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Evaluation of candidate International Standards for Vi polysaccharide from *Citrobacter freundii* and *Salmonella enterica* subspecies *enterica* serovar Typhi

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****Full details of Vi 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 **18 September 2017** 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 Insoo Shin** at email: shini@who.int

Summary

This report outlines collaborative studies (CS) 574 and 575 for the evaluation of two candidate International Standard (IS) preparations: Vi capsular polysaccharides (Vi PS) from *Citrobacter freundii* (*C. freundii*) and *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). Plain Vi PS and Vi PS conjugate vaccines are primarily evaluated by physicochemical methods to determine quality, safety and potency and ensure that batches are consistently manufactured. As different assays may be employed to quantify the PS content of final formulations and bulk intermediates, there is a need for Vi PS IS to calibrate internal references used in different laboratories. Twenty laboratories from 12 different countries participated in: CS574) to assign a mass unitage to two candidate IS of Vi PS, namely *C. freundii* and *S.* Typhi preparations (coded 12/244 and 16/126), and CS575) to assess their suitability in a variety of physicochemical assays and immunoassays used for measuring their potency.

Stability studies performed over 6 months with the candidate ISs for Vi PS demonstrated that both were stable at temperatures used for storage (-20^{o}C) and laboratory manipulation (+4^{o}C). In accelerated thermal degradation studies no size reduction was observed for either of the candidate ISs following storage at up to +56^{o}C for 6 months. The amount of polysaccharide per ampoule remained constant under all conditions. Real time stability studies of lyophilized material, reconstituted samples, and lyophilized material exposed to accelerated thermal degradation are on-going.

On the basis of the results for the two candidate ISs, it is recommended that: 1) the candidate IS 12/244 is established as the 1st IS for the *C freundii* Vi PS with a content of 1.94 ± 0.12 mg Vi PS per ampoule (expanded uncertainty with coverage factor of k=2.11 taken to correspond to a 95% level of confidence), as determined by quantitative NMR (qNMR), and 2) the candidate standard material 16/126 is established as the 1st IS for the *S.* Typhi Vi PS with a content of 2.03 ± 0.10 mg Vi PS per ampoule (expanded uncertainty with coverage factor of k=2.11 taken to correspond to a 95% level of confidence), as determined by quantitative NMR. The intended use of both candidate ISs is for the quantification of the Vi PS component of Vi PS-containing vaccines for which they are potentially suitable for use in HPAEC-PAD, ELISA, rate nephelometry and rocket immuno-electrophoresis assays.

Introduction

Typhoid fever is caused by an infection with *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi). *S.* Typhi expresses a capsular polysaccharide, Vi polysaccharide (Vi PS), which is a virulence factor and considered the main protective antigen (Felix 1934; Robbins 1984). A Vi PS capsule, which has similar physicochemical and immunological characteristics, is expressed by the soil bacterium *Citrobacter freundii* (*C. freundii*) (Szu 1991). Vaccination is the most cost effective preventative strategy to control typhoid, especially in areas where multidrug resistant strains are endemic. As plain Vi PS vaccines are unable to provide immuno-protection for young children or infants, a prototype conjugate Vi PS - recombinant *Pseudomonas aeruginosa* exoprotein A vaccine (Vi-rEPA) was developed and proved successful in clinical trials for infants under 2 year of age and in pre-school age children with highly efficacious, immunogenic, safety (Lanh 2003; Lin 2001; Szu 1994). Vaccination with Vi-rEPA induced long term protection against typhoid (Thiem 2011). Recently, two Vi PS–Tetanus Toxoid conjugate vaccines (Vi PS-TT) were licensed in India (Mohan 2015; Mitra 2016). Currently, at least nine Vi PS conjugate vaccines using a variety of carrier proteins are in development and a number of these have entered clinical trials. The World Health Organization (WHO) reference materials for Vi PS are required to allow comparison of various Vi PS conjugate vaccine formulations, either licensed or in clinical trials, by calibrated physicochemical assays and immunoassays (WHO, 2014).

Following the establishment of the 1st International Standards (ISs) for the quantitation of *Haemophilus influenza* type b (Hib) polysaccharide (2005), meningococcal serogroup C (2011), A and X (2015) saccharide contents of polysaccharide-based vaccines; we designed the Vi candidate materials to be utilized in a similar way as these ISs. The aim is to improve harmonization of the measurement of the Vi PS content of typhoid vaccines of which they are components. As both *C. freundii* Vi PS and *S.* Typhi Vi PS have a similar molecular structure but may differ in chain length and level of *O*-acetylation, and because both Vi PS preparations have been used in licensed vaccines, it was therefore decided to include these two Vi PSs in this study.

