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Evaluation of Candidate International Standards for Meningococcal Capsular Group 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 **27 September 2019** 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 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).

At the time of inception, the intention of this study was to assign unitage of these standards by quantitative nuclear magnetic resonance spectroscopy. An original report (WHO/BS/2018.2336) submitted to the WHO Expert Committee on Biological Standardization in 2018 detailed this proposal. However, an alternative proposal to assign unitage by the Resorcinol assay was also put forward at the meeting of the ECBS in November 2018. This report is now updated to reflect the change to the proposal.

Our proposals, on the basis of data from the Resorcinol assay used in this study, are: 1) candidate standard for MenW polysaccharide (16/152) to be assigned a content of 1.015 ± 0.071 mg MenW polysaccharide per ampoule (expanded uncertainty with coverage factor k = 2.13, corresponding to a 95% level of confidence) and 2) candidate standard for MenY polysaccharide (16/206) be assigned a content of 0.958 ± 0.076 mg MenY polysaccharide per ampoule (expanded uncertainty with coverage factor k = 2.26, corresponding to a 95% level of confidence).

The amount of polysaccharide per ampoule remained consistent under all conditions over a 24-month period. 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. Real time stability and accelerated thermal degradation studies are on-going.

Introduction

Meningococcal serogroups were originally defined based on the serological reactivity of their capsular polysaccharide. Nuclear magnetic resonance spectroscopy (NMR) has since enabled chemical and structural definition these polysaccharides; furthermore, the genes involved in capsule synthesis have been determined [1]. In this report, the serogroup terminology is referred to as capsular group.

Meningococcal polysaccharide conjugate vaccines have been in use for over 20 years. Prior to this, plain polysaccharide vaccines in various combinations had been available. The first licensed meningococcal conjugate vaccine only contained MenC, but since then MenA, MenW (formerly known as MenW135 [1]) and MenY polysaccharide conjugates have been added to produce tetravalent formulations, with MenX conjugates also under 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 capsular groups including MenB, MenW and MenY. In recent years there has been an increasing focus on disease caused by MenW isolates, which has been particularly notable in older age groups. Outbreaks of MenW disease have increased globally, particularly in South American countries [2], but more recently in Europe. In Europe an exponential increase in cases of MenW disease, starting in 2009, led to the introduction of the MenACWY conjugate vaccine in a number of countries [3]. Historically, disease caused by capsular group A meningococci 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 [4-6]. Although MenY features as less of a global disease burden, epidemiology is closely monitored in Europe, due to several countries reporting significant increases in the incidence of disease caused by this capsular group [3, 7] and remains the third leading cause of meningococcal disease in the United States of America, after MenB and MenC [8]. The epidemiology of the different capsular groups across different geographic regions is notable and the patterns are ever-changing, thus prevention through vaccination against as many capsular 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→ [9]. The degree of *O*-acetylation of sialic acid is variable at position C7 and C9 [10]. 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 [11] and High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) [12]. Both methods are recognized by the European Pharmacopoeia, United States Pharmacopoeia and the WHO as suitable methods for the testing of polysaccharide-conjugate vaccines [13-15].

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 capsular

group C polysaccharides were assigned in SI units, using secondary physicochemical methods, namely the Orcinol assay for determination of ribose content and the Resorcinol assay for the determination of sialic acid content, respectively [16-18]. A primary method, as defined by the Bureau International des Poids et Mesures, 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" [19, 20]. Since the MenC and Hib polysaccharides were assigned unitage using secondary methods, quantitative ^{1}H-NMR (qNMR) was 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 [21, 22]. MenA, MenX and Vi (for typhoid) polysaccharide International Standards were assigned unitage using qNMR. [23, 24]. Furthermore, the MenW and MenY polysaccharides have been extensively characterized by ^{1}H-NMR [10, 25]. The proposal to assign unitage based on 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 WHO Expert Committee on Biological Standardization (ECBS) in 2015 [26].

A collaborative study was performed by twelve laboratories to determine the polysaccharide content of candidate International Standards for meningococcal capsular group W and Y polysaccharides by qNMR and other physicochemical assays, with the intention that mass unitage assignment be determined by qNMR. A report (WHO/BS/2018.2336) was submitted to the ECBS in August 2018 with a proposal to assign unitage by qNMR and was considered in a meeting of the Committee held from 29 October to 2 November 2018. The data presented showed the expanded uncertainty on the measurement on the assigned unitage by qNMR was 14.9 and 12.8% for the MenW and MenY polysaccharides respectively. These values were higher than had been determined for the establishment of International Standards for MenA, MenX and Vi polysaccharides. Data from the Resorcinol assay were also presented at the meeting of the ECBS with an alternative option to assign unitage by this method. The close match between the assigned values from the qNMR and Resorcinol assays became apparent. The expanded uncertainty for the Resorcinol assay was, however, much lower. The reasons for the high uncertainty in the qNMR were attributed to the chemical structures of these polysaccharides which result in NMR spectra with broad peaks that are difficult to integrate, particularly when samples are not de-*O*-acetylated.

Upon review of these data, the ECBS supported the assignment of mass unitage of the MenW and MenY candidate standards by the Resorcinol assay, instead of qNMR, based on the better precision. The Committee deferred any decision on the standards and recommended that, in consultation with study participants, the study report be significantly updated with the new proposal to assign to unitage by the Resorcinol assay [27]. We hereby submit the revised report and new proposal with agreement from study participants.

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 in 2014 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 spectrometry, six laboratories performed the Resorcinol assay, six laboratories performed

HPAEC-PAD 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 evaluated as candidate 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 vaccine-grade material. 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 polysaccharides, 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 to approximately 1 mg/ml by stirring for at least 4 hours at room temperature before transferring to 4°C overnight. The polysaccharides were dispensed into glass ampoules 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 coefficients 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 the 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. Participants using other physicochemical methods were also asked to assign unitage to the candidate standards. The polysaccharide concentration was

reported in µg polysaccharide per ampoule for the purpose of data analysis, although final assignment of unitage is stated in mg per ampoule. Participants were requested to measure the polysaccharide content of four ampoules of each polysaccharide (two ampoules tested on two different days). 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. Values obtained using the candidate standards were reported after adjustment for molecular mass determination of each standard, as reported in Annex 2. The identity of each test sample was unknown to the participants at the time of the study. Sample A was a MenW 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 each candidate standard and their own in-house standards in four separate assays. Polysaccharide concentration was reported in µg polysaccharide per ml.

Methods

Quantitative ^{1}H-NMR

Quantitative ^{1}H-NMR relies on different protons 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 [28], caffeine and ascorbic acid and DSS-d_6) and 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 were amended to account for the purity of the standard used.

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.

The participants chose their own methods to perform qNMR, using different internal standards, listed in Table 3, and different peaks to integrate. Participants 1, 2, 4 and 6 all applied a treatment with sodium deuterium oxide (NaOD) to determine the percentage of *O*-acetylation. Participant 2 used the ratio of *N*-acetyl (*N*Ac) to the released free acetate to determine the percentage 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 the released free acetate to determine the percentage of *O*-acetylation of the polysaccharides [25]; both laboratories used Gal/Glc-H1 to determine the total amount polysaccharide in the ampoules. Participant 4 used the ratio of N-Ac to the released free 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 percentage 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 percentage of *O*-acetylation.

NMR data were analyzed by the individual participants' own procedures and later by a single analyst at NIBSC using the following approach. Where the material was untreated by NaOD the peak used to determine the mass of the material was 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 utmost 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.

Resorcinol assay

The resorcinol assay is the current European Pharmacopoeial method for the determination of sialic acid content in polysaccharide vaccines [29] and commonly recognized, alongside HPAEC-PAD, as a suitable method for the determination of MenC, MenW and MenY polysaccharide content in meningococcal vaccines [13-15]. It is a colorimetric assay used to measure sialic acid content after acid hydrolysis of the sample [11]. 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 color can be quantified by measuring absorption at 564 to 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 4. All the laboratories except laboratory 12 used a sialic acid standard and therefore require a conversion factor to quantify the polysaccharide content. Noticeable differences between the laboratories include the standard used and the use of extractant (butyl acetate:butanol used by Participants 7, 10 and 11).

HPAEC-PAD and other HPLC-based methods

The weakly acidic nature of 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 [12].

Six participants performed High Performance Anion Exchange Chromatography with a pulsed amperometric detector, HPAEC-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 Gal / Glc monosaccharide 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 was 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 5. Noticeable differences include the standards used and the milder acid treatment performed by two of the laboratories (Participants 7 and 8).

Participant 9 used a slightly different HPLC method, making use of the Ludger Tag 2-AA Monosaccharide Release and Labeling Kit [30]. 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, galactosamine hydrochloride, galactose, mannose, fucose and glucose.

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 [31]. 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 4, 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 [32]. When the specific antibody is added to the sample, scattered light is measured at an angle of 90° 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 rabbit polyclonal antiserum (Beckton Dickinson) 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 capsular group, using the Beckman Coulter IMMAGE 800 rate nephelometer. The final reportable result is the average of the calculated polysaccharide concentrations (n=2).

