content. Noticeable differences between the laboratories include the standard used and the use of extractants (butyl acetate:butanol) (Participants 7, 10 and 11).

Anthrone assay

The anthrone assay is a colorimetric method relying on the condensation of furfurals with anthrone under acidic conditions to form a blue-green compound [26]. The carbohydrate content is estimated through the reading absorbance at 620 nm and comparison against known concentrations of a standard curve. Furfurals, or hydroxymethylfurfurals are derived through the hydrolysis of carbohydrates, producing constituent pentose or hexose monosaccharides. The one laboratory performing the anthrone assay used glucose as a standard. Additional details of the method are given in Table 5, alongside the Resorcinol methods.

Nephelometry assay

The nephelometry assay measures the turbidity of a suspension of immune complexes formed, in this case, by the mixing of meningococcal polysaccharide samples with specific antibody [27]. When the specific antibody is added to the sample, scattered light is measured at an angle of 90°C to the light that is passed through it. Participant 10 used in-house MenW or MenY PS standard to prepare the standard curve for rate nephelometry. Standards and samples were diluted in water to desired concentration then MenW or MenY antiserum was added for reaction. Sample rate response gained through two minutes reaction time is interpolated on the standard curve to determine polysaccharide concentration for each serogroup, using the Beckman Coulter IMMAGE 800 rate nephelometer. The final reportable result is the average of the calculated polysaccharide concentrations (n=2). The results are listed in Table 11.

Stability study

Three stability studies of the candidate materials were undertaken: a real-time stability study for content of lyophilized material, an accelerated thermal degradation study for content of lyophilized material, and an accelerated thermal degradation study of the reconstituted material. The stability studies were performed at NIBSC. The MenW and MenY polysaccharide contents were determined using the HPAEC-PAD assay and the molecular integrity by High Performance Liquid Chromatography with Size Exclusion Chromatography (HPLC-SEC). The pH was also checked for all stability samples.

Monitoring of the real-time stability of the samples stored at -70^{o}C and -20^{o}C took place at 1, 2, 3, 6, 12, 24, 36, 60 and 120-month time-points. The accelerated degradation study of the candidate standards was planned at temperatures of +20^{o}C, +37^{o}C and +56^{o}C for 1, 2, 3, 6, 12, 24 and 36 months. Data are presented from real-time and accelerated stability samples stored up to 12 months for both MenW and MenY.

In the final study, the candidate standards were reconstituted in 1 ml of sterile distilled water and stored at -20^{o}C for 1, 2, 3, 6, 12, 18, 24 and 36 months (data shown up to 18 months).

The MenW or MenY polysaccharide content was determined using HPAEC-PAD with a CarboPac PA-1 analytical column. An in-house MenW or MenY polysaccharide was used as a quantitative standard (0.5–27 µg/ml). For the quantitative analysis, samples and standards were subjected to acid hydrolysis with 2.0 M TFA, for 2 h at 100^{o}C. An identical amount of L-Fucose (Sigma) was added to all standards and samples as an internal spike for normalization prior to chromatography. The eluents were mixed with a gradient pump to achieve 0–18 min, 15 mM NaOH; 18–26 min, 100 mM NaOH, 80 mM NaOAc (sodium acetate); 26-31 min, 400 mM NaOH; and, 31–41 min, 15 mM NaOH. The flow rate was 1 ml/min. The content of MenW was determined by integrating the peak arising from galactose or for MenY glucose relative to the standard curve, with signal normalized by the internal spike.

Molecular sizing analysis was performed using a Dionex U3000 HPLC system with Tosoh Bioscience TSK gel G6000 + 5000 PW_{XL} columns in series preceded by a PW_{XL} guard column. The mobile phase was phosphate buffered saline 'A' pH 7.4 at a flow rate of 0.25 ml/min. The void and total column volumes were determined using salmon DNA (Sigma) and tyrosine (Sigma), which eluted at 45 min and 100 min respectively. The 214 nm signal (Dionex VWD multi-wavelength UV Detector) was used for determining the percentage eluting at a distribution coefficient (K_D) of 0.32 for MenW and 0.29 for MenY.

Assessment of stability study samples is on-going, but at the time of writing this report, data was available for up to 18 months.

Conversion Factors

Where the concentration of Glc, Gal or NeuNAc was measured in samples using these in-house standards, the polysaccharide content was derived by applying a conversion factor of the 497.020 (for MenW PS) or 494.110 (for MenY PS) divided by the functional weight of glucose/galactose (180.156) or sialic acid (309.270). Data from participants was amended with this conversion if data had been submitted with another conversion factor applied. Participants were asked to consider moisture content of their in-house standards. One laboratory accounted for 10% moisture in their in-house polysaccharide standard when making up their standard curve for their HPAEC-PAD assay. Values obtained by qNMR were also adjusted where purity of the standard was less than 99%.

Statistical analysis

Where appropriate Dixon's Q test was applied to identify statistical outliers. Analysis of the molecular size distribution was used as a measure of stability of the candidate standards. Expressing the sizing data (% eluting before K_D 0.32 for MenW or 0.29 for MenY) relative to ampoules stored at -70°C, an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay [28] was used to predict the degradation rate when stored at -20°C.

Results

Although related, HPAEC-PAD and the HPLC method performed by Participant 9 were considered different methods for the purposes of data analysis.

Content assignment using Quantitative ^{1}H-NMR

NIBSC performed an extensive analysis of additional ampoules of each candidate standard by qNMR. Data for this study are given in Table 6 For the MenW candidate standard the mean content from ten ampoules was 944 µg MenW polysaccharide per ampoule (CV=2.0%). For the MenY candidate standard the mean content from nine ampoules was 993 µg MenY polysaccharide per ampoule (CV 1.5%)

For the main study, seven laboratories performed qNMR to determine polysaccharide content of four ampoules each of the candidate standards. Different internal reference compounds were used by the various laboratories performing qNMR: three laboratories used trimethylsialyl propionate (TSP), three laboratories used maleic acid and one laboratory each used either caffeine or 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). There appeared to be no correlation between the measured polysaccharide content and the compound used as the internal reference.

Estimates of polysaccharide content per ampoule for candidate MenW and MenY standards. Data obtained for MenW and MenY was excluded from two laboratories; Participant 3 only provided partial data (data submitted for two ampoules of each candidate standard) and there was high variation between the content measured for the four ampoules tested by Participant 5.

The mean polysaccharide content of the MenW candidate standard, as measured by qNMR by individual participants, was 865 µg/ampoule (CV=11.6%), and for the candidate MenY standard, 877 µg/ampoule, CV=13.3%)(Tables 7a and 8a).

The uncertainty of measurement determined for candidate MenW and MenY standards is presented in Tables 9 and 10, respectively. The major contributor to the uncertainty of the mean polysaccharide content was determined to be between-laboratory variability; 5.17% for MenW and 5.96% for MenY (Tables 9a and 10a). Participants were consulted on a draft report, and a decision was taken to reevaluate the data obtained by qNMR using a single analyst at NIBSC.

The mean polysaccharide content of the MenW candidate standard, as determined from the re-analysis, was 925 µg/ampoule (CV=14.6%), and for the candidate MenY standard, 950 µg/ampoule, CV=12.7%)(Tables 7b and 8b). The revised uncertainty of measurement based on the re-evaluation of the data was 6.31% for MenW and 5.42% for MenY (Tables 9b and 10b).

It was noted that laboratories using NMR spectrometers with higher field strength magnets (700 and 600 MHz) obtained higher values for polysaccharide content than those with 500 or 400 MHz spectrometers, when the data was analysed by the individual participants. This is likely due to the higher sensitivity of such instruments and the greater spectral resolution afforded by the greater field strengths which produce peaks with better resolution, thereby improving the integration that would otherwise be achieved from broader signals. However, it was later noted that when data was re-analysed by a single NIBSC analyst, the lowest estimates of polysaccharide content were obtained from Participants 2 and 7 which used 500 MHz instruments.

The coverage factor was determined using the Welch-Satterthwaite approximation for effective degrees of freedom and corresponds to a 95% level of confidence. Taking this uncertainty into account the final estimate of content for 16/152 is 0.925 ± 0.138 mg MenW polysaccharide per ampoule; expanded uncertainty with coverage factor k = 2.36. The final estimate of content for 16/206 is 0.950 ± 0.122 mg MenY polysaccharide per ampoule; expanded uncertainty with coverage factor of k = 2.36.

Estimated content of candidate standards using other methods

For comparison purposes polysaccharide content of the candidate standards was also measured by other methods. HPAEC-PAD data was submitted by six participants (with Participant 10 submitting data determined using glucose/galactose and polysaccharide in-house standards), Resorcinol data was submitted by six participants and the HPLC, Anthrone and Nephelometry assays were performed each by one participant. Participant 4 reported two sets of data (denoted as 4a and 4b in Tables 11 and 12) using their sialic acid standard. Estimates of polysaccharide content per ampoule for candidate MenW and MenY standards are given in Tables 11 and 12, respectively.

Mean estimates of polysaccharide content using the HPAEC-PAD or Resorcinol assays were around 10% higher than the proposed value for MenW, and less than less than 5% higher than the proposed value for MenY, as determined by qNMR, and were within the expanded uncertainty limits (95% confidence). Similarly, the estimates of MenY polysaccharide content determined by the Anthrone and HPLC assays were within the expanded uncertainty limits of the proposed value for MenY content determined by qNMR Mean estimates determined by Nephelometry were around 25% higher than values determined by qNMR and outside of the expanded uncertainty limits of the proposed values determined by qNMR, although it should be noted that only one laboratory each performed the Nephelometry, Anthrone and HPLC assays for this study.

Of the methods performed by more than one laboratory, HPAEC-PAD yielded the greatest inter-laboratory variability (considering all HPAEC-PAD data, CV=19.1% for MenW, CV= 21.4% for MenY), although intra-assay variability (for the measurement of four ampoules) was low. The main factors causing high inter-assay variability were the results from Participant 7 which were approximately 50% higher than the mean of all other laboratories. The mean estimates for MenW and MenY from Participant 7 could be excluded as statistical outliers; this resulted in a decrease in the CV of estimates to 11.2% and 12.1% for MenW and MenY candidate standards respectively.

The mean estimate of MenY polysaccharide content determined by Participant 12 using the Resorcinol assay was around the 25% higher than estimates obtained by other laboratories and determined to be a statistical outlier. With the exclusion of this data point, the inter-laboratory variation for the Resorcinol assay was reduced; CV=6.5% for MenW, CV= 7.4% for MenY. Participant 12 was also the only laboratory to use polysaccharide standards for the

Resorcinol assay. The lower inter laboratory variation may be attributed to the Resorcinol assay, being well-established and relatively simple, but also due to the use of the same standard material (sialic acid supplied by Sigma) by four of the six participants, whereas the HPAEC-PAD is subject to variability in the type of standard, column and processing methods. Furthermore, both HPAEC-PAD and qNMR methods rely on a degree of subjectivity in determining the baseline and limits of peaks that are integrated.

The mean MenW polysaccharide content was calculated to be 1042 µg polysaccharide/ampoule (after exclusion of Participant 7 data, CV=11.2%, n=6) using HPAEC-PAD, 1015 µg polysaccharide/ampoule (CV=6.5%, n=7) using the Resorcinol assay and 1193 µg/ml using the Nephelometry assay. Data for measurement of MenW content using the Anthrone and HPLC assays can be considered not applicable in this study as a glucose standard was used in these assays to measure galactose/sialic acid. In this case, the non-specificity of the assays is likely to have led to particularly low estimates of polysaccharide content.

Mean MenY polysaccharide content was calculated to be 991 µg/ampoule (with exclusion of Participant 7, CV = 12.1, n=6%) using HPAEC-PAD, 958 µg polysaccharide/ ampoule (after exclusion of Participant 12 data, CV=7.4%, n=6) using the Resorcinol assay, 1052 µg/ml using the HPLC assay, 1036 µg polysaccharide/ampoule using the Anthrone assay and 1156 µg/ml using the Nephelometry assay.