Vi PS is a linear homopolymer composed of single monosaccharide repeating units, α 1,4-*N*-acetylgalactosaminouronic acid (Heyns and Kiessling 1967), with a variable degree of *O*-acetylation (Baker, 1959) at the C3 position. The most commonly used methods to quantitate the content of the Vi saccharide repeating units, in final vaccines or bulk vaccine components are high performance (or high pH) anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD), rocket immuno-electrophoresis. Other methods include nuclear magnetic resonance spectrometry (NMR), an acridine orange dye binding method, rate nephelometry and enzyme-linked immunosorbent assay (ELISA). Traditionally, the Hestrin method to quantitate the *O*-acetyl content has been used to determine the potency of plain Vi PS vaccines.

The proposal for *C. freundii* and *S.* Typhi Vi PS candidate ISs was endorsed at the ECBS meeting in 2013 (WHO, 2013). These reference preparations contain a known quantity of Vi PS and can be used to cross-calibrate various methods used to quantify the Vi content of the bulk polysaccharide, bulk conjugate and final formulations. The ISs will be made available under the auspices of WHO to facilitate calibration of assays and in-house reference materials. The continuing and rapid development of new Vi PS conjugate vaccines, the establishment of programmes to distribute Vi PS vaccines in low and middle income countries, and the likelihood that more vaccine manufacturers and National Control Laboratories (NCLs) will establish

methods to evaluate these vaccines indicate that a standard for the quantification of Vi PS will be important for the introduction of these vaccines. Thus these ISs are likely to be in demand.

The WHO guidelines state that for an IS to be adopted using Système International d'Unités (SI) unitage rather than International Units, the assignment should follow ISO 17511 principles (2006). In particular, uncertainty should be assigned and unit assignment should not be based on a bioassay but rather should be derived from and traceable to a calibrators or control materials. The assignment of mass unitage to polysaccharide standards developed by NIBSC has been approached by calculation of results obtained from multiple laboratories running a single method, with the uncertainty incorporating the combined uncertainty from the different determinations (Mawas 2007; Mawas 2015; Vipond 2012).

Progressing from the use of a single secondary comparative method to assign a value to MenC and Hib polysaccharide standards, the unitages of MenA and MenX ISs were assigned by quantitative NMR (qNMR) (Vipond 2015). It was also proposed that qNMR would be used to determine the PS content of Vi candidate standards (WHO 2014). qNMR is a potential primary method for quantifying organic compounds (Bureau International des Poids et Mesures; Malz 2005) and is considered to be the most appropriate method to assign mass unitage. Therefore the mass unitage of the IS is proposed in SI units of milligrams (mg), in accordance with the ISs for Hib and meningococcal serogroups A, C and X and the measurement and specification of PS in 0.001 mg (μg) for all Vi vaccines, plain polysaccharide or conjugate.

Participants

Twenty laboratories from 12 countries participated in the study including 9 NCLs or public health laboratories, 9 manufacturers, one Pharmacopoeia laboratory and one academic laboratory. The list of participants of the Vi IS working group is included in the Annex. Participants were assigned a random code number, not corresponding to the order of listing.

Materials and Methods

The candidate international standard

Prior to carrying out major fills, a trial fill with a GMP lot of *C. freundii* Vi PS (5.2 mg/ml, donated by Novartis Vaccines Institute for Global Health (NVGH; now Glaxo Vaccines Institute for Global Health since 2014) was carried out. During the trial fill, the high viscosity of the solution caused liquid to drip from the filling needles Subsequent dilution of *C. freundii* Vi PS to 2.5 mg Vi PS/ml mitigated this effect and filling and freeze-drying of this material was successful with satisfactory cakes formed across the batch. Samples were analysed by HPAEC-PAD for Vi PS content and molecular size distribution, and no difference was observed between the freeze dried material and original liquid. The freeze-dried Vi PS was also positively identified by an in-house ELISA and a Vi capture ELISA.

NVGH donated a second GMP lot of ~5 g purified *C. freundii* Vi PS bulk in solution (2.17 mg/ml), which was stored at +4°C. A fill of 2,061 ampoules was completed at NIBSC and assigned NIBSC code "12/244".

GlaxoSmithKline (GSK) Biologicals SA, Belgium provided NIBSC with 2.4 g of bulk *S.* Typhi Vi PS which was stored at -20°C. To ensure this lyophilized Vi PS reference preparation would

remain stable over a long-term, a trial fill was performed for *S.* Typhi Vi PS dissolved in either water or 15 mM NaCl. Following lyophilization, both lyophilized formulations formed acceptable plugs. Both formulations were submitted to accelerated thermal degradation studies. Ampoules were stored at -20°C, +4°C, +40°C and +56°C for 5 weeks. Total Vi backbone saccharide content was determined on week 0 and week 5 by HPAEC-PAD using an in-house standard. Total Vi saccharide content of ampoules stored at -20°C and +56°C was also determined by qNMR at week 5. HPLC sizing was performed using a TSKgel 5000 PW_{XL} column, using 50 µg injections into 20 mM HEPES, pH 7.3 run at 0.3 ml/min. The pH of the solutions was also measured.