Stability studies

Three stability studies of the candidate materials were undertaken: real-time and accelerated thermal degradation studies were performed on the lyophilized material, and an accelerated thermal degradation study was performed on 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 samples stored at -70^{o}C and -20^{o}C was planned at 1, 2, 3, 6, 12, 24, 36, 60 and 120-month time-points. The accelerated thermal 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.

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.

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.

Data on polysaccharide content are presented from real-time, accelerated thermal degradation and reconstitued samples stored up to 24 months for both MenW and MenY.

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 24 months, depending on the study.

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 g/mol (for MenW PS) or 494.110 g/mol (for MenY PS) divided by the functional weight of glucose/galactose (180.156 g/mol) or sialic acid (309.270 g/mol). Data from participants were amended with this conversion if data had been submitted with another conversion factor. 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 in the assays. 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 [33] was used to predict the degradation rate when stored at -20°C.

Results

Polysaccharide content of candidate standards using Quantitative ^{1}H-NMR

NIBSC extended analysis of 16/152 and 16/206

NIBSC performed an extensive analysis of additional ampoules of each candidate standard by qNMR to evidence the homogeneity of the fill. Data for this study are given in Table 6. For the MenW candidate standard the mean content from ten ampoules was 945 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%)

Individual participant analysis of 16/152 and 16/206 by qNMR

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.

Data obtained for MenW and MenY from two laboratories were excluded; 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 of polysaccharide content, by qNMR, of candidate MenW and MenY standards is presented in Tables 9a and 9b, 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.

Participants were consulted on a draft report. Although responding participants agreed to the proposal of assigning unitage by qNMR set out in the draft report following analysis of the original data, two participants encouraged further analysis of the qNMR data by a single analyst at NIBSC. The expectation was that this would serve the purpose of reducing operator variability.

NIBSC analysis of qNMR data from participants

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). There was no improvement on the combined relative standard uncertainty based on the re-evaluation of the data by a single NIBSC analyst; 6.31% for MenW and 5.41% for MenY (Tables 9a and 9b).

When the data were analyzed by the individual participants, 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. This was thought 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-analyzed by a single NIBSC analyst, the lowest estimates of polysaccharide content were obtained from Participants 2 and 7 which used 500 MHz instruments. Some higher estimates were obtained using 400 MHz instruments.

The uncertainty of measurement of polysaccharide content by qNMR (Participant analysis and NIBSC analysis) is shown in Tables 9a (16/152) and 9b (16/206). 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 estimate of content for 16/152 using qNMR was 0.925 ± 0.138 mg MenW polysaccharide per ampoule; expanded uncertainty with coverage factor k = 2.36. The estimate of content for 16/206 by qNMR was 0.950 ± 0.122 mg MenY polysaccharide per ampoule; expanded uncertainty with coverage factor of k = 2.36.

Polysaccharide content of candidate standards using Resorcinol assay

Resorcinol data was submitted by six participants. Data is shown in Table 10 (16/152) and Table 11 (16/206).

The mean estimate of MenY polysaccharide content determined by Participant 12 using the Resorcinol assay was approximately 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 from 12.3% (all data) to 7.4%. For MenW the CV was 6.5% (all data). 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 a relatively simple, Pharmacopoeial method, but also due to the use of the same standard material (sialic acid supplied by Sigma) by four of the six participants.

From the data obtained using the Resorcinol assay the mean MenW polysaccharide content of 16/152 was calculated to be 1015 µg polysaccharide/ampoule (CV=6.5%, n=7); and for the mean MenY polysaccharide content of 16/206 was calculated to be 958 µg polysaccharide/ ampoule (after exclusion of Participant 12 data, CV=7.4%, n=6).

Mean estimates of polysaccharide content determined by Resorcinol assays were approximately 10% higher than the mean value obtained by qNMR for MenW. For MenY values determined by the Resorcinol assay were less than 5% higher than the mean value determined by qNMR.

Polysaccharide content of candidate standards using other methods

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) and the HPLC, Anthrone and Nephelometry assays were each performed by one participant. Estimates of polysaccharide content per ampoule for candidate MenW and MenY standards, determined by methods other than qNMR, are given in Tables 10 and 11, respectively. Participant 4 reported two sets of data (denoted as 4a and 4b) using their sialic acid standard.

Estimates of polysaccharide content for 16/152

The mean MenW polysaccharide content was calculated to be 1042 µg polysaccharide/ampoule (after exclusion of Participant 7 data, statistical outlier, CV=11.2%, n=6) using HPAEC-PAD, 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.

Estimates of polysaccharide content for 16/206

Mean MenY polysaccharide content was calculated to be 991 µg/ampoule (with exclusion of Participant 7, statistical outlier, CV = 12.1%, n=6) using HPAEC-PAD, 1011 µg/ml using the HPLC assay, 996 µg polysaccharide/ampoule using the Anthrone assay and 1156 µg/ml using the Nephelometry assay.

Similar to the Resorcinol assay, mean estimates of polysaccharide content using the HPAEC-PAD were around 10% higher than the mean value obtained by qNMR for MenW; and for MenY, values determined by HPAEC-PAD were similar to the mean value obtained by qNMR. Values determined by HPAE-PAD were within the expanded uncertainty limits determined by qNMR (95% confidence) for both candidate standards. 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 20% 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 within the same laboratory) 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 and 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 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.

Assignment of unitage to 16/152 and 16/206

The ten participants responding to the original report agreed with the proposal to have all the qNMR data re-analyzed by a single NIBSC analyst; this was duly performed, with a view to assign unitage by qNMR data analyzed by the single analyst. Overall, there was relatively 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 were limited. Although the mass unitage assignments of other recent polysaccharide International Standards have been determined by qNMR, the expanded uncertainty on the qNMR method was around twice that determined for both the Resorcinol assay and the qNMR methods used for previous studies.

Following on from discussion of the meeting of the ECBS in November 2018, the Resorcinol assay was deemed to be the preferable method by which to assign unitage, due to the lower expanded uncertainty, in combination with the close agreement of estimates for polysaccharide content determined by qNMR and Resorcinol. Consequently, we propose that assignment of polysaccharide content to the candidate standards 16/152 and 16/206 is made by the Resorcinol assay. The MenW and MenY polysaccharide standards would therefore be traceable to the sialic acid standards used to assign their values. The decision taken at the meeting of the ECBS in November 2018 was to accept this proposal.

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 determined by the Resorcinol

assay was 1.015 ± 0.071 mg MenW polysaccharide per ampoule; expanded uncertainty with coverage factor k = 2.13 (Table 12a). The final estimate of content for 16/206 determined by the Resorcinol assay was 0.958 ± 0.076 mg MenY polysaccharide per ampoule; expanded uncertainty with coverage factor of k = 2.26 (Table 12b).

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 of ten ampoules of 16/152 and nine ampoules of 16/206, and by six of seven participants also performing this method for the first part of the study. All data to determine the percentage *O*-acetylation also were re-analyzed by NIBSC using a consistent approach.

Degree of O-acetylation and functional weight 16/152

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%, Table 14a) and compares favorably to the estimate of 58.1% *O*-acetylation obtained from the extended study conducted by NIBSC on ten ampoules Table 13. Upon applying the harmonized method for determining the % *O*-acetylation, a value of 58.5% was obtained, Table 14b. 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 MenY, where the mean percent *O*-acetylation was a statistical outlier. Based on the mean percentage of *O*-acetylation of 58.5%, the functional weight of the MenW PS candidate IS is 497.020 g/mol (Annex 2).

Degree of O-acetylation and functional weight 16/206

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%, Table 15a) and is in good agreement with the estimate of 51.0% obtained from the NIBSC extended study of nine ampoules, Table 13. Upon applying the harmonized method for determining the % *O*-acetylation, a value of 51.6% was obtained, Table 15b. 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 of 51.6%, 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 vaccine-relevant 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 (polysaccharide conjugate samples) containing MenW and MenY polysaccharides respectively. Table 16 reports the polysaccharide concentration measured in each sample using the different methods. Sample B (MenA polysaccharide) 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 17.3% obtained using in-house standards (Table 16 and Figure 2). 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=18.9% and 16.3% for HPAEC-PAD and Resorcinol respectively using in-house standards; CV=9.3 and 9.4% for HPAEC-PAD and Resorcinol, respectively, using the candidate standards). The overall mean polysaccharide concentration determined by the using 16/152 (MenW polysaccharide) was around 4% higher than that obtained using in-house 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=24.8%) 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 standard over the in-house standard.

Variability in Estimates of MenY polysaccharide concentration from Sample C

With the exception of HPLC data from Participant 9 and Resorcinol data from Participants 4 and 12, greater harmonization of estimates of polysaccharide concentration in Sample C (MenY polysaccharide conjugate) was achieved using the candidate MenY standard compared to when in-house standards were used (Figure 3) and indicates a benefit to using the candidate MenY standard. Considering all HPAEC-PAD and Resorcinol data, there is a marginal decrease in variability overall from 17.2%, using the in-house standard, to 14.5% when the candidate MenY standard is used (Table 16). The high variability (CV) in the HPAEC-PAD assay using the candidate standards was significantly reduced from 17.9% (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 27% 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. 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 3 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. Mean values for the polysaccharide concentration of Sample C were essentially the same when determined using either 16/206 or in-house standards.