Overall, there was good agreement on the mean estimates of polysaccharide contents obtained by qNMR, HPAEC-PAD and Resorcinol assays. Similar good agreement was obtained with the other assays, although the data here were limited. Although the proposed content of the standards has been assigned by qNMR, where the licensure of existing vaccines is based on other assays, such as HPAEC-PAD or Resorcinol, it may be considered more appropriate to use the mean polysaccharide contents as presented in Tables 11 and 12.

Degree of *O*-acetylation of the candidate standards and functional weights

The degree of *O*-acetylation was measured by qNMR NIBSC in the extended study (Table 13) and by six of seven participants also performing this method for the main study (MenW % *O*-acetylation: Table 14a; MenY % *O*-acetylation: Table 15a). All data were re-analysed by NIBSC using a consistent approach. *O*-acetylation contents determined by the NIBSC analyst are contained in Tables 14b and 15b.

Participant 5 obtained particularly low estimates of the degree of *O*-acetylation for the MenW candidate standard and the mean value for this participant was found to be a statistical outlier. The estimate of *O*-acetylation from the remaining 5 laboratories was 56.2% (CV=2.5%) and compares favorably to the estimate of 58.1% *O*-acetylation obtained from the extended study conducted by NIBSC on ten ampoules. Upon applying the harmonized method for determining the % *O*-acetylation, a value of 58.5% was obtained. This was determined after the exclusion of the partial data provided by Participant 3 and the particularly high mean value determined from data submitted by Participant 5. Although not a statistical outlier, the mean value determined from Participant 5 data was excluded to be consistent with analysis of MenW, where the mean percent *O*-acetylation was a statistical outlier. Based on the mean percentage of *O*-acetylation, the functional weight of the MenW PS candidate IS is 497.020 g/mol (Annex 2).

For the MenY candidate standard, a particularly high value of *O*-acetylation was measured by Participant 2 on Ampoule 1. This value of 67.3% was excluded from the overall analysis as an outlier. Values for the remaining three ampoules were included. Overall with the exclusion of this individual value, the degree of *O*-acetylation was 50.2% (CV=8.7%) and is in good

agreement with the estimate of 51.0% obtained from the NIBSC extended study of nine ampoules. Upon applying the harmonized method for determining the % *O*-acetylation, a value of 51.6% was obtained. This was determined after the exclusion of the partial data provided by Participant 3 and the particularly high mean value (a statistical outlier) determined from data submitted by Participant 5. Based on the mean percentage of *O*-acetylation, the functional weight of the MenY candidate IS PS is 494.110 g/mol (Annex 2).

Suitability of the candidate standards to determine polysaccharide content in bulk conjugate samples

The second part of this study was to determine the suitability of the candidate standards to determine polysaccharide content in bulk conjugate samples and to compare the results obtained to those using the laboratories' own in-house standards. The HPAEC-PAD, HPLC, Resorcinol, Anthrone and Nephelometry assays were employed in this part of the study.

All laboratories using the HPAEC-PAD, HPLC and Nephelometry assays correctly identified Samples A and C as containing MenW and MenY polysaccharides respectively. Table 16 reports the polysaccharide concentration measured in each sample using the different methods. Sample B was correctly identified as not containing MenW or MenY polysaccharide, or that the polysaccharide concentration measured was very low / below the limit of quantitation.

Variability in estimates of MenW polysaccharide concentration from Sample A

For HPAEC-PAD and Resorcinol methods combined, a decrease in the variability of estimates obtained using candidate MenW standard was observed, CV=9.2%, compared to 17.8% obtained using in-house standards (Table 16 and Figure 1). The decrease was attributable to substantial decreases in inter-laboratory variation observed for both the HPAEC-PAD and Resorcinol assays when the candidate standards were used (CV=20.5% and 16.3% for HPAEC-PAD and Resorcinol respectively using in-house standards; CV=9.3 and 9.4% for HAPEC-PAD and Resorcinol, respectively, using the candidates standards).

As was noted for the determination of MenW polysaccharide content of the candidate standard, the Anthrone assay using a glucose standard was not suitable for the determination of MenW polysaccharide concentration in Sample A.

Use of the non-homologous MenY candidate standard to measure MenW content in the Resorcinol assay appeared to result in an increase in variability of data obtained (CV=25.5%) compared to using in-house standards. This high variability is mainly due to particularly low and high values obtained by Participants 4 and 12 respectively. After correcting for high variation in the assay, an improvement, in terms of variability, would have been achieved with the use of the candidate standards over in-house standards.

Variability in Estimates of MenY polysaccharide concentration from Sample C

With the exception HPLC data from Participant 9 and Resorcinol data from Participants 4 and 12, greater harmonization of estimates of polysaccharide concentration in Sample C was achieved using the candidate MenY standard compared to when in-house standards were used (Figure 2) and indicates that there is a benefit to using the candidate MenY standard. Considering all HPAEC-PAD and Resorcinol data, there is a marginal decrease in variability overall from 16.8% using the in-house standard to 14.5% when the candidate MenY standard is used. The high variability (CV) in the HPAEC-PAD assay using the candidate standards was significantly reduced from 18.1% (with the in-house standards) to 6.2% (with the MenY candidate standard).

Values obtained by participant 9, performing the HPLC assay with the MenY candidate standard were around half of the average value obtained by the other participants. Values determined by this laboratory using the in-house standard were also lower by around 20% compared to other laboratories/methods.

For the Resorcinol assay using the candidate MenY standard, extreme low and high estimates were obtained by Participants 4 and 12 respectively and became more divergent with the use of the candidate MenY standard (Figure 2). The data indicate that there is increase in variability by using the candidate MenY standard (CV=20.6%) over the in-house standard (16.7%). Despite this, Figure 2 demonstrates that all other participants performing the Resorcinol assay obtained results where there was a convergence in the estimate of MenY polysaccharide concentration using the candidate MenY standard as compared to using the in-house standard.

Interestingly, the data obtained for the Resorcinol assay using the candidate MenW standard to measure MenY content in Sample C was considerably less variable (CV=4.9%) than when the homologous standard was used (16.7%).

Similar estimates of the polysaccharide concentration of Sample C were obtained using the HPAEC-PAD and Resorcinol assays. Use of the non-homologous MenW candidate standard in the Anthrone assay to measure the MenY content of Sample C again produced results which were considerably higher (around double) than those obtained by other laboratories or methods, again highlighting the problem with this assay when using a standard that has a different composition to the sample tested.

Stability studies

Real-time and accelerated degradation of the candidate standards stored at -70°C, -20°C, 20°C, 37°C and 56°C was monitored at 1, 2, 3, 6 and 12 months after filling. Stability data for polysaccharide content of candidate MenW and MenY standards is reported in Tables 17 and 18, respectively. The saccharide content of both candidate standards remained relatively constant over this whole period. Similarly, saccharide content remained unaffected in reconstituted samples that were stored at -20°C for up to 12 months for MenW and MenY. Due to its stability, it was not possible to predict a percentage loss per year based on the polysaccharide content.

The molecular size distribution of MenW and MenY polysaccharides had profiles consistent with high quality, meningococcal polysaccharide. Both eluted as a single peak with a K_D of 0.32 for MenW and 0.29 for MenY. The molecular size distribution of MenW and MenY polysaccharides were affected by high temperature storage. Figures 3 and 4 demonstrate a decrease in molecular size distribution after storage at 37°C and 56°C from the one-month time point.

As with other studies of polysaccharides, the molecular size distribution is considered a good indicator of stability. Use of the sizing data (% eluting before K_D =0.32 for MenW or 0.29 for MenY) gave predicted losses per month of 0.001% for 16/152 MenW and 0.019% for 16/206 MenY, when stored at -20°C.

Measured across all time points, the pH of MenW polysaccharide 16/152 has a pH range of 6.3-7.1 when reconstituted with 1 ml sterile distilled water. The pH range of MenY polysaccharide 16/152 is 6.2-7.4 when reconstituted with 1 ml sterile distilled water.

Discussion

Physicochemical assays are primarily used to estimate total polysaccharide in meningococcal polysaccharide/conjugate vaccines. Differences in assays used, details of methodology and standards used lead to variation in the estimates of polysaccharide content. Previous studies of candidate International Standards for various polysaccharides have demonstrated that greater harmonization of results can be achieved if International Standards are used as compared to laboratories' own in-house standards.

In this study qNMR has been used to assign the polysaccharide content to candidate International Standards for MenW and MenY. Our data show that higher estimates of polysaccharide content were obtained by NMR spectrometers with higher magnetic field strength, when analysis was performed by the individual participants. However, the lower estimates of polysaccharide content were obtained on 500 MHz instruments following analysis performed by the NIBSC analyst. A proposed general chapter of the United States Pharmacopoeia [29] suggests that a 400 MHz spectrometer at a minimum should be sufficient. Although higher field strengths contribute to the production of sharper, more well-defined peaks, exclusion of data obtained from lower field strength spectrometers would be unjustified on this point alone, since there are other factors that contribute to assay variability.

The type and quality of the internal standard used in qNMR are important contributory factors to the accuracy of NMR data. It is essential that internal standards are of high purity and SI-traceability should be maintained; this is achieved if the internal standard is a) a CRM where the value and purity have been assigned by a National Measurement Institute; b) the CRM is produced by a Reference Material Provider accredited to ISO17034:2016 requirements; c) is a high purity material subject to a validated measurement procedure for purity assignment by qNMR using, as an internal standard, a CRM of type a) or b) [18]. Although NIBSC obtained highly consistent data for the extended study on the candidate standards, the internal standard did not satisfy the requirement above and so it would not be appropriate for content to be assigned based on data from only one laboratory. Furthermore, high levels of moisture in the standard may have an influence and ideally, moisture content should be accounted for when performing calculations. In the case of the internal standard used for the NIBSC measurements, TSP was lyophilized before use, ensuring that the contribution of moisture was minimized. Full information on the quality of the standards used for the study was not available. However, Participant 1 had noted that the thermogravimetric analysis of the maleic acid indicated that it was anhydrous, and the maleic acid used by Participant 5 was a CRM. In addition to NIBSC, two other participants used TSP as an internal standard and another laboratory used DSS, which is similar to TSP. The latter though has a better performance under varying ranges of pH and interacting less with proteins, which is irrelevant to this study. Several WHO International Standards have been produced by NIBSC using qNMR as the primary determinant of unitage, including Men A and Vi (for typhoid vaccines) polysaccharide, in both these studies NIBSC used TSP as the internal standard for unitage assignment.

By comparison, the variability in estimates for polysaccharide content by qNMR was higher than obtained for measurement of other polysaccharide standards (MenA, MenX, Vi) [19,20]. This may be attributable to the non-homogeneity of the instrumentation used. Further, additional complexity has been introduced to this study of partially *O*-acetylated disaccharide subunits, whereas MenA, MenX and Vi polysaccharides are composed of repeating monosaccharide subunits. Furthermore, the capsular polysaccharides of groups W and Y are known to have variable *O*-acetylation [30,31,32], and as batch-to-batch variation may occur during manufacturing, it is important to be able to make accurate measurements. The re-analysis of the *O*-acetylation and backbone of the standards was undertaken in an attempt to obtain more accurate measurements.

Alongside the choice of standard, sample preparation, NMR parameters used and equipment operation (for example the number of scans and shimming to correct for inhomogeneity in the magnetic field) and the peaks chosen for integration may also contribute to inter-laboratory variation. Although qNMR is proposed as a primary method for the quantification of organic compounds, there is a necessity to define and integrate the signals from resonances which relies on the judgement and experience of the operator. This might be particularly difficult where integrals of interest overlap with each other; for example, the proximity of the peak for H1 hexose to the peak for H7 (OAc), or where the peaks are broad as often seen with the presence of calcium. Such judgements on how data is processed are also applicable to the analysis of data obtained from the HPAEC-PAD method and could explain why the greatest variability obtained in this study arose from these assays.