Compared to the formulation prepared from Vi PS in water the formulation prepared from Vi PS in saline showed higher variation in Vi PS content with only 50% of target Vi PS content present in the samples exposed to +37°C and the +56°C . The pH of this formulation was about 0.25 pH units lower and showed more variation in pH between temperatures. Thus, it was decided to use water as the diluent for the definitive fill of the candidate *S.* Typhi Vi PS. A total of 960 ampoules were filled and assigned NIBSC code "16/126".

Information from the Certificates of Analysis provided by the manufacturers for Vi PS from *C. freundii* and *S.* Typhi is given in Tables 1a and 1b, respectively. The candidate ISs are compliant with WHO Recommendations (WHO, 2006) and the European Pharmacopoeia (2011) for purified bulk PS for use in vaccines.

Both 12/244 and 16/126 complied with WHO recommendations for the preparation, characterization and establishment of reference standards (WHO 2006). Details of the filling and freeze-drying cycles of 12/244 and 16/126are given in Table 2. At NIBSC 1500 ampoules of 12/244 and 700 ampoules of 16/126 are stored at -20°C and available for distribution. NIBSC will act as the WHO custodian for these materials.

Study design

The study has been split into two separate parts, coded Collaborative Study (CS) CS574 and CS575, which were run sequentially. In CS574, 8 laboratories analysed the Vi PS content and *O*-acetyl content of candidate IS 12/244 and candidate IS 16/126 by qNMR and mass unitage was assigned to these two candidates. This was followed by CS575, where 16 laboratories used four commonly methods to evaluate the suitability of candidate ISs in these assays to determine the concentration of Vi PS and *O*-acetyl content in vaccine-relevant samples.

Study materials

Participants of study CS574 and CS575 were sent standard and test samples according to Table 3. In CS574, to assign a unitage, participants received 2 sets of candidate ISs, with each set containing 2 ampoules coded A for *C. freundii* Vi PS 12/244, and 2 ampoules coded B for *S.* Typhi Vi PS 16/126 respectively. The samples were used to determine the Vi PS content and *O*-acetyl content in two analytical sessions performed on different days. Participants were required to sonicate the materials after they were reconstituted in D_2O, deuterium oxide, to improve the solubility of the polysaccharide prior to qNMR. Sonication, sodium deuteroxide addition to prepare the de-*O*-acetylated sample, and qNMR were required to be done on the same day.

Laboratories that participated in CS575, to study the fitness of purpose of the candidate ISs, were sent 1 ampoule of each Vi PS candidate IS 12/244 and 16/126 with a supplied assigned mass

unitage derived from CS574 of 1.97±0.11 mg Vi PS/ampoule for 12/244, and 2.02±0.11 mg Vi PS/ampoule for 16/126 (Note: these mass unitages were slightly revised following the final analysis). A set of samples coded A (*S.* Typhi Vi PS), B (*C. freundii* Vi conjugate), C (MenW PS) and D (*S.* Typhi Vi conjugate), were also included. Participants would use the candidate ISs and their in-house standard to determine the Vi PS content in samples A, B, C and D.

Participants performing the Hestrin method, using their in-house acetylcholine standard, would also determine *O*-acetyl content in both the candidate ISs and the coded samples. Upon receipt, participants were asked to store the standards at -20 °C and samples, A-D at +4°C. Instruction of standard preparation was provided to participants which was to reconstitute with 1.97 ml water for 12/244 (*C. freundii*) and 2.02 ml water for 16/126 (*S.* Typhi) to reach a final concentration of 1 mg PS/ml. After reconstitution, the ampoule should be wrapped well with parafilm to avoid evaporation and the polysaccharides should be left at room temperature for 2 hours, with gentle shaking to aid resuspension, and then kept at 2-8°C overnight for complete dissolution. The standard could then be transferred to screw-capped tubes, or aliquoted, and stored at 4°C for further use. Prior to use in assays, samples were to be left at room temperature for 1 hour and gently but thoroughly mixed, and stored at 4°C for the duration of the study. It was recommended to complete the study within 2 weeks of the beginning of laboratory work, if possible.

Assigning functional weight to Vi PS

For the determination of the functional weight of the candidate ISs, the mean values of the degree of *O*-acetylation as determined by NMR of the base-catalyzed de-*O*-acetylated samples (as per Berti and Ravenscroft, 2015) by 8 laboratories were considered. The Materials and Instructions for CS574 specified that the well homogenized samples should be incubated for 60 minutes with 200 mM NaOD to de-*O*-acetylate the PS. NMR instruments with field strengths of 400 MHz (one), 500 MHz (four) and 600 MHz (three) magnetic fields, were utilized, with parameters listed in Table 3. The ratios of the intensity of the acetate integral at 1.9 ppm to that of the *N*-acetate integral at 2.1 ppm (Lemercinier *et al*., 2000) were calculated. From the mean of the eight laboratories, the *C. freundii* sample contained 94% *O*-acetylation, and the *S.* Typhi sample was 103% *O*-acetylated. The overestimate was probably due to some residual free acetate in the sample. The *S.* Typhi Vi PS is considered to be fully *O*-acetylated. No adjustment was made to the measured level of *O*-acetylation of the *C. freundii* candidate standard. The functional weight of the repeating unit of the *C. freundii* PS was calculated to be 278.674 g/mol, and the functional weight of the *S.* Typhi PS was calculated to be 281.196 g/mol. Full details are given in Annex 2.