Interestingly, the data obtained for the Resorcinol assay using the candidate MenW standard to measure MenY content in Sample C were considerably less variable (CV=4.9%) than when the homologous, MenY candidate standard was used (20.6%).

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, 12 and 24 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 lyophilized candidate standards remained relatively constant over this period when stored at -20°C, the designated storage temperature. Similarly, saccharide content remained unaffected in reconstituted samples that were stored at -20°C for up to 24 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 both candidate standards remained stable after storage at 20°C for 12 months but was affected by high temperature storage. Figures 4 and 5 demonstrate a decrease in the percent polysaccharide eluting before the designated coefficient (K_D) after storage at 37°C and 56°C for 1-12 months, due to depolymerization.

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 6.0-7.1 when reconstituted with 1 ml sterile distilled water. The pH range of MenY polysaccharide 16/152 is 5.2-7.5 when reconstituted with 1 ml sterile distilled water. There was no discernible trend over time.

Discussion

Physicochemical assays are primarily used to estimate total polysaccharide in meningococcal polysaccharide/conjugate vaccines. Differences in assays performed, 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 six methods were utilized across the 12 laboratories to measure the polysaccharide content of candidate materials 16/152 and 16/206. Of these methods, seven laboratories performed qNMR, six laboratories each performed HPAEC-PAD and the Resorcinol assay, with only one laboratory each running the HPLC, Anthrone or Nephelometry assays; thereby ruling out the latter three as methods to define the unitage.

NIBSC have produced seven WHO International Standards for bacterial polysaccharides. Those most recently produced, Men A, Men X and the Vi polysaccharides, were assigned unitage based on the qNMR data of multiple laboratories [23, 24]. As unitage is assigned in SI units (mg/vial) and not International Units of activity, a primary method should be used for the measurement directly determining the quantity of polysaccharide in the ampoule rather than measuring as a ratio of another quantity. Quantitative NMR can be considered a primary method and the quantitative estimations can be made without the requirement for a specific reference standard. ECBS therefore endorsed qNMR as a preferred approach for assigning unitage, in mg, to polysaccharides and this method was intended to be used for the W and Y polysaccharides.

As unitage assignment by a secondary method deviates from the philosophy held at NIBSC that a primary method should provide a more robust and accurate means to assign unitage, underlying problems which are responsible for the variability seen in the qNMR measurements were investigated. 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. Although a proposed general chapter of the United States Pharmacopoeia [34] suggests, at a minimum, a 400 MHz spectrometer should be sufficient, the lowest estimates of polysaccharide content were obtained on 500 MHz instruments, following analysis performed by the NIBSC analyst. Higher field strengths contribute to the production of sharper, more well-defined peaks. However, 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. Such variability in the use of the qNMR technique and the necessity for a standardized method has previously been recognized [35].

The type and quality of the internal standard used in qNMR are important contributory factors to the accuracy of NMR data. The production of high-quality NMR standards, or certified reference materials (CRMs), is a growing field [21, 28]. Of crucial importance is that the internal standards used have a defined and high purity, defined uncertainty and are SI-traceable. These attributes are 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) [28]. Although NIBSC obtained highly consistent data for the extended study on the candidate standards, the internal standard did not satisfy the requirement above, thereby making value assignment based on data from only one laboratory inappropriate. Furthermore, high levels of moisture in the standards used may have an influence and ideally, moisture content should be accounted for when performing calculations. Full information on the quality of the standards used for the study was not available. In the case of the internal standard used for the NIBSC measurements (this study and MenA and Vi polysaccharide studies), TSP was lyophilized before use, ensuring that the contribution of moisture was minimized. Likewise, to minimize moisture content, 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. Alongside NIBSC, two other participants used TSP as an internal standard and another laboratory used DSS, which is similar to TSP.

Alongside the choice of standard, the sample preparation, NMR parameters used and equipment operation (for example the number of scans and shimming to correct for non-homogeneity 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 (*O*-acetylated), 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.

By comparison, the variability in estimates for polysaccharide content by qNMR was higher than obtained for measurement of other polysaccharide standards (MenA, MenX, Vi) [23, 24]. Although this may be attributable to the non-homogeneity of the instrumentation used for this study compared to those instruments used in previous studies, there are likely several factors contributing to the overall variability. Additional chemical complexity of the analytes has been introduced to this particular study; MenW and MenY are partially *O*-acetylated disaccharide subunits, whereas MenA, MenX and Vi polysaccharides are composed of repeating monosaccharide subunits [10]. Furthermore, the capsular polysaccharides of groups W and Y are known to have variable *O*-acetylation [36-38], 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 with the expectation that more accurate measurements would be obtained.

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 (WHO/BS/2018.2336) following on from analysis of the original data. Two participants encouraged further analysis of the qNMR data by NIBSC. It was agreed that re-analyzed 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. Re-analysis of the data by a single analyst did not reduce the overall variability in the estimates of polysaccharide content. Data from all assays were considered by the ECBS in November 2019 and the decision taken to assign the polysaccharide content to 16/152 and 16/206 using data obtained from the Resorcinol assay rather than qNMR [27].

The lack of improvement in the variability of measurements of polysaccharide content after re-analysis suggested that the sources of variability must primarily lie within sample preparation (sample recovery and standard preparation, for example), as well, to a lesser extent, the parameters used to collect the spectral data. All factors affecting the performance of qNMR should be further explored and consideration given to how these may be better controlled with regard to the analysis of complex, heterogenous polysaccharides. 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, the use of system suitability tests (SSTs) performed before the participant moves 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 spectrometer was performing optimally, guarantying the quality of the shim, for example, thereby ensuring the collected data was of the highest quality. 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 analyzed. In such scenarios it is difficult to know whether the standard will react in the same or similar manner to the sample and introduces 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 vaccine-relevant samples; either polysaccharide or polysaccharide-conjugate. 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 24 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

Based on the Resorcinol assay data presented here we propose a content of 1.015 ± 0.071 mg MenW polysaccharide per ampoule for candidate standard 16/152 (expanded uncertainty with coverage factor k = 2.13, corresponding to a 95% level of confidence) and 0.958 ± 0.076 mg MenY polysaccharide per ampoule for candidate standard 16/206 (expanded uncertainty with coverage factor k = 2.26, corresponding to a 95% level of confidence).

The storage condition for both candidate standards is -20ºC.

The data demonstrate that using 16/152 Meningococcal capsular group 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. A significant benefit of using 16/206 Meningococcal capsular group 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 levels 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.

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Participants' comments before the meeting of the ECBS 2018

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-analyzed using your procedures-and considering points 2) and 3), may reduce the variability of the qNMR results.

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

Comment 1: We all agree that there are minor typos and just a few necessary edits.

Comment 2: It is not clear to me why the potency value assignments are based on qNMR only and the results obtained by other methods were not considered. I am wondering how many users in regulated industry uses qNMR for quantitation of MenW and MenY for lot release and stability studies, where the assigned value of the standards are critically important. To the best of my knowledge, HPAEC-PAD is used most widely. Therefore, the values obtained using HPAEC-PAD is most critical. Has it been established that qNMR, HPAEC-PAD and other methods give comparable results? If different results were obtained by different methods, NIBSC/WHO should assign different values for the standard for different methods, as has been widely done for other NIBSC/WHO biological reference standards.

Response: *We agree the HPAEC-PAD and Resorcinol are widely used methods for quantitation of polysaccharide for lot release purposes. However, from the point of view of standardization and metrology, a primary method has the highest metrological properties and it is possible to define the uncertainty of the measurement in SI units. It is also important that the uncertainty of the unitage assigned to International Standards is reduced. qNMR has been proposed as a primary method and therefore deemed to be the most accurate method. In this study it has proven not be as precise as has previously been demonstrated with the MenA, MenX and Vi studies and hence why have looked to see whether the variability and therefore the uncertainty, could be decreased by re-analyzing the qNMR data. The data from this study show that qNMR, HPAEC-PAD and Resorcinol give comparable results. Variability was highest with HPAEC-PAD and qNMR, whereas the Resorcinol assay produced the least variable data.*

Participant 8

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

Participant 9

It’s better to 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.

Participants' comments in the revised report with the proposal to assign unitage by the Resorcinol assay

The revised report with the proposal to assign unitage by the Resorcinol assay was circulated to all participants.

Participant 3

Thanks a lot for your patience and allow us the opportunity to participate in this collaborative study. For my side we don't have any new comment.

Participant 5

Comment 1: Many thanks again for the entire work. We really appreciated working with you. No particular objections or issues from our side.

Comment 2: Many thanks for all the work you have done, the report is fine with me.

Participant 6

In agreement with the proposal to assign unitage by the Resorcinol assay.

Participant 10

We agree with you to assign unitage of the candidate standards using results from the Resorcinol assay.

Participant 12

I am fine with the report.