Original data received from the collaborative study participants revealed that there was high inter-laboratory variation obtained from laboratories performing the qNMR method. All the responding participants agreed to the proposal initially set out in the draft report following on from analysis of the original data. Two participants encouraged further analysis of the qNMR data by NIBSC. It was agreed that re-analysed data by one analyst would still capture the different methods used by different laboratories but would serve the purpose of reducing operator variability in the analysis. For future studies a means of minimizing variability could be to propose stricter control of the collaborative study, setting the internal standard used for the unitage assignment and suggesting the concentration of the internal standard solution to be used. Furthermore, in future studies the use of system suitability tests (SSTs) performed before the participant move on to measuring the study materials could be encouraged; this would be a sealed sample distributed to the participants containing known amounts of an internal standard and reference polysaccharide. In addition to SSTs, spectral performance parameters could be set, for example the width at half height of the internal standard; in the case of the TSP this could be ≤ 1 Hz width at half height. This would ensure the quality of the spectral data gathered, guarantying the quality of the shim, for example. The combination of the above proposals would improve the consistency of the spectral data collected.

The use of a glucose standard in Anthrone assay in this study produced results for the determination of MenW content that deviated the most from data produced from other laboratories using other methods. Similarly, measurement of MenY polysaccharide content by the Anthrone assay using the MenW candidate standard, also resulted in inaccurate estimates of content. This highlights the drawback of using standards that are not matched well to the samples being analysed. In such scenarios it is difficult to know whether the standard will react in the same or similar manner to the sample and also brings in the additional requirement for conversion factors needed to convert the measured quantity of one substance to another.

The candidate standards were demonstrated to be fit for purpose for accurately measuring the polysaccharide content of conjugate vaccine samples. The distribution of estimates for the concentration of Samples A and C, determined from in-house standards was wider than when the candidate standards were used. Although the benefit of using the candidate MenY polysaccharide standard, over the use of in-house standards, in the Resorcinol assay, was less clear, the convergence of estimates for polysaccharide concentration obtained by four of six participants (with the exception of Participants 4 and 12) is clearly seen in Figure 2. There was insufficient data in this study to determine the fitness for purpose of the candidate standards in the nephelometry assay; although for the one participant performing this assay, the estimates of polysaccharide content were closer to the group mean obtained from all participants when the candidate standards were used, than when in-house standards were used.

Stability studies are on-going, but data collected up to 12 months do not indicate any stability issues under normal storage conditions. From this study, it was not possible to predict a percentage loss of polysaccharide content. A decrease in molecular size distribution was only observed at 37°C and 56°C, which are outside the normal temperature range for use. Data obtained on ampoules stored at +20°C, a temperature at which shipment of ampoules may take place, did not raise any concerns; it is unlikely that short term storage at this temperature would have any deleterious effect on the polysaccharides.

Proposal

Following on from previous studies to assign unitage to the first International Standards for MenA, MenX and Vi polysaccharide standards, we propose that assignment of polysaccharide content to the candidate standards 16/152 and 16/206 is made by qNMR. The ten participants responding to the draft report agreed with the proposal to have all the qNMR data re-analysed by a single NIBSC analyst; this was duly performed and unitage for each candidate polysaccharide standard was proposed.

Based on the data presented here we propose a content of 0.925 ± 0.138 mg MenW polysaccharide per ampoule for candidate standard 16/152 (expanded uncertainty with coverage factor k = 2.36, corresponding to a 95% level of confidence) and 0.950 ± 0.122 mg MenY polysaccharide per ampoule for candidate standard 16/206 (expanded uncertainty with coverage factor k = 2.36, corresponding to a 95% level of confidence). Mean estimates of polysaccharide content were also obtained using other methods; the HPAEC-PAD and Resorcinol assays were performed by multiple laboratories. Where polysaccharide content of currently licensed vaccines is determined using these methods it may be appropriate to refer to polysaccharide contents of the candidate standards as determined by these methods, rather than by qNMR.

The data demonstrate that using 16/152 Meningococcal serogroup W polysaccharide offers benefit to the harmonization of data gained using the HPAEC-PAD and Resorcinol assays as compared to when in-house standards are used. The benefit of using 16/206 Meningococcal serogroup Y polysaccharide in the Resorcinol assay was less clear, although greater harmonization of results was achieved with the use of this standard in the HPAEC-PAD assay. On the basis of results from the one laboratory performing the Nephelometry assay, the candidate standards also offered a similar benefit over the in-house standards by lowering the mean estimate of polysaccharide concentration to a level that were more in line with those obtained by other methods/laboratories. Therefore, we conclude that candidate standards 16/152 and 16/206 are suitable to aid the harmonization of the measurement of MenW and MenY polysaccharide content from vaccine samples using HPAEC-PAD and Resorcinol assays. With the use of these standards, measurement of polysaccharide content should be adjusted to account for the degree of *O*-acetylation, and therefore the expected molecular weight, of the polysaccharide in the sample.

The storage condition for both candidate standards is -20^{o}C.

Acknowledgements

We would like to thank: GSK Vaccines s.r.l. for their generous donation of meningococcal MenW and MenY capsular polysaccharides used in the production of 16/206 and 16/152 candidate standards; the members of the MenW and MenY IS Working Group for participating in this study; Dr Barbara Bolgiano and Dr Caroline Vipond for a wealth of experience and advice on this study; Sara-Jane Holmes and staff at the CBRM, NIBSC for the production and processing of the standards; Paul Matejtschuk, Kiran Malik and Chinwe

Duru of the Standardisation Science Department at NIBSC for performance of trial fills/lyophilization and additional testing for moisture content; Mark Harris for the coordination of shipments to study participants.

Reference List

[1] Invasive meningococcal disease. Annual epidemiological report for 2015. Stockholm: European Centre for Disease Prevention and Control (ECDC); 2017.

[2] Epidemic meningitis control in countries of the African meningitis belt, 2017. Weekly Epidemiological Record; 93(14):173-84. Geneva: World Health Organization; 2018.

[3] Epidemic meningitis control in countries of the African meningitis belt, 2016. Weekly Epidemiological Record; 92(13):145–54). Geneva: World Health Organization; 2017.

[4] Meningitis control in countries of the African meningitis belt, 2015. Weekly Epidemiological Record; 91(16):209-16.). Geneva: World Health Organization; 2016.

[5] Abad R, López EL, Debbag R, Vázquez JA. Serogroup W meningococcal disease: Global spread and current affect on the Southern Cone in Latin America (Review). Epidemiology and Infection 2014;142(12):2461-70.

[6] Araya P, Fernández J, Del Canto F, Seoane M, Ibarz-Pavón AB, Barra G, et al. *Neisseria meningitidis* ST-11 clonal complex, Chile 2012. Emerg Infect Dis 2015;21(2):339-41.

[7] Bröker M, Emonet S, Fazio C, Jacobsson S, Koliou M, Kuusi M, et al. Meningococcal serogroup Y disease in Europe: Continuation of high importance in some European regions in 2013. Hum Vaccin Immunother 2015;11(9):2281-6.

[8] Enhanced Meningococcal Disease Surveillance Report, 2016. Atlanta (GA): Centers for Disease Control and Prevention; 2016.

[9] Bhattacharjee AK, Jennings HJ, Kenny CP, Martin A, Smith IC. Structural determination of the polysaccharide antigens of *Neisseria meningitidis* serogroups Y, W-135, and BO1. Can J Biochem 1976;54(1):1-8.

[10] Svennerholm L. Quantitative estimation of Sialic acids II. A colourimetric Resorcinol-hydrochloric acid method. Biochemica et Physica Acta 1957;24(3):604-611.

[11] Bardotti A, Averani G, Berti F, Berti S, Galli C, Giannini S, et al. Size determination of bacterial capsular oligosaccharides used to prepare conjugate vaccines against *Neisseria meningitidis groups* Y and W135. Vaccine 2005;23(16):1887-99.

[12] Mawas F, Bolgiano B, Rigsby P, Crane D, Belgrave D, Corbel MJ. Evaluation of the saccharide content and stability of the first WHO International Standard for *Haemophilus influenzae* b capsular polysaccharide. Biologicals 2007;35(4):235-45.

[13] Vipond C, Mulloy B, Rigsby P, Burkin K, Bolgiano B; MenC IS Working Group. Evaluation of a candidate International Standard for Meningococcal Group C polysaccharide. Biologicals 2012;40(5):353-63.
[14] Mawas F, Burkin K, Dougall T, Saydam M, Rigsby P, Bolgiano B, et al. International collaborative study for establishment of the 2nd WHO International Standard for *Haemophilus influenzae* type b polysaccharide. Biologicals 2015;43(6):492-503.
[15] Com. Cons. Quantit´e de Mati`ere (CCQM): Rapport de la 1re session. Sèvres Cedex: Bureau International des Poids et Mesures (BIPM); 1995.
[16] Milton MJ, Quinn TJ. Primary methods for the measurement of amount of substance. Metrologia 2001;38(4):289-296.
[17] King B. The practical realization of the traceability of chemical measurement standards. Accred Qual Assur 2005;5(10):429–36.
[18] Westwood S, Stoppacher N, Garrido B, Huang, Saito T, Un I,Yamazaki T, et al. qNMR Internal Standard Reference Data (ISRD): Maleic Acid [BIPM ISRD-01]. Rapport BIPM – 2018/01 Available from: https://www.bipm.org/utils/common/pdf/rapportBIPM/RapportBIPM-2018-01.pdf
[19] Vipond C, Swann CJ, Dougall TW, Rigsby P, Gao F, Beresford NJ, et al. Evaluation of candidate international standards for meningococcal serogroups A and X polysaccharide. Biologicals 2017;47:33-45.
[20] Gao F, Swann C, Rigsby P, Lockyer K, Logan A, Rijpkema S, et al. Evaluation of candidate International Standards for Vi polysaccharide from *Citrobacter freundii* and *Salmonella enterica* subspecies *enterica* serovar Typhi. WHO/BS/2017.2310. Available from: http://www.who.int/biologicals/expert_committee/BS2310_Vi_PS_Report_for_WHO_Final.pdf?ua=1
[21] Lemercinier X, Jones C. Full 1H NMR assignment and detailed *O*-acetylation patterns of capsular polysaccharides from *Neisseria meningitidis* used in vaccine production. Carbohydr Res 1996;296:83-96.
[22] Jones C, Lemercinier X. Use and validation of NMR assays for the identity and *O*-acetyl content of capsular polysaccharides from *Neisseria meningitidis* used in vaccine manufacture. J Pharm Biomed Anal 2002;30(4):1233-47.
[23] WHO Technical Report Series, Sixty sixth report 999:80-81. Geneva: WHO Expert Committee on Biological Standardization (ECBS); 2015.
[24] Product Guide for LudgerTagTM 2-AA (2-aminobenzoic acid). Monosaccharide Release and Labeling Kit. https://www.ludger.com/docs/products/lt/lt-mono-96/ludger-lt-mono-96-guide.pdf.
[25] European Directorate for the Quality of Medicines & Healthcare (EDQM). European Pharmacopoeia version 8.8: Sialic acid in polysaccharide vaccines (Monograph 2.05.23).
[26] Mokrasch LC. Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. J Biol Chem 1954;208(1):55-9.

[27] Lee CJ. The quantitative immunochemical determination of pneumococcal and meningococcal capsular polysaccharides by light scattering rate nephelometry. J Biol Stand 1983;11(1):55-64.

[28] Kirkwood TB. Predicting the stability of biological standards and products. Biometrics 1977; 33(4):736-42.

[29] United States Pharmacopeia (USP). Pharmacopeial Forum; 43(2): ⟨198⟩ Nuclear Magnetic Resonance Spectroscopy Identity Testing of Bacterial Polysaccharides Used in Vaccine Manufacture.

[30] Longworth E, Fernsten P, Mininni TL, Vogel U, Claus H, Gray S et al. *O*-Acetylation status of the capsular polysaccharides of serogroup Y and W135 meningococci isolated in the UK. FEMS Immunol Med Microbiol 2002;32(2):119-23.

[31] Vogel U, Claus H, Frosch M. Genetic Lineages and their Traits in *Neisseria meningitidis*. Int J Med Microbiol 2004;294:75-82.

[32] Berti F, De Ricco R, Rappuoli R. Role of *O*-Acetylation in the Immunogenicity of Bacterial Polysaccharide Vaccines. Molecules (Review) 2018;23:1340-9.