Assays for evaluation of the candidate international standards

Quantitative ^{1}H-NMR

Non-overlapping resolved resonances from ^{1}H-NMR spectra were used to estimate the molar concentration of the material in the ampoules, using a precisely known concentration of an internal reference compound (Ravenscroft 1999). The area under the reference material peak and resonances corresponding to the Vi polysaccharide in the spectrum were integrated and the normalised intensity values used to determine the final concentration of each of the ampoules. Eight laboratories in total performed qNMR (1- 8) using in-house methods, the experimental parameters of which are summarised in Table 4.

General method

In general the internal standard of choice was dissolved in deuterium oxide and then a known amount was transferred to each ampoule to give an accurate concentration. The sealed ampoules were left to stand at room temperature for two hours before being stored overnight at 4°C to allow complete dissolution. All participating laboratories sonicated the ampoules for 1 hour before NMR acquisition with the exception of laboratory 3. Laboratory 3 lyophilized the samples and the data were excluded from the study. A range of internal standards were used from participating laboratories which include malic acid, 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), Sodium trimethylsilyl-2,2,3,3-2H-propionate (TSP-d_4) and sodium formate. Each laboratory was asked to optimise the delay (d1) and 90° pulse width (p1), spectra were to be recorded at 50°C, with the aim of achieving a signal-to-noise ratio of greater than 500:1. The parameters are summarised in Table 4.

NIBSC method

To complete the determination of uncertainty, qNMR was performed at NIBSC using 14 ampoules of *C. freundii* Vi PS (12/244) and 14 ampoules of *S.* Typhi Vi PS (16/126). Deuterated TSP-d_4 (Sigma-Aldrich, 269913) was weighed on a calibrated balance (12.80 mg) and dissolved in a freshly opened 100 g bottle of D_2O (99.9%D). One ml of the TSP-d_4/D_2O solution was transferred to the ampoules ensuring the contents were fully dissolved. The ampoules were sealed and the contents were allowed to fully dissolve (see 'General Method').

Then 700 µl from each sample was then transferred into labelled 535-PP 5 mm NMR tubes and noesy1d experiments were performed using the optimised parameter-sets outlined in Table 4. The first measurement confirmed the identity of the Vi PS samples. The NMR tubes were then treated with 14 µl sodium deuteroxide (10M) to de-*O*-acetylate the polysaccharide. This method has been validated and described by Lemercinier *et al*., (2000). The ampoules were allowed to stand for 60 minutes for full de-*O*-acetylation (DeOAc) to occur, before a second spectrum was acquired using the same parameters, the spectrum was used for the qNMR analysis and determining the degree of *O*-acetylation.

All ^{1}H NMR spectra were recorded using a Bruker AVANCE III HD 500 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a BBI probe and Topspin software 3.5. The process was automated using ICON-NMR, which as well as recording the spectra performed the following tasks automatically; spectral phasing and baseline correction as well as calibrating the spectra with respect to the resonance of TSP-d_4. One-dimensional ^{1}H nuclear Overhauser effect spectroscopy (NOESY) NMR experiments were performed on each sample using specific optimized parameters for *C. freundii* or *S.* Typhi Vi PS (Table 5). For *C. freundii* Vi PS, NMR spectra were acquired at 323 K, 16 scans were collected, each spectrum was comprised of 65k data points with a spectral width of 15 ppm, a 10 ms NOESY mixing time and a relaxation delay of 20 s. For *S.* Typhi, NMR spectra were acquired at 323 K, 32 scans were collected, each spectrum was comprised of 65k data points with a spectral width of 15 ppm, a 10 ms NOESY mixing time and a relaxation delay of 28 s.

Hestrin assay

Ten laboratories (4, 8, 9, 10, 12, 13, 14, 15, 17 and 19) ran a colorimetric Hestrin assay to determine the quantity of *O*-acetylation present in the candidate materials. Exposure to sodium hydroxide causes *O*-acetyl groups to react with hydroxylamine to form hydroxamic acid. The acid reacts with ferrous ions in ferric chloride to form a yellow colored complex (Hestrin 1949). The absorbance of the samples is measured and is directly proportional to the concentration of

O-acetyl, since one mole of acetylcholine chloride is equivalent to one mole of *O*-acetyl. Nine out of ten laboratories (8, 9, 10, 12, 13, 14, 15, 17 and 19) used acetylcholine chloride from Sigma (catalogue number A6625), and Laboratory 4 used acetylcholine chloride from Sigma (catalogue number A9101). A range of standard curves were used across the laboratories, with five laboratories (8, 10, 13, 15 and 19) using a standard curve of 7 points or more and five laboratories (4, 9, 12, 14 and 17) using a standard curve of 5 or 6 points. The lowest concentration used was 0.055 μmol acetyl choline chloride/ml (Laboratory 8 and 12).