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

Name	Laboratory	Country
Dr. Wim Van Molle Dr. Valérian Bunel Dr. Geneviève Waeterloos	Sciensano, Quality of Vaccines and Blood Products, Juliette Wytsmanstraat 14, Brussels 1050	Belgium
Dr. Filipe Soares Quirino da Silva Dr. Jochen Junker	Departamento de Química, Instituto Nacioanal de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz Avenida Brasil 4365, Manguinhos, Rio de Janeiro	Brazil
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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Dr. Jose Luis Marcelo Sainz Makis Y.Torres Toledo, Gleysin Caberera Herrera, Satomy Pousa Ramirez	Centre for Genetic Engineering and Biotechnology (CIGB), PO Box 6162, Ave 31e/158y 190 Cubanacan, Playa, Havana CP10600	Cuba
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Dr. Sunil Kumar Goel, Dr. Sunil Gairola, Pankaj Sharma, Mr Sameer Kale, Shantanu Kale, Prasad Shinde, Dr Swapnil Phugare	Serum Institute of India Ltd, 212/2, Hadapsar, Pune, Maharashtra	India
Dr. Sanath K. Goud, Dr. S Gururaj Dr. Arunima Pola, Avadesh K. Pandey	United States Pharmacopoeia Convention India (P) Ltd, Plot No D6 & D8, IKP Knowledge Park, Genome Valley, Rangareddy District TG	India
Dr. Suk Bae Lee, Dr. Sang Ja Ban	Vaccines Division, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety (MFDS)187 Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si 28159	Korea
Prof. Neil Ravenscroft	Department of Chemistry, University of Cape Town, Rondebosch 7701	South Africa
Justine Vionnet, Dr. Willie Vann, Dr. Salil K Ghosh, Dr Hugo Azurmendi, Dr. Daron Freedberg, Dr. Lokesh Bhattacharyya	CBER/FDA 10903 New Hampshire Ave, WO 52/72 Room G218 Silver Spring, Maryland 20993	USA
Dr. Hannah Chan, Dr. Nicola Beresford, Dr. Fang Gao, Dr. Caroline Vipond, Dr. Barbara Bolgiano	Division of Bacteriology, NIBSC, Blanche Lane, Potters Bar, Hertfordshire EN6 3QG	UK
Dr. Tim Rudd, Mr Peter Rigsby	Analytical and Biological Sciences Division, NIBSC, Blanche Lane, Potters Bar, Hertfordshire EN6 3QG	
Ms. Sara-Jane Homes, Dr. Paul Matejtschuk, Ms. Kiran Malik, Ms. Chinwe Duru	Centre for Biological Reference Materials (CBRM), NIBSC, Blanche Lane, Potters Bar, Hertfordshire EN6 3QG	

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**	
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 capsular group 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
K_D 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 capsular group W polysaccharide and 16/206 – Meningococcal capsular group Y polysaccharide

	MenW polysaccharide	**MenY polysaccharide**
Date of reconstitution	26 May 2016	01 September 2016
Date of filling and start date of lyophilization	27 May 2016	02 September 2016
End date of lyophilization and date of sealing	31 May 2016	06 September 2016
Label	16/152 Meningococcal Capsular Group W Polysaccharide	16/206 Meningococcal Capsular Group 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 and end of filling) 0 (start and end of sealing)	0 (start and end of filling) 0 (start and end of sealing)
Mould/yeast colony Count (cfu/ml)	0 (start and end of filling) 0 (start and end of sealing)	0 (start and end of filling) 0 (start and 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)
Purity (%)	≥ 99			98	99.99		
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 Avance III 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		203	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 Resorcinol (Participants 4, 6, 7, 10, 11) and Anthrone (Participant 9) assay 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	80 µg/ml	95 mg/L NANA 55mg/L Glc/Gal	~ 2 mg/ml	2 mg/ml
Storage conditions of working stock	-20ºC	-20ºC	4°C	2-8℃	-10 to -25ºC	-20ºC	-20ºC
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	1.0 ml	0.5 ml	1.0 ml	0.5 ml	0.4 ml	0.2 ml	1.0 ml

Reagent (Resorcinol or Anthrone)	0.2 % resorcinol (0.1 % final with sample)	0.2 % resorcinol (or 18.2 mM) (0.1 % final with sample)	0.2 % resorcinol (0.1 % final with sample)	0.1 mol/l $CuSO_4$ 0.5 ml, 4%Resorcin 5ml, HCl 80ml, add water to 100ml	0.2 % resorcinol (0.14 % final with sample)	0.6 % resorcinol (0.086 % final with sample)	1 mg/ml Anthrone
Volume reagent	1.0 ml	0.5 ml reagent	1.0 ml reagent	0.5 ml	1.0 ml	1.2 ml	3.0 ml
Copper sulphate	0.25 mM	0.25 mM	0.25 mM		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 ml	1 ml		
Incubation temp, time, method	100ºC, 20 mins	110ºC, 15 mins	100ºC, 15 mins	water bath,100℃,15min	98ºC, 30 mins	100ºC, 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 5 Details of HPAEC-PAD / HPLC methodology, instrumentation and parameters used in the study. Although related, HPAEC-PAD and the HPLC method performed by Participant 9 were considered different methods for the purposes of data analysis. 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	D-(+)-Gal D-(+)-Glc	MenW PS MenY PS	Monosacchairde mix of Gal, Glc, GlcN, GalN, Man, Fuc
Source	In house	In house	Sigma G0750 (Gal) Sigma G8270 (Glc)	Sigma PHR1206 (Gal) PHR1000 (Glc)	Sigma G0750 (Gal) Sigma G8270 (Glc) MenW/MenY PS	In house	Ludger
Purity (%)	-	-	≥99 (Gal) ≥99 (Glc)	99.97 (Gal) 99.8 (Glc)	≥99 (Gal) ≥99.5 (Glc)	-	99 (Gal) 100 (Glc)
Moisture (%)	-	Dried	≥99 (Gal)	0.004 (Gal) 0.03 (Glc)	0.32 (Gal) 0.25 (Glc)	10.07 (MenW) 10.97 (MenY)	Not known
Concentration of Standard	1.1143 mg/ml (MenW) 1.1235 mg/ml (MenY)	1.0 mg/ml	1.0 mg/ml working stock	1.0 mg/ml	100 µg/ml	0.25 mg/ml	10 mg/ml (Gal) 2 mg/ml (Glc)
Diluent	0.005 Tween 20 in MQW	dH_2O	dH_2O	PBS	dH_2O	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)	0.167-9µg/ml (5) for Gal/Glc 0.5-27 µg/ml (5) for PS	0.17-27 µg/ml (6)	0.1-1.5 nmol (Gal) 0.1-2.0 nmol (Glc)
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, 100ºC	2h, 100ºC	1h, 100ºC	1h, 100ºC	2h, 100ºC	2h, 100ºC	45 min, 80ºC
Instrument	Dionex ICS3000	Dionex ICS3000	Dionex ICS-3000	Dionex ICS5000	ICS5000 and ICS5000+	ICS5000	
Reference electrode	Ag/AgCl	Ag/AgCl	Ag/AgCl	Ag/AgCl	Ag/AgCl	Ag/AgCl	
Working electrode	Au	Au	Au	Au		Au	
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**	1 ml/min 40 min	1 ml/min 41 min	1 ml/min 60 min	 50 min	1 ml/min 45 min	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	0-22 min, 15m M NaOH 22-27 min, 200 mM NaOH, 200mM NaOAc 27-40 min, 15mM NaOH	0-18 min, 15 mM NaOH 18-26 min, 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, 100 mM 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, 0.6-1.0 M NaOAc 31-35 min, 200 mM NaOH, 1000-0 mM NaOAc 35-43 min, 200 mM NaOH 43-50 min 9 mM NaOH	0-32 min, 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 For conversion from Gal/Glc to polysaccharide			N/A	MenW: 497.020/180.156 MenY: 494.110/180.156

Table 6 Extended qNMR study at NIBSC – measurement of polysaccharide content of MenW (16/152) and MenY (16/206) candidate standards. Values shown were corrected for the mass of the repeating unit calculated with an average percent *O*-acetylation of 58.50% for 16/152) and 51.58% for 16/206 (determined from data presented in Tables 14b and 15b, see section of the report "Degree of *O*-acetylation of the candidate standards and functional weights".