Participants' comments

The draft report was submitted to all participants and comments on the draft report were received from Participants 1, 2, 4, 5, 6, 7, 9, 11 and 12.

Participant 1

Re: discussion on high variability of qNMR results compared to that obtained for other polysaccharide…"These are the results obtained and I think it also reflects the complexity of Y and W compared to the monomeric A, C and Vi" "…complexity of the partially *O*-acetylated disaccharide repeating unit of MenW and MenY polysaccharides? Other monosaccharides". The peaks chosen for integration also contribute to the inter-laboratory variation.

Some points

1) I am not sure how many labs routinely perform qNMR for MenY/W. If I were to do the qNMR assay more regularly or several samples like NIBSC I would expect to produce results with reduced variability. Thus this study by several groups on a few samples may not do justice to the power of the qNMR approach.
2) MHz effect. one certainly gets sharper peaks at higher field and for me this made a big difference when I have tested qNMR on 300, 400 versus 600- my highest values were at 600 MHz. USP considers 400 to be sufficient, however, this is more for CPS identity, not qNMR. We cannot exclude lower MHz data from this study. How one performs the integration of the broad CPS peaks versus sharp internal standard peak can make a big difference (at lower field one would probably have to choose a wider region to integrate).
3) The MenY/W NMR spectrum is complex because of the close proximity of the H1 hexose and the HCOAc signal. This means variability in integration in selecting the exact region to integrate and that appropriate integration would be more easily performed at higher field (with better separation of these almost overlapping signals).

What about NIBSC asking all the groups to send their data. This could be re-analysed using your procedures-and considering points 2) and 3), may reduce the variability of the qNMR results.

Re: Resorcinol assay. "Perhaps mention (again) that this is the current EP method for the PS content"

Re: the use of Extractant in the Resorcinol assay. "Old" method typically no longer used. Adds variability.

Response: *A re-analysis of all of the data was carried out by the analyst at NIBSC and the revised results are presented in sections b) of Tables 7, 8, 14 and 15.*

Re: 50% lower values obtained by Participant 9 using HPLC assay. "Cannot think of a scientific reason for this Gal bonds are not as stable as Glc bonds. What is odd is the standard concentrations used: 2 mg/ml Glucose, 10mg/ml Galactose"

"The HPLC method can be grouped together with H-PAD, however, it is such a different method that I don't think the results should be assessed together. I cannot explain the low Gal obtained by Lab 9."

Response: *Thank you for your observations. It was noted that the working concentrations of the two standards were different, although for the production of the standard curve, it appeared that they were diluted to a similar range. The HPLC and HPAEC-PAD results have not been grouped together for statistical analysis.*

Re: Resorcinol assay. “Perhaps mention (again) that this is the current EP method for the PS content”

Re: the use of Extractant in the Resorcinol assay. “Old” method typically no longer used. Adds

variability.

Labs 7, 10 and 11 did use an extractant step, and this did not lead to higher variability (see Table 12).

Participant 2

The report is fine with us including Annex I.

Participant 3

No comments received on the draft report.

Participant 4

I looked at the table and notice that participants (not us) who determined the amount of the deacetylated PS obtained narrower CV and closer results. This is more likely due to the fact that the lines are narrower for the de-OAc than the fully acetylated PS making the integration more precise. If I recall, the PS was first quantified, then de-acetylated, therefore spectra of the de-acetylated PS could be integrated, and better numbers should be obtained. That is interesting as my tendency is to quantify to the unaltered sample (full OAc) but this suggest that it is better to get narrower lines as these do provide more precise integrations.

Higher field also helped in getting better resolved line. One more look and the 700MHz results of participant 6 are better than participant 1 (600MHz). It will be interesting to see if re-analysis of our data (600MHz) will provide better final results.

Response: *The data originally presented for Participants 1 and 6 should have been on the unaltered sample (before de-O-acetylation). This has now been changed. A re-analysis was carried out as explained in the response to Participant 1.*

Participant 5

for MenW particularly the result of Ampoule #4 appears out of trend. By comparing the NMR profile of MenW analytical samples, normalizing the intensity of maleic acid reference signal the H3eq signal of sialic acid clearly show higher intensity for Ampoule #4.

Participant 6

Variation due to differences in methods of analysis might help to reduce the overall inter-laboratory variation obtained with the NMR.

Participant 7

We all agree that there are minor typos and just a few necessary edits.

Participant 8

No comments received on the Report. Moisture contents are not included in the calculation of polysaccharide contents.

Participant 9

We agree with your idea, it’s better separate the groups (HPLC from HPAEC-PAD) due to the treatment and the condition are so different.

Participant 10

Participant 10 provided the text for the Nephelometry method section of the report. No comments received on the draft report.

Participant 11

We are okay with the Collaborative Study Draft Report and have no points for correction or discussion.

Participant 12

Typographical and grammatical errors were reported.

Annex 1. Participants of the MenW/MenY IS Working Group

Name	Laboratory	Country
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Ms. Sherri Boucher Dr. Yves Aubin Ms. Mary Abraham Mr. Derek J. Hodgson	Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Canada, 251 Sir Frederick Banting Driveway, Tunney's Pasture, AL 2201E, Ottawa, ON K1A 0K9	Canada
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Annex 2 Calculation of MenW PS (16/152) and MenY PS (16/206) standard residue weights

MenW/Y saccharide residue weight				
	Calcium salt, fully *O*-acetylated		**Calcium salt, de-*O*-acetylated**	
Atom	**No. Atoms**	**Mass**	**No. Atoms**	**Mass**
Carbon	19	228.209	17	204.187
Oxygen	14	223.986	13	207.987
Hydrogen	28	28.224	26	26.208
Nitrogen	1	14.007	1	14.007
Calcium	0.5	20.039	0.5	20.039
Total Mass		**514.465**		**472.428**

Using IUPAC, 2009 atomic masses	
Atom	**Mass**
Carbon	12.011
Oxygen	15.999
Hydrogen	1.008
Nitrogen	14.007
Calcium	40.078

	Water (H_2O)		***O*-acetyl ($COCH_3$) – 1 H (for adding)**	
Atom	**No. Atoms**	**Mass**	**No. Atoms**	**Mass**
Carbon	0	0	2	24.022
Oxygen	1	15.999	1	15.999
Hydrogen	2	2.016	2	2.016
Total Mass		**18.015**		**42.037**

MenW PS standard is 58.5% *O*-acetylated and has a residue weight of 497.020 g/mol.

472.428 g/mol + (0.585 *42.037 g/mol) = 497.020 g/mol

MenY PS standard is 51.58 % *O*-acetylated and has a residue weight of 494.110 g/mol.

472.428 g/mol + (0.5158 *42.037 g/mol) = 494.110 g/mol

Table 1 Summary of data supplied from the manufacturer's Certificate of Analysis for the donated meningococcal serogroup MenW and MenY polysaccharides.

	Specification	**MenW polysaccharide**	**MenY polysaccharide**
Identity	Positive	Positive for MenW	Positive for MenY
Sialic acid content (mg/g dry weight)	≥ 560	633	596
Protein content (mg/g dry weight)	≤ 10	< 1	< 1
Nucleic acid content (mg/g dry weight)	≤ 10	< 0.00003	< 0.00003
Endotoxin content (IU/µg)	≤ 10	< 0.0005	< 0.0005
Dry weight (%) Thermogravimetric analysis	≥ 79%	87	88
Kd HPLC	≤ 0.7	0.4	0.4
***O*-acetylation (mmol/g)**	≥ 0.3	0.9	0.9

Table 2 Preparation and production dates, and post-fill assessment of 16/152 – Meningococcal serogroup W polysaccharide and 16/206 – Meningococcal serogroup MenY polysaccharide

	MenW polysaccharide	**MenY polysaccharide**
Date of reconstitution	26 May 2016	01 September 2016
Date of filling and start date of lyophilisation	27 May 2016	02 September 2016
End date of lyophilisation and date of sealing	31 May 2016	06 September 2016
Label	16/152 Meningococcal serogroup W polysaccharide	16/206 Meningococcal serogroup Y polysaccharide
Final no. containers in use	9304	9180
Mean mass of fill (g), CV (%) (Number of ampoules)	1.0068 (0.23) (330)	1.0082 (0.25) (359)
Mean dry weight (g), CV (%) (Number of ampoules)	0.00055 (32.82) (6)	0.00066 (30.39) (6)
Moisture content: Karl-Fischer (%) CV (%) (number of ampoules)	3.02 (45.6)(3)	2.79 (7.8)(3)
Moisture content (%): Thermogravimetric analysis (number of pooled ampoules)	Run 1: 0.37 (2) Run 2: 0.37 (3)	Run 1: 0.69 (3) Run 2: 0.74 (3)
Mean oxygen headspace (%) CV (%) (number of ampoules)	0.35 (30.4) (12)	0.27 (36.47) (12)
Bacterial colony count (cfu/ml)	0 (start of filling run) 0 (end of filling run) 0 (start of sealing) 0 (end of sealing)	0 (start of filling run) 0 (end of filling run) 0 (start of sealing) 0 (end of sealing)
Mould/yeast colony Count (cfu/ml)	0 (start of filling run) 0 (end of filling run) 0 (start of sealing) 0 (end of sealing)	0 (start of filling run) 0 (end of filling run) 0 (start of sealing) 0 (end of sealing)

Table 3 Details of NMR spectrometer instrumentation and parameters used in the study

Participant	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Spectral reference	Maleic acid	TSP-d_4	Caffeine / Ascorbic acid	TSP-d_4	Maleic acid	TSP-d_4	DSS-d_6 (0.01% w/v)
Temperature	303 K			308 K	298 K	303.2 K	300 K
Instrument	Bruker Avance III with BBO Prodigy cryoprobe	Bruker Avance III HD	Bruker Avance III HD Prodigy BBFO probe	Bruker Avance III	Bruker DRX400	Bruker Avance NEO	Bruker Avance IIII QXI temperature probe
Field (MHz)	600	500	400	600	400	700	500
Number of scans	64	256	32 and 64	512/1024	128	128	256
T1	5.99			5.07 s (MenW) 4.35 s (MenY)	5.3, the longest one (Maleic acid)		3.66 s (DSS-d_6)
D1 relaxation delay	30 s	18 s (MenW), 30 s (MenY)	30 s	26 s (MenW) 22 s (MenY)	30 s	35 s	20 s
Receiver gain (RGA)	57-80.6		50.8-57			101	256
Pulse width P1 (µsec)	13.76-14.86 µs (MenW) 14.13-15.28 µs (MenY)	90°	11.5 µs	23.0 µs	8 µs	8.0 µs	10.45 µs
Pulse sequence	ZG	ZG	ZG	ZG	ZG	ZG	ZG

Table 4 Details of HPAEC-PAD / HPLC methodology, instrumentation and parameters used in the study. PS - polysaccharide