Laboratory 10, 15 and 17 prepared their samples using a "blank and no blank" method, whereby each sample would have a blank subtracted from it. The other laboratories appear to have used a one blank and subtracted that absorbance from both samples and standards. Where the "blank and no blank" method was used samples were prepared in duplicate. Laboratory 6 and 19 also read samples in duplicate. Laboratory 8 and 12 prepared samples and standards in duplicate. Further details from laboratories performing Hestrin assays are included in Table 6.

HPAEC-PAD

Seven laboratories performed HPAEC-PAD (4, 8, 10, 11, 13, 15 and 16). For PS depolymerization prior to chromatography, six laboratories adopted the method of Micoli *et al.* (2011) using 2 M NaOH at 110°C for 4 hours. Laboratory 15 increased to 4 M NaOH with the same heating conditions as the previous laboratories. This alkaline hydrolysis approach is used to measure the content of Vi polysaccharide backbone, that is, the de-*O* and de-*N*-acetylated saccharide repeating units of galactosaminouronic acid. All laboratories, except Laboratory 11, have established in-house Vi PS standards, either from *S.* Typhi or *C. freundii*. Two laboratories used internal spike, *N*-acetyl-glucosamine-6-phosphate or glucosamine-1-phosphate, to harmonise detector variation. The laboratories used CarboPac PA1 columns, and detected the relevant peak at similar elution times using an eluent containing 100 mM NaOH and up to 150 mM sodium nitrate. Further details of the HPAEC-PAD assays are included in Table 7.

Rate nephelometry

Laboratory 3 and 18 performed rate nephelometry to quantitate the Vi PS content in the PS and conjugate samples. This method relies on the rate of change in light scattering caused by formation of an antibody-antigen complex, in this case by the binding of Vi PS to anti-Vi antibodies. This method is used to determinethe saccharide content in polyvalent pneumococcal polysaccharide vaccines, pneumococcal conjugate vaccines and Vi PS vaccines (Katkocin, 2000; Lee, 2002). Both laboratories used an in-house standard based on Vi PS sourced from the manufacturer: Laboratory 18 used the manufacturer's *S.* Typhi Vi PS vaccine to prepare the standard curve while Laboratory 3 used a lyophilized purified *S.* Typhi Vi PS bulk. Both laboratories constructed a similar standard curve range and obtained comparable rate units, using the Beckman Coulter IMMAGE 800 rate nephelometer. Sample buffers and sample preparation varied between laboratories. Further details are included in Table 8.

Rocket immuno-electrophoresis

In-house rocket immuno-electrophoresis was carried out by Laboratory 9, 17, 18 and 20. Dilutions of the test samples and the reference samples are loaded on to an agarose gel containing antibody directed against the Vi PS. Samples are electrophoresed into the gel and run during which time interaction between Vi and anti-Vi IgG occurs. Once an equivalence point is reached the antigen-antibody complex will precipitate. The rocket heights of the reference antigen are measured on the Coomassie Blue stained gels, and the mean calculated for each dilution to produce the dose response curve. The rocket heights of the test samples are measured against the reference curve and concentrations are calculated from this (Walker, 1984).

Laboratories 9 and 20 used 100 µg Vi PS /ml as reference whereas laboratories 17 and 18 started from 50 or 65 µgVi PS/ml respectively. All four laboratories used different running times and voltages for electrophoresis. Laboratory 18 and 20 used saline and purified water for rinsing whereas laboratory 9 used purified water only. Laboratory 17 did not rinse the gel. Further details of this procedure are given in Table 9.

ELISA

Laboratory 7 used a capture ELISA to determine Vi content in samples A, B, C and D, and compared these samples with candidate ISs Vi PS 12/244 and 16/126 and an in-house standard. Vi PS was captured by a rabbit anti-Vi PS antibody immobilized on the microtiter plate surface and detected by the same anti-Vi PS antibody conjugated to horseradish peroxidase. The intensity of the colorimetric signal detected is proportional to the amount of Vi PS in the sample. The sample value was determined from a standard curve generated from samples of known Vi PS concentration.

Stability Studies

To determine the stability of the candidate IS Vi PS 12/244 and 16/126, three studies were undertaken: a real-time stability, an accelerated degradation and a stability study of the reconstituted material. Monitoring of the real-time stability of samples stored at -20°C was planned for 6, 12, 30, 60 and 120 month time-points using the -70°C sample as baseline.

The accelerated degradation study of the candidate ISs was planned at temperatures of +4°C, +20°C , +37°C and +56°C, using a -20°C baseline for 1, 2, 4, 6, 12, and 24 months. Following storage at the desired temperature and duration, the samples were transferred to -20°C prior to analysis, and were analyzed in a single session.