16/152 Meningococcal capsular group W polysaccharide	
Ampoule	**µg polysaccharide /ampoule**
1	939
2	931
3	952
4	907
5	978
6	960
7	940
8	943
9	951
10	947
Mean	945
SD	18.7
CV	2.0%

16/206 Meningococcal capsular group Y polysaccharide	
Ampoule	**µg polysaccharide /ampoule**
1	992
2	984
3	1022
4	972
5	976
6	1002
7	997
8	994
9	999
Mean	993
SD	15.0
CV	1.5%

Table 7 MenW polysaccharide content of 16/152 determined by qNMR. Analysis was carried out by individual study participants (7a) and re-analyzed by one NIBSC analyst (7b). Values given as µg MenW polysaccharide per ampoule. * Determined from de-*O*-acetylated samples. Values presented were adjusted for their mass according to their degree of *O*-acetylation, see section of the report "Degree of *O*-acetylation of the candidate standards and functional weights". 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	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participants 3 and 5)	
				Overall		**Overall**	
1*	937	12.7	1.4%	**mean**	854	**mean**	865
2	783	5.5	0.7%	**SD**	85.6	**SD**	100.1
3	797	n/c	n/c	**CV**	10.0%	**CV**	11.6%
4	892	36.7	4.1%	**95% LCL**	775	**95% LCL**	741
5	859	167.0	19.4%	**95% UCL**	934	**95% UCL**	989
6*	972	9.1	0.9%	**n**	7	**n**	5
7	739	9.8	1.3%				

7b) Analysis by NIBSC analyst

Lab	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participant 3)	
				Overall		**Overall**	
1*	952	21.5	2.3%	**mean**	936	**mean**	925
2	762	19.7	3%	**SD**	127.0	**SD**	135.1
3	1005	n/c	n/c	**CV**	13.6%	**CV**	14.6%
4	1003	50.2	5.0%	**95% LCL**	819	**95% LCL**	783
5	1097	140.6	12.8%	**95% UCL**	1054	**95% UCL**	1066
6*	971	9.3	1.0%	**n**	7	**n**	6
7	763	25.6	3.4%				

Table 8 MenY polysaccharide content of 16/206 determined by qNMR. Analysis was carried out by individual study participants (8a) and re-analyzed by one NIBSC analyst (8b). Values given as µg MenY polysaccharide per ampoule. * Determined from de-*O*-acetylated samples. Values presented were adjusted for their mass according to their degree of *O*-acetylation, see section of the report "Degree of *O*-acetylation of the candidate standards and functional weights". 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	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participants 3 and 5)	
1*	989	35.0	3.5%	**Overall mean**	800	**Overall mean**	877
2	736	32.8	4.4%	**SD**	181.3	**SD**	116.9
3	468	n/c	n/c	**CV**	22.7%	**CV**	13.3%
4	904	35.5	3.9%	**95% LCL**	632	**95% LCL**	732
5	747	48.8	6.5%	**95% UCL**	967	**95% UCL**	1022
6*	981	6.2	0.6%	**n**	7	**n**	5
7	773	24.0	3.1%				

8b) Analysis by NIBSC analyst

Lab	Mean	SD	CV	Summary Statistics (All data)		Summary Statistics (Exc. Participant 3)	
1*	1007	33.0	3.3%	**Overall mean**	956	**Overall mean**	950
2	776	17.0	2.2%	**SD**	110.8	**SD**	120.2
3	990	n/c	n/c	**CV**	11.6%	**CV**	12.7%
4	1060	130.5	12.3%	**95% LCL**	853	**95% LCL**	824
5	1051	28.9	2.7%	**95% UCL**	1058	**95% UCL**	1076
6*	980	6.1	0.6%	**n**	7	**n**	6
7	826	22.7	2.7%				

Table 9 Determination of uncertainty assigned to MenW polysaccharide content of 16/152 (9a) and MenY polysaccharide content of 16/206 (9b), as determined for qNMR (Participant and NIBSC analysis).

9a) MenW (16/152)

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 Additional NIBSC qNMR, mass adjusted (Table 6, MenW 16/152)	944.75 µg	18.75	1.99%
Between-laboratory variability				
i) Participant analysis	SEM (calculated from Table 7a)	864.91 µg	44.75	5.17%
ii) NIBSC analysis	SEM (calculated from Table 7b)	924.65 µg	55.15	5.96%
Combined relative standard uncertainty				
i) Participant analysis				**5.57%**
ii) NIBSC analysis				**6.31%**

9b) MenY (16/206)

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 Additional NIBSC qNMR, mass adjusted (Table 6, MenY 16/206)	993.20µg	14.97	1.51%
Between-laboratory variability				
i) Participant analysis	SEM (calculated from Table 8a)	876.71 µg	52.27	5.96%
ii) NIBSC analysis	SEM (calculated from Table 8b)	950.08 µg	49.09	5.17%
Combined relative standard uncertainty				
i) Participant analysis				**6.18%**
ii) NIBSC analysis				**5.41%**

Table 10 MenW polysaccharide content of 16/152 determined by other methods. Values given as µg MenW polysaccharide per ampoule. Values determined using sialic acid (#) or Gal (*) standards were converted to MenW polysaccharide with a mean value of 58.50% *O*-acetylation corresponding to a residue weight of 497.020 g/mol (see section of the report "Degree of *O*-acetylation of the candidate standards and functional weights"). † Polysaccharide standards.

Method	Lab	Mean	SD	CV	Summary Statistics			
Resorcinol	**4a**#	1001	21.6	2.2%	**Overall mean (Resorcinol,**			
	4b#	1020	44.4	4.4%	**All data)**	1015		
	6#	1069	87.7	8.2%	**SD**	66.3		
	7#	993	37.7	3.8%	**CV**	6.5%		
	10#	1015	22.0	2.2%	**95% LCL**	953		
	11#	897	39.4	4.4%	**95% UCL**	1076		
	12†	1109	47.1	4.2%	**n**	7		
HPAEC-PAD	**5**†	1080	37.0	3.4%	**Overall mean (HPAEC-PAD,**		**Overall mean (HPAEC-PAD,**	
	6†	1126	39.1	3.5%	**All data)**	1112	**Exc. Participant 7)**	1042
	7*	1530	56.8	3.7%	**SD**	212.6	**SD**	116.5
	8*	870	30.2	3.5%	**CV**	19.1%	**CV**	11.2%
	10*	953	42.0	4.4%	**95% LCL**	915	**95% LCL**	920
	10†	1190	85.2	7.2%	**95% UCL**	1309	**95% UCL**	1165
	11†	1036	20.9	2.0%	**n**	7	**n**	6
HPLC	**9***	501	19.3	3.9%				
Anthrone	**9***	516	15.3	3.0%				
Nephelo-metry	**10**	1193	41.7	3.5%				

Table 11

MenY polysaccharide content of 16/206 determined by other methods. Values given as µg MenY polysaccharide per ampoule. Values determined using sialic acid (#) or Glc (*) standards were converted to MenY polysaccharide with a mean value of 51.58% *O*-acetylation corresponding to a residue weight of 494.110 g/mol (see section of the report "Degree of *O*-acetylation of the candidate standards and functional weights"). †Polysaccharide standards.

Method	Lab	Mean	SD	CV	Summary Statistics		Summary Statistics	
Resorcinol	**4a**#	1032	21.9	2.1%	**Overall mean (Resorcinol**		**Overall mean (Resorcinol**	
	4b#	949	145.5	15.3%	**All data)**	998	**Exc. Participant 12)**	958
	6#	985	46.7	4.7%	**SD**	122.4	**SD**	71.3
	7#	908	27.1	3.0%	**CV**	12.3%	**CV**	7.4%
	10#	1027	43.5	4.2%	**95% LCL**	884	**95% LCL**	884
	11#	849	13.7	1.6%	**95% UCL**	1033	**95% UCL**	1033
	12†	1233	298.8	24.2%	**n**	7	**n**	6
HPAEC-PAD	**5†**	1021	56.2	5.5%	**Overall mean (HPAEC-PAD**		**Overall mean (HPAEC-PAD**	
	6†	1077	40.1	3.7%	**All data)**	1067	**Exc. Participant 7)**	991
	7*	1521	25.6	1.7%	**SD**	228.1	**SD**	119.9
	8*	855	36.4	4.3%	**CV**	21.4%	**CV**	12.1%
	10*	834	22.4	2.7%	**95% LCL**	856	**95% LCL**	865
	10†	1127	45.9	4.1%	**95% UCL**	1278	**95% UCL**	1117
	11†	1032	14.4	1.4%	**n**	7	**n**	6
HPLC	**9***	1011	86.73	8.6%				
Anthrone	**9***	996	17.1	1.7%				
Nephelometry	**10†**	1156	39.4	3.4%				

Table 12 Determination of uncertainty assigned to MenW polysaccharide content of 16/152 (12a) and MenY polysaccharide content of 16/206 (12b), as determined for Resorcinol assay.

12a) MenW (16/152)

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Purity of sialic acid standard	Using a rectangular distribution for calculation of standard uncertainty	99.00%	0.58%	0.59%
Weighing/mass of standard used	From manufacturer data for balance used to weight standard at NIBSC, assuming a triangular distribution for calculation of standard uncertainty	20.20 mg	0.14	0.71%
Between-ampoule homogeneity (from qNMR)	SD Additional NIBSC qNMR, mass adjusted (Table 6, MenW 16/152)	944.75 µg	18.75	1.98%
Between-laboratory variability	SEM (calculated from Table10)	1014.58 µg	25.06	2.47%
Combined relative standard uncertainty				**3.30%**

12b) MenY (16/206)

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Purity of sialic acid standard	Using a rectangular distribution for calculation of standard uncertainty	99.00%	0.58%	0.59%
Weighing/mass of standard used	From manufacturer data for balance used to weight standard at NIBSC, assuming a triangular distribution for calculation of standard uncertainty	20.20 mg	0.14	0.71%
Between-ampoule homogeneity	SD Additional NIBSC qNMR, mass adjusted (Table 6, MenY 16/206)	993.20 µg	14.97	1.51%
Between-laboratory variability	SEM (calculated from Table 11)	958.40 µg	29.11	3.04%
Combined relative standard uncertainty				**3.51%**

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 capsular group W polysaccharide		**16/206 Meningococcal capsular group 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 (16/152) determined by qNMR. Analysis was carried out by individual study participants (14a) and re-analyzed by one NIBSC analyst (14b).