Participant	**5**	**6**	**7**	**8**	**10**	**11**	**9 HPLC**
Standard	MenW PS MenY PS	MenW PS MenY PS	Galactose (MenW) Glucose (MenY)	D-(+)-Gal D-(+)-Glc	Galactose / Glucose MenW PS MenY PS	MenW PS MenY PS	Galactose (MenW) Glucose (MenY)
Source	In house	In house	Sigma G0750 (Gal) Sigma G8270 (Glc)	Sigma PHR1206 (Gal) PHR1000 (Glc)	Sigma (Glc/Gal) (MenW/MenY PS)	In house	LudgerTag 2-AA Monosaccharide Release ad Labeling Kit
Purity (%)	-	-	≥99%	99.97 99.8	≥99.5%/≥99% (Glc/Gal)	-	
Moisture (%)	-	Dried	Not known	0.004 (Gal) 0.03 (Glc)	0.25/0.32 (Glc/Gal)	10.07 (MenW) 10.97 (MenY)	
Concentration of Standard	1.1143 mg/ml (MenW) 1.1235 mg/ml (MenY)	1 mg/ml	1 mg/ml working stock	1 mg/ml	100 µg/ml	0.25 mg/ml	2 mg/ml Glucose 10 mg/ml Galactose
Diluent	0.005 Tween 20 in MQW	dH_2O	dH_2O	PBS	ultrapure water	dH_2O	
Standard Curve (number of points)	0.5-10 µg/ml (5)	0.5-27 µg/ml (5)	1-5 µg/ml (5)	0.2-3 µg/ml (5)	0.5-27 µg/ml (5)	0.17-27 µg/ml (6)	0.1-2.0 nmol (Glc) 0.1-1.5 nmol (Gal)
Internal spike	-	Fucose	-	Fucose	Fucose	Fucose	Xylose
Hydrolysis	2.0 M TFA	2.0 M TFA	0.6 M TFA	0.6 M TFA	final concentration 2.0 M TFA	2.0 M TFA	2M TFA
Incubation	2h, 100^0 C	2h, 100^0 C	1h, 100^0 C	1h, 100^0 C	2h, 100^0 C	2h, 100^0 C	45 min, 80^0 C
Instrument	Dionex ICS3000	Dionex ICS3000	Dionex ICS-3000	Dionex ICS5000	ICS5000 and ICS5000+	ICS5000	
Electrode	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl	AgCl reference electrode gold working electrode	Au, Ag/AgCl	
Waveform		Quadruple	Quadruple		Gold,Carbo,Quad	Quadruple	
Column	CarboPac PA1	CarboPac PA1	CarboPac PA1 or PA100	CarboPac PA10	PA1	CarboPac PA1	

Guard column	PA1 Guard Amino Trap	Amino Trap	PA1 or PA100 Guard	PA10 Guard	Aminotrap 4×50mm	Amino Trap	
Flow rate **Run time**	40 min	1 ml/min 41 min	1 ml/min 60 min	50 min	1ml/min 45min	1 ml/min 60 min	
Eluting time (min)	14.3 (Gal) 16.0 (Glc)	13.5 (Gal) 14.4 (Glc)	14.5 (Gal) 15.8 (Glc)	17.3 (Gal) 18.6 (Glc)	20.5 (Gal) 23.5 (Glc)	15.3 (Gal) 16.2 (Glc)	
Elution conditions		0-18 min, 15 mM NaOH 18-26, 100 mM naOH, 80 mM NaOAc 26-31 min, 400 mM NaOH 31-41 min, 15 mM NaOH	0-22 min, 16 mM NaOH 22-27 min, 100mM NaOH, 200 mM NaOAc 27-60 min, 16 mM NaOH	0-30 min, 9-124 mM NaOH, 0-600 mM NaOAc 30-31 min, 124-200 mM NaOH, 600-1000mM NaOAc 31-35 min, 200 mM NaOH, 1000-0 mM NaOAc 35-43 min, 200 mM NaOH 43-50 min 9 mM NaOH	0-32min 10mM NaOH 32-40 min 200mM NaOH 40-45 min 10mM NaOH	0-16 min, 18 mM NaOH 16-18 min, 18-100 mM NaOH, 0-100 mM NaOAc 18-42 min, 100 mM NaOH, 100-224 mM NaOAc 42-52 min, 400 mM NaOH 52-65 min, 18 mM NaOH	
Conversion factor	N/A	N/A	MenW: 497.020/180.156 MenY: 494.110/180.156			N/A	

Table 5 Details of Resorcinol (Participants 4, 6, 7, 10, 11) and Anthrone (Participant 9) methodology and parameters used in the study

Participant	**4 (Resorcinol)**	**6 (Resorcinol)**	**7 (Resorcinol)**	**10 (Resorcinol)**	**11 (Resorcinol)**	**12 (Resorcinol)**	**9 (Anthrone)**
Standard	Sialic acid	Sialic acid	Sialic acid	Sialic acid	Sialic acid with D-(+)-gal Or Sialic acid with D-(+)-Glc	MenW PS MenY PS	D-(+)-Glucose
Source	Sigma A2388 SLBK1108V	Sigma A2388 SLBS7252	Nacalai USA	Sigma	Sigma A2388 sialic acid Lot: SLBK1108V Sigma G0750 Gal Lot: SLBG8191V Sigma G5767 Glc Lot: BCBF4689V	Vaccine-grade	Sigma G7021 Lot: BCBC0541
Purity (%)	99	99	99.6	≥98	99% (Sialic acid) N/A (Gal) N/A (Glc)	-	100
Moisture (%)	1.1	1.1	3.0	0.35	1.1 (NANA) 1.1 (Gal) 0.05 (Glc)	-	-
Concentration	1 mg/ml	1 mg/ml	1 mg/ml	80µg/ml	95 mg/ L NANA 55mg/L Glc / Gal	~ 2 mg/ml	2 mg/ml
Storage conditions of working stock	-20°C	-20°C	4°C	2-8°C	-10 to -25°C	-20°C	-20°C
Range of standard curve (number of levels on curve)	0-25 µg / ml	0- 40 µg / ml	0-70 µg / ml	0-64 µg/ml (6)	0-47.5 µg / ml	0-200 µg / ml	0-60 µg/ml (6)
Replicates	2 standards 4 samples)	3	3 standards 2 samples	2	2 standards	3	2
Volume of standard/ sample	1 ml	0.5 ml	1 ml	0.5 ml	0.4 ml	0.2 ml	1 ml
Reagent (Resorcinol or Anthrone)	0.2% resorcinol (0.1% final with	0.2% resorcinol (or 18.2 mM)	0.2% resorcinol (0.1% final with	0.1mol/l $CuSO_4$ 0.5ml,	0.2% resorcinol (0.14% final with	0.6% resorcinol (0.086% final	1 mg/ml Anthrone

	sample)	(0.1% final with sample)	sample)	4%Resorcin 5ml, HCl 80ml, add water to 100ml	sample)	with sample)	
Volume reagent	1 ml	0.5 ml reagent	1 ml reagent	0.5ml	1 ml	1.2 ml	3 ml
Copper sulphate	0.25 mM	0.25 mM	0.25mM		0.25 mM	?	
HCl	29.6%	29.6%	29.6% (160 ml of 37% in 200 ml total)	29.6% (80 ml of 37% in 100 ml total)	20.7%		
Extractant			85:15 v/v 1-butyl acetate/1-butanol	n-Butanol 15ml, add Butyl acetate to 100ml	Butanol:Butyl acetate – 1:4		
Volume of extractant			2 ml	1.0ml	1 ml		
Incubation temp, time, method	100°C, 20 mins	110°C, 15 mins	100°C, 15 mins	water bath,100℃,15min	98°C, 30 mins	100°C, 30 mins	
Other steps			Add extraction solvent, vortex for 2 seconds. Remove the organic layer for analysis from the aqueous phase.	ice-bath,10min	Add extraction solvent, vortex for 20 seconds. Remove the organic layer for analysis from the aqueous phase.		
Wavelength	580 nm	564 nm	580 nm	585 nm	580 nm (subtract 450 nm value)	430 nm	585 nm
Conversion factor	MenW: 497.020 / 309.27 MenY: 494.110 / 309.27					N/A	MenW: 497.020 / 309.27 MenY: 494.110 / 309.27

Table 6 Extended qNMR study at NIBSC – measurement of polysaccharide content of MenW (16/152) and MenY (16/206) candidate standards. Values shown were individually corrected for the mass of the repeating unit calculated using the percent *O*-acetylation determined for each ampoule.

16/152 Meningococcal serogroup W polysaccharide	
Ampoule	**µg polysaccharide /ampoule**
1	938
2	930
3	951
4	907
5	978
6	961
7	940
8	942
9	950
10	947
Mean	944
SD	18.7
CV	2.0%

16/206 Meningococcal serogroup Y polysaccharide	
Ampoule	**µg polysaccharide /ampoule**
1	993
2	982
3	1021
4	971
5	976
6	1001
7	997
8	994
9	999
Mean	993
SD	15.0
CV	1.5%

Table 7 MenW polysaccharide determined by qNMR. Analysis was carried out by individual study participants (7a) and re-analysed by one NIBSC analyst (7b). Values given as µg MenW polysaccharide per ampoule and determined before de-*O*-acetylation. Values presented in 7a) were adjusted for a mean of 58.50% *O*-acetylation value corresponding to a residue weight of 497.020 g/mol. Values presented in 7b) were individually corrected for the mass of the repeating unit calculated using the percent *O*-acetylation determined for each ampoule.

7a) Analysis by individual participants

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participants 3 and 5)	
								Overall		**Overall**	
1	945	931	923	951	937	12.7	1.4%	**mean**	854	**mean**	865
2	785	786	785	775	783	5.5	0.7%	**SD**	85.6	**SD**	100.1
3	789	805	n/t	n/t	797	n/c	n/c	**CV**	10.0%	**CV**	11.6%
4	891	853	885	942	892	36.7	4.1%	**95% LCL**	775	**95% LCL**	741
5	741	770	822	1105	859	167.0	19.4%	**95% UCL**	934	**95% UCL**	989
6	981	974	960	975	972	9.1	0.9%	**n**	7	**n**	5
7	743	750	738	727	739	9.8	1.3%				

7b) Analysis by NIBSC analyst

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participant 3)	
								Overall		**Overall**	
1	967	939	929	973	952	21.5	2.3%	**mean**	936	**mean**	925
2	764	739	787	757	762	19.7	3%	**SD**	127.0	**SD**	135.1
3	1011	1000	n/t	n/t	1005	n/c	n/c	**CV**	13.6%	**CV**	14.6%
4	990	959	989	1075	1003	50.2	5.0%	**95% LCL**	819	**95% LCL**	783
5	1105	913	1113	1256	1097	140.6	12.8%	**95% UCL**	1054	**95% UCL**	1066
6	980	973	958	974	971	9.3	1.0%	**n**	7	**n**	6
7	725	780	775	772	763	25.6	3.4%				

Table 8 MenY polysaccharide determined by qNMR. Analysis was carried out by individual study participants (8a) and re-analysed by one NIBSC analyst (8b). Values given as µg MenY polysaccharide per ampoule and determined before de-*O*-acetylation. Values presented in 8a) were adjusted for a mean of 51.58 % *O*-acetylation value corresponding to a residue weight of 494.110 g/mol. Values presented in 8b) were individually corrected for the mass of the repeating unit calculated using the percent *O*-acetylation determined for each ampoule.