In the final study, the candidate ISs were reconstituted in 2 ml of sterile distilled water and stored in 0.3 ml aliquots at +4°C for 0.5, 1, 2, 6, 12 and 18 months and -20°C for 1, 2, 6, 12, 18, 24 and 30 months. Frozen samples were thawed at room temperature for 1 hour prior to analysis. For all three stability studies, data is presented up to 6 months for both Vi 12/244 and 16/126.

The Vi PS content was determined by HPAEC-PAD and the molecular integrity by HPLC-SEC with duplicated injections. For qNMR the NIBSC method was used on stability samples stored for 6 months. The pH was also checked for all stability samples.

The HPAEC-PAD method was performed with a CarboPac PA-1 (2mm) analytical column. Vi polysaccharide from *C. freundii* (12/244) was used as a quantitative standard (0.5–27 µg/ml) with unitage assigned on a dry weight basis. For the quantitative analysis, samples and standards were subjected to alkaline hydrolysis with 4.0 M NaOH, for 4 h at 110°C. The eluents were mixed with a gradient pump to achieve 0–2 min, 100 mM NaOH, 40 mM $NaNO_3$ (sodium nitrate, Sigma 31440); 2–22 min, 100 mM NaOH, 40–150 mM $NaNO_3$; and, 22–31 min, 100 mM NaOH, 40 mM $NaNO_3$. The flow rate was 0.25 ml/min. The content of Vi was determined by integrating the peak arising from galactosaminouronic acid relative to the standard curve. Chromeleon software version 7.2 was used for analysis.

Molecular sizing analysis was performed using a Thermo Dionex ICS5000+ HPLC system with Tosoh Bioscience TSKgel 5000 PW_{XL} column preceded by a PW_{XL} guard column. The mobile phase was 20 mM HEPES, pH 7.3 (Sigma H3375) buffer at a flow rate of 0.3 ml/min. The void and total column volumes were determined using salmon DNA (Sigma) and tyrosine (Sigma),

which eluted at 18 min and 42 min respectively. The RI signal was used for determining the % eluting at a distribution coefficient (K_D) of 0.11.

Results

Determination of *C. freundii* Vi PS and *S*. Typhi Vi PS in candidate international standards 12/244 and 16/126

Details of all the data, from collaborating laboratories including NIBSC, for the estimation of the Vi PS content per ampoule are summarised in Tables 10 & 11a and Figures 1 & 2a.

Quantitative ^{1}H-NMR

Eight laboratories returned qNMR data (see Table 11a and Figure 2a). There was a fairly good agreement between laboratories with the exception of Laboratory 1 and 3, whose data were both excluded from further analysis: results from Laboratory 1 showed a high level of variability and the values for the Vi PS content calculated by Laboratory 3 were too low, which may indicate loss of material following lyophilization, a step which was not included in the prescribed qNMR method. Half of the laboratories chose to use deuterated TSP and maleic acid as the internal standard and two laboratories used sodium formate and DSS. Laboratories which used maleic acid as an internal reference standard, in particular Laboratory 4 and 5, calculated a higher Vi PS content for sample A compared to Laboratory 2 and 8, which used TSP as an internal standard and Laboratory 7 that chose to use sodium formate. This phenomenon was not observed for sample B, with the exception of Laboratory 7 and 2 which used sodium formate and TSP, respectively. The unitage values for *S.* Typhi (sample B) from Laboratory 6 were more variable than the other laboratories, but the mean was close to the overall mean.

Data used to determine uncertainty of measurement are shown in Tables 10, 12 and 13. The estimated content of candidate IS 12/244 by qNMR across six laboratories is 1.94 ± 0.12 mg VI PS per ampoule (expanded uncertainty with coverage factor of k=2.11 taken to correspond to a 95% level of confidence; coverage factor was determined using Welch-Satterthwaite approximation for effective degrees of freedom). Candidate IS 16/126 has an estimated content of 2.03 ± 0.10 mg per ampoule (expanded uncertainty with coverage factor of k=2.11).

Determination of *O*-acetyl content in candidate international standards 12/244 and 16/126

Quantitative ^{1}H-NMR

The ratio of the two integrated resonances (*N*-Acetyl 2.07 ppm) and acetate (1.910 ppm) were used to calculate the percentage of *O*-acetylation. The *O*-acetylation contents are summarised in Table 11b and Figure 2b. Overall the results for the *O*-acetyl content were close throughout the eight laboratories. The estimated mean value of the *O*-acetyl content of candidate IS 12/244 for Vi *C. freundii* PS across eight laboratories is 94.3% per ampoule with a CV of 5.9%. The candidate IS 16/126 for Vi *S*. Typhi PS has an estimated *O*-acetyl content of 103.0% per ampoule with a CV of 3.2%.