14a) Participant analysis

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) NIBSC analysis

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 (16/206) determined by qNMR. Analysis was carried out by individual study participants (15a) and re-analyzed by one NIBSC analyst (15b). *Ampoule 1 from Participant 2

15a) Participant analysis

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) NIBSC analysis

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%				

Table 16 Estimate of polysaccharide contents of Sample A and Sample C (µg/ml) using candidate polysaccharide and in-house standards.[#]Glc/Gal [†]polysaccharide

		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	412	428	406	425	418	2.5														
	6	482	531	511	506	507	4.0														
	7	369	400	395	387	388	3.6			434	9.3										
	8	413	465	469	440	447	5.8														
	10	443	431	411	412	424	3.7														
	11	434	398	440	416	422	4.4	426	9.2												
Resorcinol	4	254	309	369	508	360	30.3					210	251	317	434	303	32.3				
	6	405	423	414	399	411	2.5					343	376	337	344	350	5.1				
	7	414	377	449	449	422	8.1			417	9.4	351	319	345	356	343	4.8	331	4.9	331	4.9
	10	423	434	474	456	447	5.0					332	318	348	331	332	3.7				
	11	361	408	421	380	393	6.9					301	334	351	321	327	6.4				
	12	458	492	367	563	470	17.3					327	352	285	366	332	10.7				
HPLC	9	362	453	453	426	424	10.1			424		N/A	N/A	N/A	N/A	n/c	n/c				
Anthrone	9	446	397	333	338	379	14.1			379		855	687	600	615	689	16.9			689	
Nephelometry	10	400	420	454	427	425	5.2			425		N/A	N/A	N/A	N/A	n/c	n/c				
ii) MenY candidate standard																					
HPAEC-PAD	5											348	357	357	359	355	1.4				
	6											385	355	369	361	367	3.5				
	7											319	332	345	318	329	3.8			358	6.2
	8											332	329	346	343	337	2.4				
	10[#]											364	356	386	368	369	3.5				
	11											401	410	371	374	389	5.0	346	14.5		
Resorcinol	4	218	254	285	331	272	17.7					180	205	245	283	228	19.8				
	6	401	390	397	386	394	1.7					326	336	344	326	333	2.6				
	7	399	425	436	460	430	5.9	417	24.8	417	24.8	332	340	338	366	344	4.3			335	20.6
	10	420	412	437	431	425	2.6					340	349	335	320	336	3.5				
	11	425	368	395	371	390	6.7					357	304	322	304	322	7.6				
	12	556	662	536	616	592	9.6					418	482	424	458	446	6.7				
HPLC		N/A	N/A	N/A	N/A	n/c	n/c					200	185	171	144	175	13.6			175	
Anthrone	9	218	156	168	176	179	15.0			179		418	258	286	308	318	22.0			318	
Nephelometry	10	N/A	N/A	N/A	N/A	n/c	n/c					313	332	343	334	331	3.8			331	
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			419	18.9	339	346	350	345	345	1.3				
	8	321	324	341	327	328	2.6					263	235	256	262	254	5.0			360	17.9
	10[#]	399	381	373	401	388	3.5					323	324	329	321	324	1.0				
	10[†]	501	443	455	444	461	5.9					445	413	420	425	426	3.2				
	11	413	343	413	382	388	8.6	409	17.3			393	420	344	403	390	8.4	348	17.2		
Resorcinol	4	256	273	299	335	291	11.7					212	222	256	298	247	15.8				
	6	420	443	417	427	427	2.7					347	357	375	358	359	3.3				
	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	353	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	240	178	216	207	210	12.2			210		460	297	383	372	378	17.7			360	
Nephelometry	10	449	508	496	459	478	5.9			478		434	409	400	391	408	4.5			408	

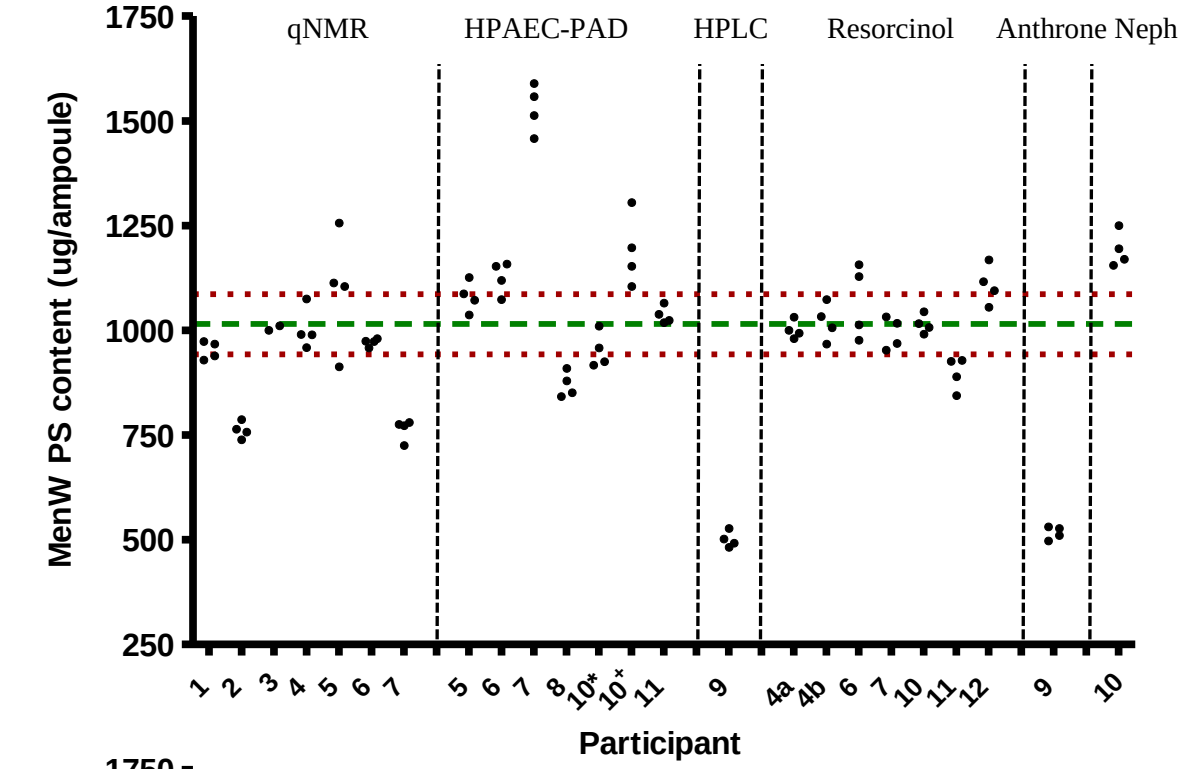
Table 17 MenW polysaccharide content (% of baseline -70ºC sample) of 16/152 by HPAEC-PAD for the lyophilized real-time, accelerated thermal degradation and reconstituted samples.

Storage time (months)	MenW PS content (µg MenW PS/ampoule) -70ºC	MenW PS content (mg MenW PS/ampoule) % -70ºC sample				
		-20ºC	+20ºC	+37ºC	+56ºC	-20ºC Reconstituted
0	1190					
1	1110	106.3	104.1	102.3	103.2	101.4
2	1389	88.5	90.6	83.0	88.2	88.8
3	1356	97.1	98.1	94.5	99.6	100.1
6	1285	107.7	100.7	104.4	107.8	107.0
12	1282	82.9	97.6	98.8	104.2	103.7
24	1181	105.6	107.7	103.9	115.1	117.0

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

Storage time (months)	MenY PS content (µg MenY PS/ampoule) -70ºC	MenY PS content (mg MenY PS/ampoule) % -70ºC sample				
		-20ºC	+20ºC	+37ºC	+56ºC	-20ºC Reconstituted
0	1121					
1	1239	99.9	98.6	105.0	106.5	104.2
2	0999	105.4	111.0	113.2	112.1	105.5
3	1165	91.1	97.4	97.5	96.4	98.5
6	1233	98.5	95.3	95.4	97.3	96.6
12	1076	100.5	97.2	97.3	101.7	101.6
24	1237	99.5	100.5	101.2	103.8	99.3