8a) Analysis by individual participants

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participants 3 and 5)	
1	1041	976	965	974	989	35.0	3.5%	**Overall mean**	800	**Overall mean**	877
2	785	713	723	724	736	32.8	4.4%	**SD**	181.3	**SD**	116.9
3	501	434	n/t	n/t	468	n/c	n/c	**CV**	22.7%	**CV**	13.3%
4	914	950	874	879	904	35.5	3.9%	**95% LCL**	632	**95% LCL**	732
5	681	794	771	741	747	48.8	6.5%	**95% UCL**	967	**95% UCL**	1022
6	977	978	979	990	981	6.2	0.6%	**n**	7	**n**	5
7	782	788	738	787	773	24.0	3.1%				

8b) Analysis by NIBSC analyst

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participant 3)	
1	1029	1042	976	983	1007	33.0	3.3%	**Overall mean**	956	**Overall mean**	950
2	800	761	773	770	776	17.0	2.2%	**SD**	110.8	**SD**	120.2
3	974	1006	n/t	n/t	990	n/c	n/c	**CV**	11.6%	**CV**	12.7%
4	1021	1252	1003	962	1060	130.5	12.3%	**95% LCL**	853	**95% LCL**	824
5	1071	1071	1010	1054	1051	28.9	2.7%	**95% UCL**	1058	**95% UCL**	1076
6	975	978	979	989	980	6.1	0.6%	**n**	7	**n**	6
7	794	845	839	825	826	22.7	2.7%				

Table 9 Determination of uncertainty assigned to MenW polysaccharide content of 16/152: determined from data submitted by study participants (9a) or from data re-analysed by a single NIBSC analyst (9b)

9a)

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Weight of the TSP-d4 reference material	From calibration data (±0.012 mg, assuming triangular distribution)	12.700 mg	0.005 mg	0.04%
Purity of the reference material	Estimated as 99% ± 1% (assuming rectangular distribution)	99%	0.58%	0.59%
Amount of deuterated water added to the sample	From calibration data (±0.019 mg, assuming triangular distribution)	1000 mg	<0.1 mg	<0.01%
Random error and between-ampoule homogeneity	SD (Table 6, MenW 16/152)	944.40	18.75	1.99%
Between-laboratory variability	SEM (calculated from Table 7)	864.91	44.75	5.17%
Combined relative standard uncertainty				**5.57%**

9b)

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Weight of the TSP-d4 reference material	From calibration data (±0.012 mg, assuming triangular distribution)	12.700 mg	0.005 mg	0.04%
Purity of the reference material	Estimated as 99% ± 1% (assuming rectangular distribution)	99%	0.58%	0.59%
Amount of deuterated water added to the sample	From calibration data (±0.019 mg, assuming triangular distribution)	1000 mg	<0.1 mg	<0.01%
Random error and between-ampoule homogeneity	SD (Table 6, MenW 16/152)	944.40	18.75	1.99%
Between-laboratory variability	SEM (calculated from Table 7)	924.65	55.15	5.96%
Combined relative standard uncertainty				**6.31%**

Table 10 Determination of uncertainty assigned to MenY polysaccharide content of 16/206 determined from data submitted by study participants (10a) or from data re-analysed by a single NIBSC analyst (10b)

10a)

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Weight of the TSP-d4 reference material	From calibration data (±0.012 mg, assuming triangular distribution)	12.700 mg	0.005 mg	0.04%
Purity of the reference material	Estimated as 99% ± 1% (assuming rectangular distribution)	99%	0.58%	0.59%
Amount of deuterated water added to the sample	From calibration data (±0.019 mg, assuming triangular distribution)	1000 mg	<0.1 mg	<0.01%
Random error and between-ampoule homogeneity	SD (Table 6, MenY 16/206)	992.72	15.05	1.52%
Between-laboratory variability	SEM (calculated from Table 8)	876.71	52.27	5.96%
Combined relative standard uncertainty				**6.18%**

10b)

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Weight of the TSP-d4 reference material	From calibration data (±0.012 mg, assuming triangular distribution)	12.700 mg	0.005 mg	0.04%
Purity of the reference material	Estimated as 99% ± 1% (assuming rectangular distribution)	99%	0.58%	0.59%
Amount of deuterated water added to the sample	From calibration data (±0.019 mg, assuming triangular distribution)	1000 mg	<0.1 mg	<0.01%
Random error and between-ampoule homogeneity	SD (Table 6, MenY 16/206)	992.72	15.05	1.52%
Between-laboratory variability	SEM (calculated from Table 8)	950.08	49.09	5.17%
Combined relative standard uncertainty				**5.42%**

Table 11 MenW polysaccharide content determined by other methods. Values given as µg MenW polysaccharide per ampoule. [#]Values from these participants were determined using Glc, Gal or sialic acid standards and were converted to MenW polysaccharide with a mean value of 58.50% *O*-acetylation corresponding to a residue weight of 497.020 g/mol. *Glc/Gal standard; [†] Polysaccharide standards;

Method	Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics		Summary Statistics	
HPAEC-PAD	**5**	1087	1126	1072	1037	1080	37.0	3.4%	**Overall mean (HPAEC-PAD**		**Overall mean (HPAEC-PAD**	
	6	1119	1073	1153	1158	1126	39.1	3.5%	**All data)**	1112	**Exc. Participant 7)**	1042
	7[#]	1589	1558	1458	1513	1530	56.8	3.7%	**SD**	212.6	**SD**	116.5
	8[#]	851	842	879	909	870	30.2	3.5%	**CV**	19.1%	**CV**	11.2%
	10[#]*****	925	1010	958	917	953	42.0	4.4%	**95% LCL**	915	**95% LCL**	920
	10[†]	1197	1305	1153	1105	1190	85.2	7.2%	**95% UCL**	1309	**95% UCL**	1165
	11	1024	1018	1038	1065	1036	20.9	2.0%	**n**	7	**n**	6
HPLC	**9**[#]	482	492	527	502	501	19.3	3.9%				
Resorcinol	**4a**[#]	993	1000	1031	980	1001	21.6	2.2%	**Overall mean (Resorcinol**			
	4b[#]	1033	1073	1006	967	1020	44.4	4.4%	**All data)**	1015		
	6[#]	1013	976	1128	1157	1069	87.7	8.2%	**SD**	66.3		
	7[#]	1017	1032	953	969	993	37.7	3.8%	**CV**	6.5%		
	10[#]	1016	1007	1044	991	1015	22.0	2.2%	**95% LCL**	953		
	11[#]	889	844	926	928	897	39.4	4.4%	**95% UCL**	1076		
	12	1055	1116	1095	1168	1109	47.1	4.2%	**n**	7		
Anthrone	**9**[#]	527	531	510	497	516	15.3	3.0%				
Nephelo-metry	**10**	1155	1250	1170	1195	1193	41.7	3.5%				

Table 12 MenY polysaccharide content determined by other methods. Values given as µg MenY polysaccharide per ampoule. [#]Values from these participants were determined using Glc, Gal or sialic acid standards and were converted to MenY polysaccharide with a mean value of 51.58% *O*-acetylation corresponding to a residue weight of 494.110 g/mol. * Glc/Gal standard [†] Polysaccharide standards

Method	Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics		Summary Statistics	
HPAEC-PAD	**5**	1060	1070	1006	948	1021	56.2	5.5%	**Overall mean (HPAEC-PAD**		**Overall mean (HPAEC-PAD**	
	6	1031	1057	1114	1108	1077	40.1	3.7%	**All data)**	1067	**Exc. Participant 7)**	991
	7[#]	1535	1550	1499	1499	1521	25.6	1.7%	**SD**	228.1	**SD**	119.9
	8[#]	847	829	908	836	855	36.4	4.3%	**CV**	21.4%	**CV**	12.1%
	10[#]*****	858	847	820	810	834	22.4	2.7%	**95% LCL**	856	**95% LCL**	865
	10[†]	1174	1159	1095	1082	1127	45.9	4.1%	**95% UCL**	1278	**95% UCL**	1117
	11	1018	1052	1027	1032	1032	14.4	1.4%	**n**	7	**n**	6
HPLC	**9**[#]	934	1132	1011	966	1011	86.73	8.6%				
Resorcinol	**4a**[#]	1036	1024	1061	1009	1032	21.9	2.1%	**Overall mean (Resorcinol**		**Overall mean (Resorcinol**	
	4b[#]	1096	1050	847	803	949	145.5	15.3%	**All data)**	998	**Exc. Participant 12**	958
	6	1004	1029	920	987	985	46.7	4.7%	**SD**	122.4	**SD**	71.3
	7[#]	899	920	876	939	908	27.1	3.0%	**CV**	12.3%	**CV**	7.4%
	10[#]	1039	1032	1070	966	1027	43.5	4.2%	**95% LCL**	884	**95% LCL**	884
	11[#]	855	861	851	829	849	13.7	1.6%	**95% UCL**	1033	**95% UCL**	1033
	12	1050	1144	1060	1676	1233	298.8	24.2%	**n**	7	**n**	6
Anthrone	**9**[#]	1008	1013	989	976	996	17.1	1.7%				
Nephelometry	**10**	1140	1110	1175	1200	1156	39.4	3.4%				

Table 13 Extended qNMR study at NIBSC – measurement of degree of *O*-acetylation of MenW (16/152) and MenY (16/206) candidate standards after de-*O*-acetylation with sodium deuterium oxide.

16/152 Meningococcal serogroup W polysaccharide		**16/206 Meningococcal serogroup Y polysaccharide**	
Ampoule	**% *O*-acetylation**	**Ampoule**	**% *O*-acetylation**
1	58.071	1	51.981
2	58.188	2	49.431
3	57.841	3	50.707
4	58.614	4	50.507
5	58.512	5	51.337
6	59.027	6	50.718
7	58.385	7	51.507
8	57.100	8	52.045
9	57.193	9	51.039
10	57.635		
Mean	58.056	**Mean**	51.030
SD	0.621	**SD**	0.813
CV	1.1%	**CV**	1.6%

Table 14 Degree of *O*-acetylation (%) of MenW polysaccharide determined by qNMR. Analysis was carried out by individual study participants (14a) and re-analysed by one NIBSC analyst (14b).

14a)

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participant 5)	
1	57.60	54.60	55.80	58.60	56.65	1.8	3.2%	**Overall mean**	52.7	**Overall mean**	56.2
2	56.97	57.35	56.70	58.13	57.29	0.6	1.1%	**SD**	8.6	**SD**	1.4
4	54.69	55.87	53.45	51.63	53.91	1.8	3.4%	**CV**	16.3%	**CV**	2.5%
5	32.10	36.50	34.10	38.70	35.35	2.9	8.1%	**95% LCL**	43.7	**95% LCL**	54.5
6	57.18	57.50	56.83	57.25	57.19	0.3	0.5%	**95% UCL**	61.8	**95% UCL**	57.9
7	56.00	54.00	56.00	58.00	56.00	1.6	2.9%	**n**	6	**n**	5

14b)

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (Exc. Participant 3)		Summary Statistics (Exc. Participant 3 and 5)	
1	55.42	60.83	55.44	56.89	57.14	2.6	4.5%	**Overall mean**	61.6	**Overall mean**	58.50
2	49.92	52.62	49.07	50.16	50.44	1.5	3.0%	**SD**	9.2	**SD**	5.6
3	64.24	62.28	n/t	n/t	63.26	n/c	n/c	**CV**	14.9%	**CV**	9.6%
4	63.51	65.37	57.99	65.14	63.00	3.4	5.5%	**95% LCL**	52.0	**95% LCL**	51.5
5	88.50	71.62	75.65	73.41	77.30	7.6	9.9%	**95% UCL**	71.3	**95% UCL**	65.5
6	57.18	57.50	56.83	57.25	57.19	0.3	0.5%	**n**	6	**n**	5
7	61.99	63.70	65.31	67.88	64.72	2.5	3.9%				

Table 15 Degree of *O*-acetylation (%) of MenY polysaccharide determined by qNMR. Analysis was carried out by individual study participants (15a) and re-analysed by one NIBSC analyst (15b). *Ampoule 1 from Participant 2

15a)

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. *)	
1	52.20	52.10	54.90	51.00	52.55	1.7	3.2	**Overall mean**	50.6	**Overall mean**	50.2
2	67.25*****	56.18	54.76	55.88	58.52	5.9	10.0	**SD**	5.2	**SD**	4.4
4	49.26	52.17	53.29	52.68	51.85	1.8	3.4	**CV**	10.3%	**CV**	8.7%
5	41.00	43.50	43.40	45.60	43.38	1.9	4.3	**95% LCL**	45.2	**95% LCL**	45.6
6	49.49	51.55	51.69	50.51	50.81	1.0	2.0	**95% UCL**	56.1	**95% UCL**	54.8
7	47.00	46.00	47.00	47.00	46.75	0.5	1.1	**n**	6	**n**	6

15b)

Lab	Ampoule 1	Ampoule 2	Ampoule 3	Ampoule 4	Mean	SD	CV	Summary Statistics (Exc. Participant 3)		Summary Statistics (Exc. Participants 3 and 5)	
1	53.22	50.78	52.56	50.17	51.68	1.4	2.8%	**Overall mean**	54.1	**Overall mean**	51.6
2	52.10	46.78	47.25	49.73	48.97	2.5	5.0%	**SD**	6.3	**SD**	1.9
3	47.27	46.35	n/t	n/t	46.81	n/c	n/c	**CV**	11.7%	**CV**	3.6%
4	54.18	51.03	56.85	54.02	54.02	2.4	4.4%	**95% LCL**	47.4	**95% LCL**	49.2
5	63.35	63.22	63.69	75.87	66.53	6.2	9.4%	**95% UCL**	60.7	**95% UCL**	53.9
6	49.49	51.55	51.69	50.51	50.81	1.0	2.0%	**n**	6	**n**	5
7	51.50	52.62	53.95	51.61	52.42	1.1	2.2%				