Hestrin Assays

Ten laboratories returned data estimating the *O*-acetyl content of the Vi saccharide using the Hestrin assay, which are summarised in Table 14. The mean value for Vi PS 12/244 was 3.17 µmole *O*-acetyl/mg saccharide, with a CV of 24.1%, and a mean value for Vi PS 16/126 was

3.24 µmole *O*-acetyl/mg saccharide, with a CV of 29.1%. Laboratory 12 obtained comparatively lower results than the other participating laboratories but no reason could be identified from the method details provided. Excluding their results would give values of 3.33 µmole *O*-acetyl/mg saccharide (CV of 17.6%) for Vi PS 12/244 and 3.48 µmole *O*-acetyl/mg saccharide (CV of 16.2%) for Vi PS 16/126. In order to easily compare the *O*-acetyl content obtained from the Hestrin assay with the qNMR method the results can be converted to a percentage of *O*-acetyl content; this would equate to 88.3% *O*-acetylated for the *C. freundii* Vi PS 12/244 and 91.1% *O*-acetylated for the *S.* Typhi Vi PS 16/126. The estimated values determined by the Hestrin method are about 6-12% lower than those obtained by qNMR, compared with 5-17% lower as reported in the literature (Lemercinier 2000).

Participating laboratories returned results of *O*-acetyl content with good consistency for all Vi PS relevant samples (A, B and D). Of the test samples analysed by the Hestrin method Sample A (*S.* Typhi Vi PS) gave a mean value of 0.84 µmole *O*-acetyl/ml (CV 17.2%), Sample B (*C. freundii* Vi conjugate) gave a mean value of 0.81 µmole *O*-acetyl/ml (CV 19.7%), Sample C (MenW PS) gave a mean value of 0.30 µmole *O*-acetyl/ml (CV 63.9%) and Sample D (*S.* Typhi Vi conjugate) gave a mean value of 0.68 µmole *O*-acetyl/ml (CV 14.8%). The between-laboratory assay variation for Vi PS conjugate samples was in line with that determined for the two candidate Vi PS standards by this method.

Determination of Vi saccharide in bulk conjugate samples

The purpose of this part of the study was to determine if the candidate ISs could be used to accurately determine the Vi saccharide content in vaccine samples, in each of the commonly used methods. The use of polysaccharide standards to determine the saccharide content of oligo- or polysaccharide –protein conjugate vaccines has been demonstrated for Hib, Meningococcal and Pneumococcal classes of vaccines (Rosskopf , 2016; Gao, 2013; Lee, 2002). Whether the NMR-derived unitage would give similar results to that obtained by in-house standards whose unitages may have been determined by other commonly used methods was also of importance. By comparing the saccharide content values determined across laboratories when the candidate standards were used with that determined when the in-house standards were used allowed not only a comparison of the means to be compared, but also the variability. The use of a common, lyophilized and ampouled standard would be expected to reduce variability between (and within) laboratories. Laboratory mean estimates (µg Vi saccharide/ml) and CV values obtained for the bulk conjugate sample are summarised in Table 15 and Figure 3. A detailed description of the results obtained from each method is given below.

HPAEC-PAD

Seven laboratories returned data for the determination of Vi content in the supplied samples. Of these, five laboratories used both candidate standards and their in-house standard. Exceptions were Laboratory 11 which returned results using the two candidate ISs only and Laboratory 16, which returned data using its in-house standard only. Results from Laboratory 16 were omitted from the respective statistical analysis.

The use of the *C. freundii* Vi PS candidate standard 12/244 gave a slightly lower mean Vi content for samples A (*S.* Typhi PS), B (*C. freundii* Vi conjugate) and D (*S.* Typhi Vi conjugate), compared to the mean values determined from using the *S.* Typhi Vi PS candidate standard 16/126 and in-house standards (See Table 15). The CV for sample A (*S.* Typhi Vi PS) decreased from 14.4% to 5.4% or 4.0%, when using either the *Citrobacter* Vi PS 12/244 or the homologous 16/126 candidate standards, respectively. For estimation of Vi content in sample B and sample D,

there was no difference in CV values from the two candidate standards and in-house standard, however, it was noticed that Laboratory 10 and 15 underestimated Vi content in these two samples, compared with the other four laboratories. If the data from Laboratory 10 and 15 are excluded, then the CV using the two Vi candidate ISs is less than 5% for sample B and less than 10% for sample D, which indicates the possibility that the relatively lower values from Laboratory10 and 15 biased the CV values of candidate ISs.

Variation in the mean Vi content determined for sample D (*S.* Typhi Vi conjugate) was higher than those obtained for samples A and B. This infers that the conditions for hydrolysis may need to be optimised for this Vi PS conjugate, due to differences in conjugation chemistry and carrier protein. As all laboratories adopted the HPAEC-PAD method described for the analysis of *C. freundii* Vi PS and its conjugate (Micoli *et al.* 2011), then the increased variation observed for sample D is more likely to be caused by the method rather than the reference reagent. In general, the HPAEC-PAD assay showed improved inter-laboratory agreement when candidate ISs instead of in-house standards were used.