A



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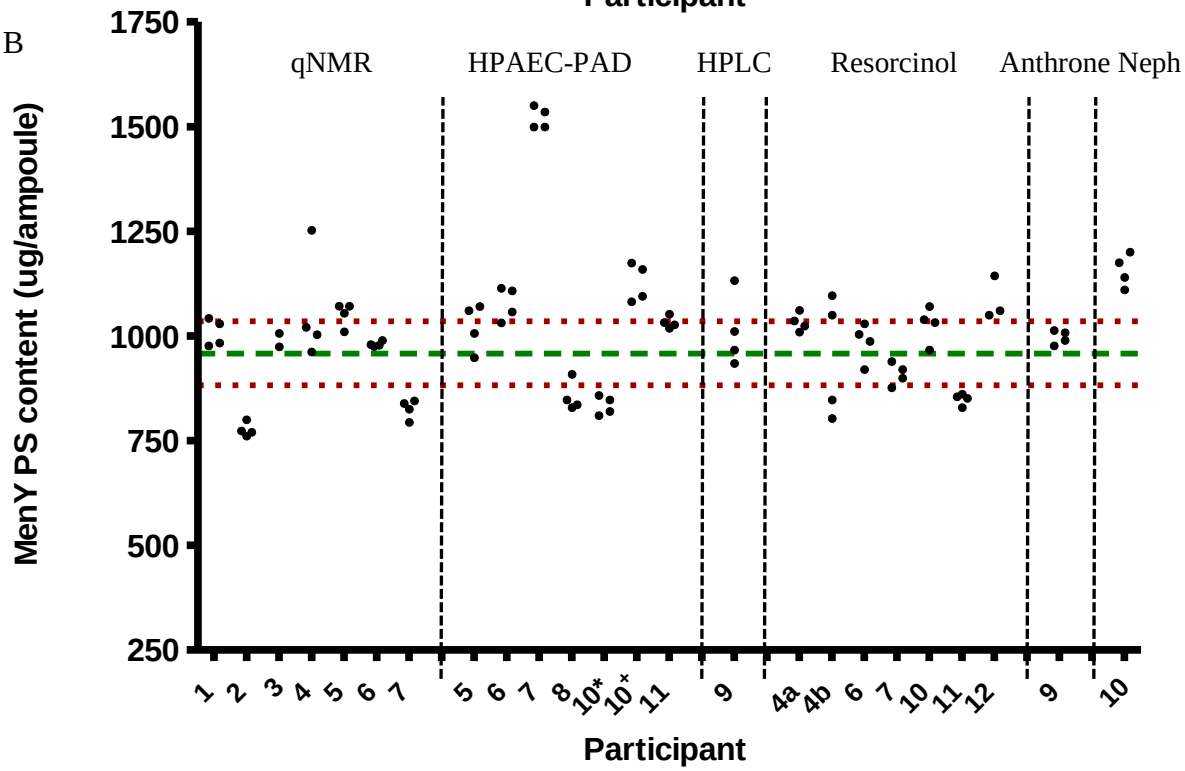
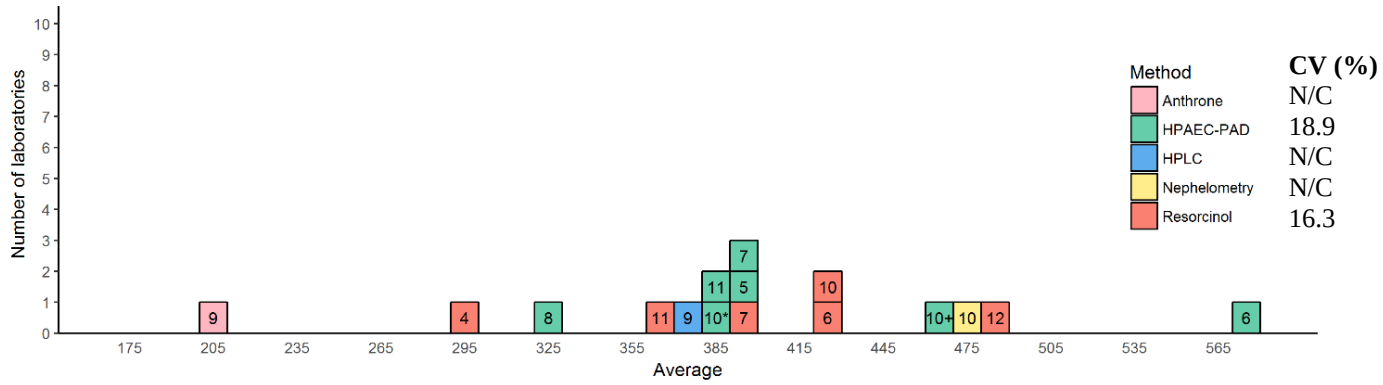


Figure 1 Polysaccharide content estimates for A) 16/152 (MenW) and B) 16/206 (MenY) by all methods. Green line - mean polysaccharide content determined by Resorcinol assay. Red line – expanded uncertainty (95% confidence). *Monosaccharide standard. †Polysaccharide standard.

A



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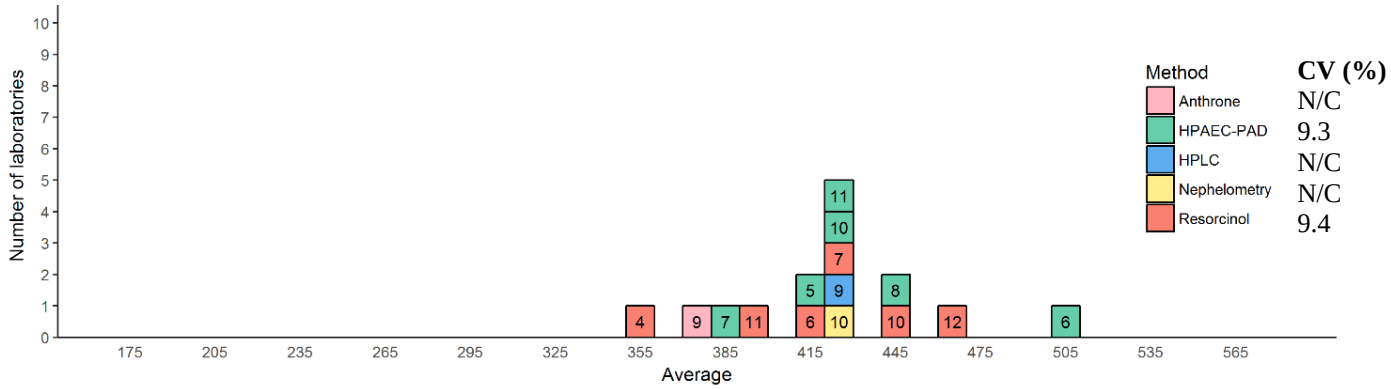
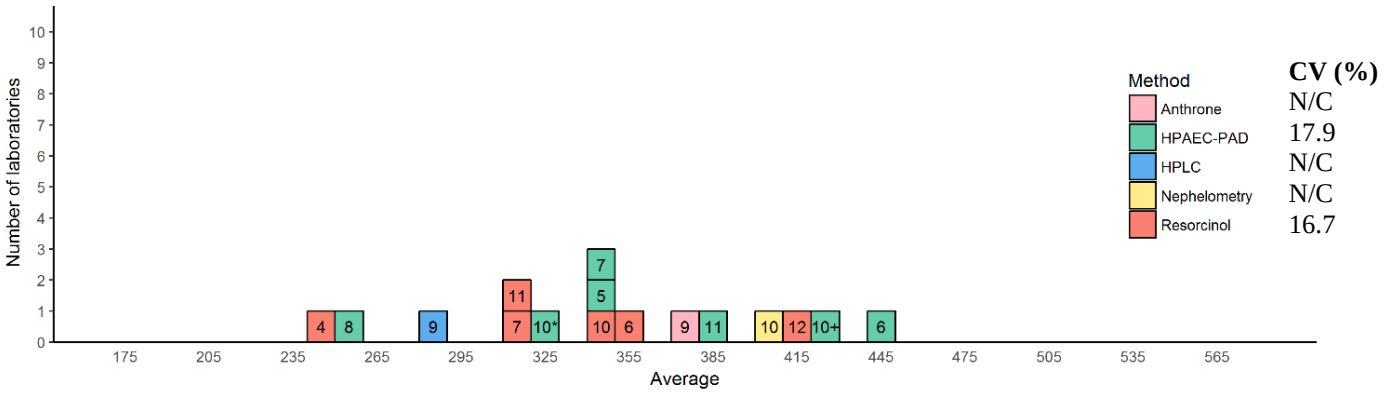


Figure 2 Distribution and frequency of estimates for MenW polysaccharide concentration (µg polysaccharide/ml) for Sample A using in-house standards (A) and candidate standard 16/152 (B). *Monosaccharide standard. †Polysaccharide standard. Coefficient of variation (CV)(%) is given for HPAEC-PAD and Resorcinol methods; CV is non-calculable (N/C) for other methods that were performed by one laboratory only.