		Sample A (MenW)										Sample C (MenY)									
Method using		**(1)**	**(2)**	**(3)**	**(4)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**	**(1)**	**(2)**	**(3)**	**(4)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**
i) MenW candidate standard																					
HPAEC-PAD-	5	406	422	400	419	412	2.5														
	6	475	523	504	498	500	4.0														
	7	363	395	389	381	382	3.6			428	9.3										
	8	407	458	462	433	440	5.8														
	10	436	425	405	406	418	3.7														
	11	428	393	433	410	416	4.4	419	9.2												
Resorcinol	4	250	305	363	501	355	30.3					207	248	312	428	299	32.3				
	6	399	417	408	394	404	2.5					338	371	332	339	345	5.1				
	7	408	371	442	442	416	8.1			411	9.4	346	314	340	351	338	4.8	326	4.9	326	4.9
	10	417	428	467	449	440	5.0					327	313	343	326	327	3.7				
	11	356	402	414	375	387	6.9					296	329	345	317	322	6.4				
	12	451	485	362	554	463	17.3					322	346	281	361	327	10.7				
HPLC	9	357	447	446	420	418	10.1			418		N/A	N/A	N/A	N/A	n/c	n/c				
Anthrone	9	440	391	329	333	373	14.1			373		842	677	591	606	679	16.9			679	
Nephelometry	10	395	414	447	421	419	5.2			419		N/A	N/A	N/A	N/A	n/c	n/c				
ii) MenY candidate standard																					
HPAEC-PAD	5											363	373	373	375	371	1.4				
	6											402	370	385	377	383	3.5				
	7											333	347	360	332	343	3.8			373	6.2
	8											347	344	361	358	352	2.4				
	10[#]											380	371	403	385	385	3.5				
	11											419	428	387	390	406	5.0	361	14.5		
Resorcinol	4	227	265	297	346	284	17.7					188	214	255	296	238	19.8				
	6	418	407	415	403	411	1.7					340	351	359	341	348	2.6				
	7	416	444	455	480	449	5.9	437	25.5	437	25.5	347	355	353	382	359	4.3			349	20.6
	10	439	430	457	450	444	2.6					355	364	350	334	351	3.5				
	11	443	384	412	387	406	6.7					372	318	336	318	336	7.6				
	12	580	691	600	643	628	7.8					437	503	442	478	465	6.7				
HPLC		N/A	N/A	N/A	N/A	n/c	n/c					208	193	178	150	182	13.6			182	
Anthrone	9	227	163	176	183	187	15.0			187		436	269	299	322	331	22.0			331	
Nephelometry	10	N/A	N/A	N/A	N/A	n/c	n/c					327	347	359	349	345	3.8			345	
iii) In-house standard																					
HPAEC-PAD	5	400	410	390	389	397	2.5					339	332	347	350	342	2.4				
	6	519	636	552	597	576	8.9					472	437	442	416	441	5.2				
	7	390	390	392	395	392	0.5			412	20.5	339	346	350	345	345	1.3			349	18.1
	8	321	324	341	327	328	2.6					263	235	256	262	254	5.0				
	10[#]	399	381	373	401	388	3.5					323	324	329	321	324	1.0				
	11	413	343	413	382	388	8.6	405	17.8			393	420	344	403	390	8.4				
Resorcinol	4	256	273	299	335	291	11.7					212	222	256	298	247	15.8	341	16.8		
	6	427	417	436	427	427	1.8					353	392	347	357	362	5.6				
	7	398	373	415	413	400	4.8			399	16.3	334	294	315	324	317	5.4			333	16.7
	10	419	423	420	428	422	0.9					360	340	341	336	344	3.2				
	11	372	375	377	352	369	3.1					321	313	317	306	314	2.0				
	12	493	462	416	564	484	12.8					446	389	436	385	414	7.6				
HPLC	9	324	404	404	380	378	9.9			378		328	302	278	230	285	14.6			285	
Anthrone	9	229	169	206	197	200	12.2			200		439	283	365	355	360	17.7			360	
Nephelometry	10	449	508	496	459	478	5.9			478		434	409	400	391	408	4.5			408	

Table 16 Estimate of concentrations of Sample A and Sample C using candidate polysaccharide and in-house standards.[#]Glc/Gal in-house standards

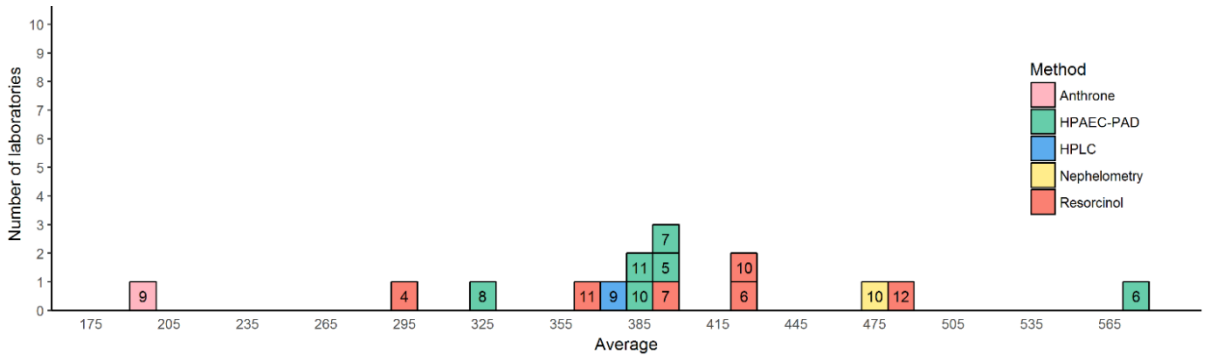
Table 17 MenW polysaccharide content (% of baseline -70°C sample) of 16/152 by HPAEC-PAD for the lyophilized real-time, accelerated thermal degradation and reconstituted samples.

Storage time (months)	MenW PS content (mg MenW PS/ampoule)	MenW PS content (mg MenW PS/ampoule) % -70°C sample				
	-70°C	-20°C	+20°C	+37°C	+56°C	-20°C Reconstituted
0	1.190					
1	1.110	106.3	104.1	102.3	103.2	101.4
2	1.389	88.5	90.6	83.0	88.2	88.8
3	1.356	97.1	98.1	94.5	99.6	100.1
6	1.285	107.7	100.7	104.4	107.8	107.0
12	1.282	82.9	97.6	98.8	104.2	103.7

Table 18 MenY polysaccharide content (% of baseline -70°C sample) of 16/206 by HPAEC-PAD for the lyophilized real-time, accelerated thermal degradation and reconstituted samples.

Storage time (months)	MenY PS content (mg MenY PS/ampoule)	MenY PS content (mg MenY PS/ampoule) % -70°C sample				
	-70°C	-20°C	+20°C	+37°C	+56°C	-20°C Reconstituted
0	1.121					
1	1.239	99.9	98.6	105.0	106.5	104.2
2	0.999	105.4	111.0	113.2	112.1	105.5
3	1.165	91.1	97.4	97.5	96.4	98.5
6	1.233	98.5	95.3	95.4	97.3	96.6
12	1.076	100.5	97.2	97.3	101.7	101.6

A



B

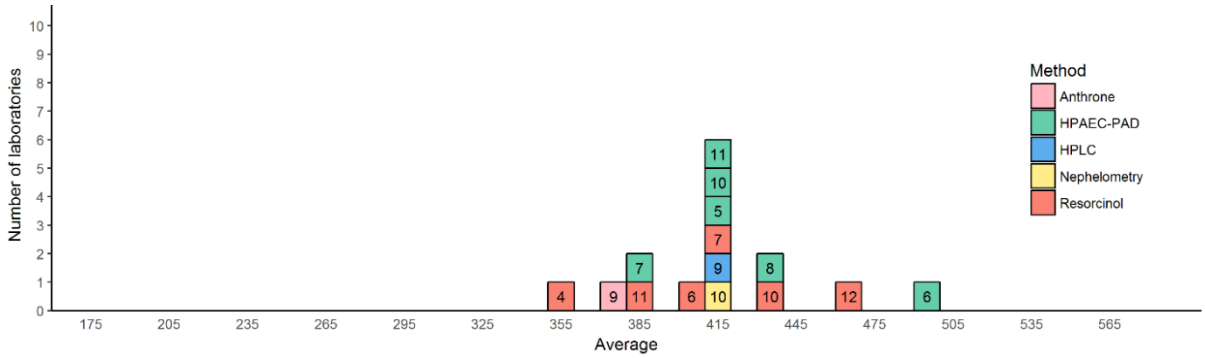


Figure 1 Distribution and frequency of estimates for MenW polysaccharide concentration (µg polysaccharide/ml) for Sample A using in-house standards (A) and candidate standard 16/152 (B)

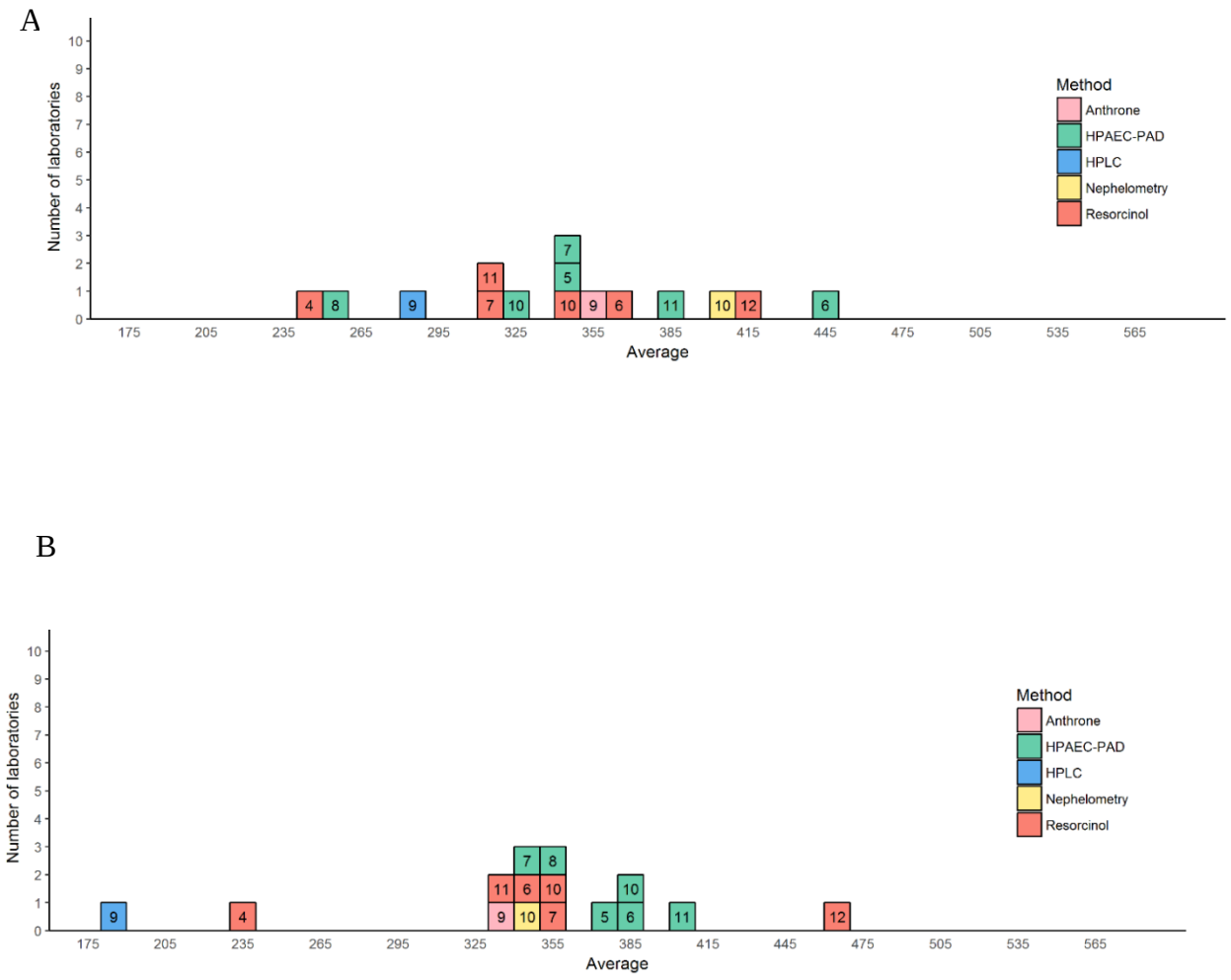


Figure 2 Distribution and frequency of estimates for MenY polysaccharide concentration (µg polysaccharide/ml) for Sample C using in-house standards (A) and candidate standard 16/206 (B)