A



B

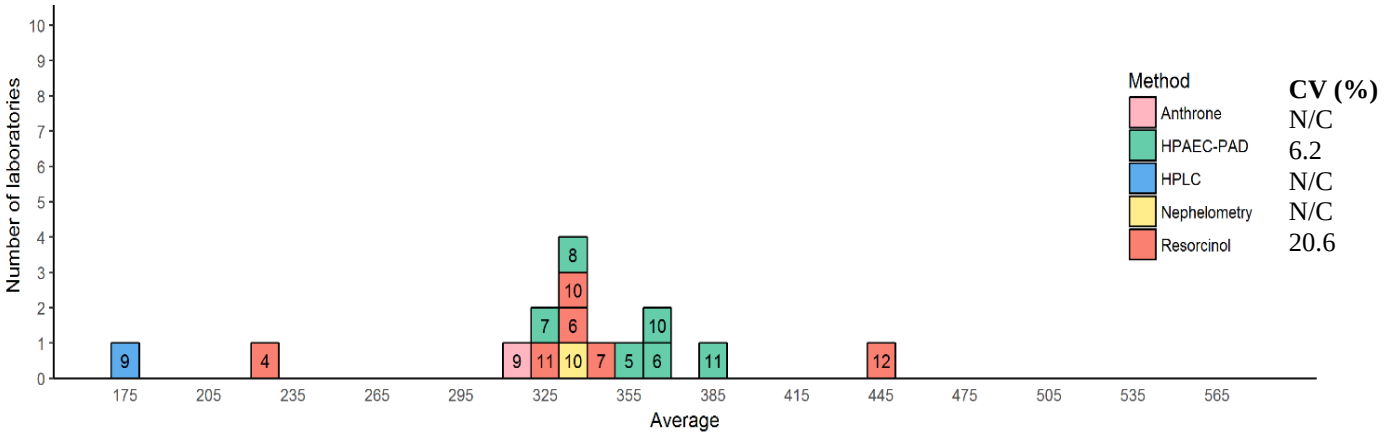


Figure 3 Distribution and frequency of estimates for MenY polysaccharide concentration (µg polysaccharide/ml) for Sample C using in-house standards (A) and candidate standard 16/206 (B). *Monosaccharide standard. †Polysaccharide standard. Coefficient of variation (CV)(%) is given for HPAEC-PAD and Resorcinol methods; CV is non-calculable (N/C) for other methods that were performed by one laboratory only.

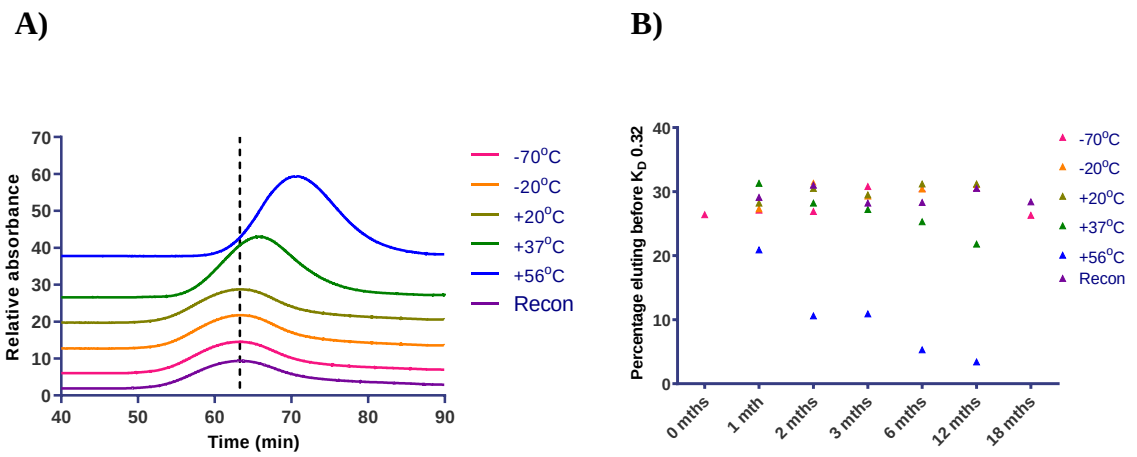


Figure 4 Molecular sizing chromatograms of the 16/152 MenW polysaccharide at 12 months following real-time, accelerated thermal degradation and reconstituted MenW polysaccharide (A). Using V_o and V_t from the column markers on the HPLC-SEC, the percentage of MenW polysaccharide eluted by a K_D of 0.32 (as indicated by the vertical line in Figure A) can be determined for each storage temperature (B).

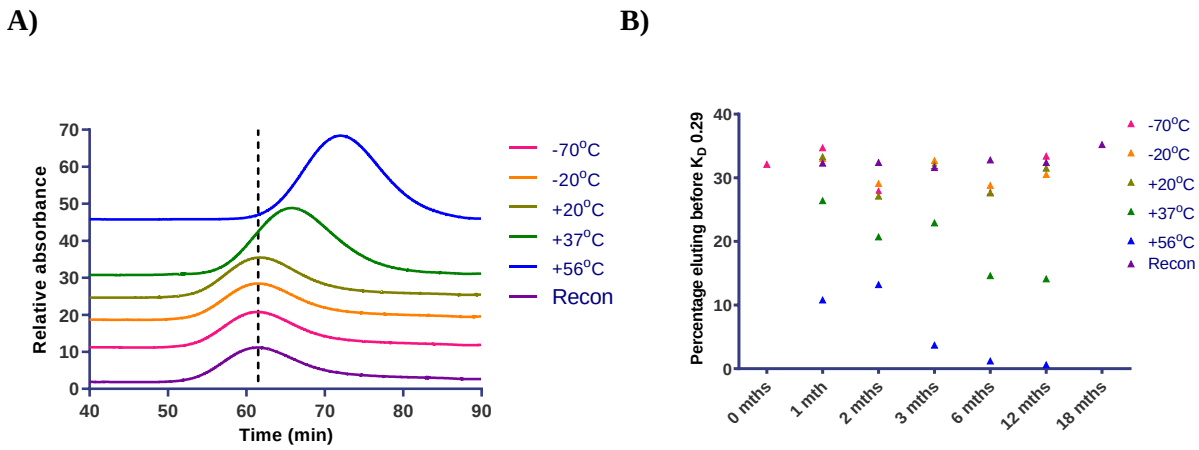


Figure 5 Molecular sizing chromatograms of the 16/206 MenY polysaccharide at 12 months following real-time, accelerated thermal degradation and reconstituted MenY polysaccharide (A). Using V_o and V_t from the column markers on the HPLC-SEC, the percentage of MenY polysaccharide eluted by a K_D of 0.29 (as indicated by the vertical line in Figure A) 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 Capsular Group 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 capsular group W (serogroup W, formerly known as Men W135) 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 polysaccharide content in SI units and to evaluate its suitability for use as a standard for quantification of MenW in bulk MenW polysaccharide conjugate material. Quantitative 1H (proton) nuclear magnetic resonance spectroscopy, Resorcinol and HPAEC-PAD were the main assays performed for the study. Other assays (HPLC, Anthrone and Nephelometry) were performed also, 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 Capsular Group W polysaccharide 16/152, has a content of 1.015 ± 0.071 mg polysaccharide/ampoule as determined by the Resorcinol assay. The residue weight of MenW polysaccharide is 497.020 g/mol with a degree of O-acetylation of 58.5%.

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

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 lyophilised standard to be stable up to 24 months at 37ºC (as determined by HPAEC-PAD to measure the polysaccharide content of the material reconstituted with water). Storage of the lyophilised standard at 20ºC for up to 12 months did not affect the molecular size distribution as determined by HPLC-SEC.
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, Nicola Beresford, Timothy Rudd, Peter Rigsby, Caroline Vipond, Fang Gao, Barbara Bolgiano and the MenW/MenY IS Working Group. Evaluation of Candidate International Standards for Meningococcal Capsualr Group W and Y Polysaccharides. WHO/BS/2019.2374.

Harrison OB, Claus H, Jiang Y, Bennett JS, Bratcher HB, Jolley KA, et al. Description and nomenclature of Neisseria meningitidis capsule locus. Emerg Infect Dis. 2013;19:566-73.

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:
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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.

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

14. MATERIAL SAFETY SHEET

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.

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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Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
Ist WHO International Standard for Meningococcal Capsular Group 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 capsular group Y (sergroup Y) 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 and to evaluate its suitability for use as a standard for quantification of MenY in bulk MenY polysaccharide conjugate material in mainly. Quantitative 1H (proton) nuclear magnetic resonance spectroscopy, Resorcinol and HPAEC-PAD were the main assays performed for the study. Other assays (HPLC, Anthrone and Nephelometry) were performed also, 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 Capsular Goup Y polysaccharide 16/206, has a content of 0.958 ± 0.076 mg polysaccharide /ampoule as determined by the Resorcinol assay. The residue weight of MenY PS is 494.110 g/mol with a degree of O-acetylation of 51.6%.

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 51.6% 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

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 lyophilised standard to be stable up to 24 months at 37ºC (as determined by HPAEC-PAD to measure the polysaccharide content of the material reconstituted with water). Storage of the lyophilised standard at 20ºC for up to 12 months. did not affect the molecular size distribution as determined by HPLC-SEC.
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, Nicola Beresford, Timothy Rudd, Peter Rigsby, Caroline Vipond, Fang Gao, Barbara Bolgiano and the MenW/MenY IS Working Group. Evaluation of Candidate International Standards for Meningococcal Capsular Group W and Y Polysaccharides. WHO/BS/2019.2374.

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http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
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Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

14. MATERIAL SAFETY SHEET

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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Net weight: 5g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No

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