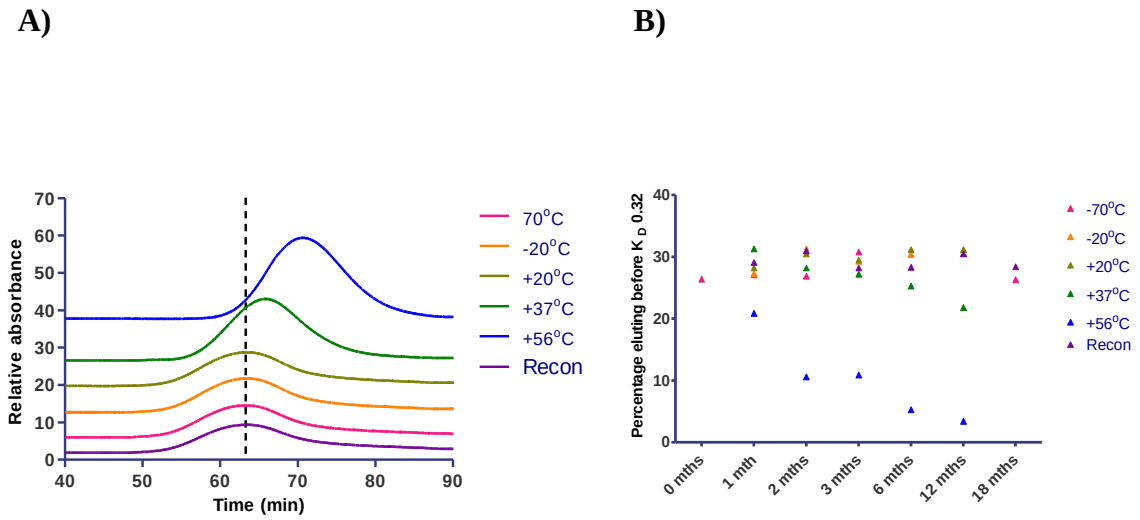


Figure 3 Molecular sizing chromatograms of the 16/152 MenW PS at 12 months following real-time, accelerated thermal degradation and reconstituted MenW PS (A). Using V_o and V_t from the column marker on the HPLC-SEC, the percentage of MenW PS eluted by a K_D of 0.32 (as indicated by the vertical line in Figure 3A) can be determined for each storage temperature B).

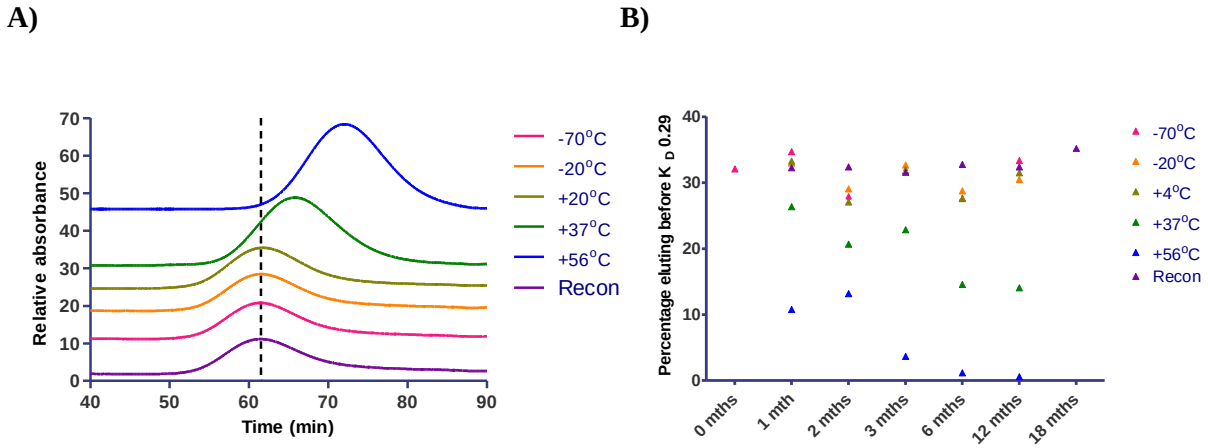


Figure 4 Molecular sizing chromatograms of the 16/206 MenY PS at 12 months following real-time, accelerated thermal degradation and reconstituted MenY PS (A). Using V_o and V_t from the column marker on the HPLC-SEC, the percentage of MenY PS eluted by a K_D of 0.29 (as indicated by the vertical line in Figure 4A) can be determined for each storage temperature B).

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
1st WHO International Standard for Meningococcal Serogroup W Polysaccharide
NIBSC code: 16/152
Instructions for use
(Version 1.00, Dated)

Not for in vitro diagnostic use

1. INTENDED USE

The freeze-dried preparation of Neisseria meningitidis serogroup (capsular group) W capsular polysaccharide (MenW), provided by GSK Vaccines S.r.l., Italy was prepared in ampoules in 2016 at the Centre for Biological Reference Materials (CBRM), NIBSC and coded 16/152. A collaborative study was carried out on this material by twelve laboratories in 2017/2018 to determine the MenW content in SI units by quantitative 1H (proton) nuclear magnetic resonance spectroscopy, and to evaluate its suitability for use as a standard for quantification of MenW in bulk MenW polysaccharide conjugate material in mainly HPAEC-PAD and Resorcinol assays. Other assays (HPLC, Anthrone and Nephelometry) were performed also for the study, although users should verify its suitability and determine the uncertainly of measurement in their specific assay. NIBSC, Potters Bar, UK is the custodian and distributor of this material.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

Not human or bovine source material

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The first WHO International standard for Meningococcal Group W polysaccharide 16/152, has a content of 0.925 ± 0.138 mg polysaccharide/ampoule as determined by quantitative 1H (proton) nuclear magnetic resonance spectroscopy. The residue weight of MenW PS is 497.020 g/mol with a degree of O-acetylation of 58.50%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each ampoule contains the freeze dried powder of 1 ml of MenW PS in water, at a nominal concentration of 1 mg/ml. The moisture content is 0.37%, as determined by thermogravimetric analysis.

5. STORAGE

Ampoules should be stored at or below -20ºC
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Din Ampoule
Please complete this section when choosing 'other' from the drop-down above

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
Resuspend the contents of the ampoule in 1 ml of distilled water. To ensure complete solubilisation of the material, reconstitute the material 24 hours prior to use. Allow to dissolve for 4 hours at room temperature then transfer to 4ºC for the remaining time. The reconstituted material should be aliquoted and frozen at or below -20ºC. The standard can be used directly as a reference in the physico-chemical assays or for calibrating of secondary standards.
This MenW standard is 58.5% O-acetylated, and is appropriate for the measurement of the MenW content of material that has a similar O-acetylation level. If the standard is to be used for measuring the MenW content of a sample with a different degree of O-acetylation, a correction factor will have to be used.

8. STABILITY (Add or amend as necessary)

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.
Accelerated degradation studies revealed the freeze dried standard to be stable up to one year at 37ºC (as determined by HPAEC-PAD to measure the polysaccharide content of the material reconstituted with water).
Real-time and extended accelerated thermal degradation studies are on-going.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

Hannah Chan, Timothy Rudd, Peter Rigsby, Fang Gao, Nicola Beresford and the MenW/MenY IS Working Group. Evaluation of Candidate International Standards for Meningococcal Serogroup W and Y Polysaccharides. WHO/BS/2018.2336.

10. ACKNOWLEDGEMENTS

We would like to thank GSK Vaccines S.r.l. Italy for their gift of the polysaccharide used to make this standard.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

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In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

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14. MATERIAL SAFETY SHEET (Add or amend as necessary)
Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried, white powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	No special handling precautions		
Toxicological properties			
Effects of inhalation:	Not established, avoid inhalation		
Effects of ingestion:	Not established, avoid ingestion		
Effects of skin absorption:	Not established, avoid contact with skin		
Suggested First Aid			
Inhalation:	Seek medical advice		
Ingestion:	Seek medical advice		
Contact with eyes:	Wash with copious amounts of water. Seek medical advice		
Contact with skin:	Wash thoroughly with water		
Action on Spillage and Method of Disposal			
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.			

15. LIABILITY AND LOSS
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Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 5g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS
NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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NIBSC Confidence in Biological Medicines

WHO International Standard
Ist WHO International Standard for Meningococcal Serogroup Y Polysaccharide
NIBSC code: 16/206
Instructions for use
(Version 1.00, Dated)

Not for in vitro diagnostic use

1. INTENDED USE

The freeze-dried preparation of Neisseria meningitidis serogroup (capsular group) Y capsular polysaccharide (MenY), provided by GSK Vaccines S.r.l., Italy was prepared in ampoules in 2016 at the Centre for Biological Reference Materials (CBRM), NIBSC and coded 16/206. A collaborative study was carried out on this material by twelve laboratories in 2017/2018 to determine the MenY content in SI units by quantitative 1H (proton) nuclear magnetic resonance spectroscopy, and to evaluate its suitability for use as a standard for quantification of MenY in bulk MenY polysaccharide conjugate material in mainly HPAEC-PAD and Resorcinol assays. Other assays (HPLC, Anthrone and Nephelometry) were performed also for the study, although users should verify its suitability and determine the uncertainly of measurement in their specific assay. NIBSC, Potters Bar, UK is the custodian and distributor of this material.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

Not human or bovine source material

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The first WHO International standard for Meningococcal Group Y polysaccharide 16/206, has a content of 0.949 ± 0.121 mg/ampoule as determined by quantitative 1H (proton) nuclear magnetic resonance spectroscopy. The residue weight of MenY PS is 494.110 g/mol with a degree of O-acetylation of 51.58%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each ampoule contains the freeze dried powder of 1 ml of MenY polysaccharide in water, at a nominal concentration of 1 mg/ml. The moisture content is 0.72%, as determined by thermogravimetric analysis.

5. STORAGE

Ampoules should be stored at or below -20ºC
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Din Ampoule
Please complete this section when choosing 'other' from the drop-down above

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
Re-suspend the contents of the ampoule in 1 ml of distilled water. To ensure complete solubilisation of the material, reconstitute the material 24 hours prior to use. Allow to dissolve for 4 hours at room temperature then transfer to 4ºC for the remaining time. The reconstituted material should be aliquoted and frozen at or below -20ºC. The standard can be used directly as a reference in the physico-chemical assays or for calibrating of secondary standards.
This MenY standard is 51.58% O-acetylated, and is appropriate for the measurement of the MenY polysaccharide content of material that has a similar O-acetylation level. If the standard is to be used for measuring the MenY content of a sample with a different degree of O-acetylation, a correction factor will have to be used,

8. STABILITY (Add or amend as necessary)

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.
Accelerated degradation studies revealed the freeze dried standard to be stable up to one year at 37ºC (as determined by HPAEC-PAD to measure polysaccharide content of the material reconstituted in water).
Real-time and extended accelerated thermal degradation studies are on-going.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

Hannah Chan, Timothy Rudd, Peter Rigsby, Fang Gao, Nicola Beresford and the MenW/MenY IS Working Group. Evaluation of Candidate International Standards for Meningococcal Serogroup W and Y Polysaccharides. WHO/BS/2018.2336.

10. ACKNOWLEDGEMENTS

We would like to thank GSK Vaccines S.r.l. Italy for their gift of the polysaccharide used to make this standard.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

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14. MATERIAL SAFETY SHEET (Add or amend as necessary)
Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried, white powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	No special handling precautions		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS
In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
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