Rate nephelometry

Laboratory 3 and 18 returned results using the two candidate standards and their own in-house standards (*S.* Typhi Vi PS). Laboratory 18 obtained slightly lower values and higher CV values than Laboratory 3. This difference may be explained by the fact that Laboratory 3 used a sample buffer with Tween-80, and vortexed samples during the preparation stages, which may have resulted in lower CV values.

Both laboratories obtained the lowest CV values when candidate IS 16/126 (*S.* Typhi Vi PS) was used to determine the content of sample A (*S.* Typhi Vi PS). In this case the mean Vi contents were comparable to those determined with HPAEC-PAD and rocket immuno-electrophoresis. The PS content of sample D (*S.* Typhi bulk conjugate) wasbetween the values obtained by HPAEC-PAD and rocket immuno-electrophoresis.

Determination of the PS content of the *C. freundii* Vi PS- bulk conjugate using either of the candidate ISs or in-house standards gave significantly lower PS contents by rate nephelometry than by the HPAEC-PAD or rocket immuno-electrophoresis, and a slightly lower contents as obtained by ELISA. Slightly lower Vi contents were determined using the *C. freundii* standard for both the *S.* Typhi Vi samples A and D, whether PS or conjugate. Thus it appears that for rate nephelometry, an homologous Vi PS standard is better suited to determine the Vi PS contents of either plain Vi PS or Vi conjugate vaccines.

Rocket immuno-electrophoresis

Candidate IS 16/126 (*S.* Typhi Vi PS) gave a value for sample A (*S.* Typhi Vi PS) by rocket immuno-electrophoresis, which was comparable to that generated by in-house standards and with similar CV value. When Vi PS 12/244 (*C. freundii*) was used, the value for sample A was lower when compared to the in-house standard, though with a lower CV value. The Vi saccharide contents obtained for the bulk conjugate samples B (*C. freundii* Vi conjugate) and D (*S.* Typhi Vi conjugate) using candidate IS 16/126 were comparable to those when in-house standards were used. Saccharide contents obtained with candidate IS 12/244 were lower for both bulk conjugates, samples B and D. Both candidate ISs had similar CV values compared to in-house standards for samples B and D, except sample D determined using candidate Vi PS 12/244. The Vi content of Sample D was much lower when compared to other methods used for this study. This may be influenced by the characteristics of the conjugate, the carrier protein, and the solubility or viscosity of the Vi PS, rather than being solely the source of Vi PS.

ELISA

Laboratory 7 tested the samples by ELISA. The concentrations for samples A, B and D were similar when tested with both candidate ISs but were lower for all samples when compared to data produced when tested using in-house standards (Table 15).

Stability of the candidate international standards 12/244 and 16/126

Stability studies were performed by Laboratory 9. The amount of Vi PS per ampoule was compared over time using the HPAEC-PAD in the three types of stability studies: real-time, and accelerated thermal degradation studies of the lyophilized material and of the reconstituted sample. The amount of Vi PS in the -20°C baseline ampoule remained within 97% of the baseline sample at Month 6 (See Table 16). Lyophilised material stored up to +56°C showed no decrease in the content up to Month 4 and only a slight decrease at Month 6, with the contents remaining within 99% of the baseline at Month 6 (See Table 16). Due to the between-assay variability of the HPAEC-PAD assay (6% CV) any trends will need to be confirmed by additional data points. Thus due to its apparent stability, the current time points do not allow prediction of the percentage of Vi PS content lost during prolonged storage at -20°C.

Analysis of the molecular size of stability samples of both Vi PS 12/244 and 16/126 did not show significant temperature dependent changes at Month 6. Overall, *C. freundii* Vi PS had a 10% higher amount eluting by the column distribution coefficient, K_D, of 0.11 (Figures 4 and 5), suggesting that it may have a slightly higher hydrodynamic volume.

The Vi PS content of reconstuted material remained constant following storage at -20°C or +4°C for up to 2 weeks. At Month 6 the content appeared to decrease for storage at both -20°C and +4°C. The values were in line with lyophilized samples stored at -70°C and -20°C (See Table 17). This indicates the discrepancy may originate from intra-assay variation.

The pH of both Vi 12/244 and 16/126 samples remained stable at all temperatures up to month 6 (Figure 6). The pH of the *C. freundii* sample was 6.6, and that of the *S.* Typhi sample was 7.1.

Both candidate ISs had profiles consistent with clinical grade Vi PS. The stability study is ongoing and further sizing and viscosity studies will be performed to compare these characteristics in the two candidate ISs.

Discussion

Vi PS-based typhoid vaccines rely on physicochemical and serological methods to provide an estimation of the polysaccharide content as a measure of vaccine potency. However, the methods used are not well standardized between individual laboratories and worldwide different reference materials are used. The candidate ISs Vi PSs sourced from clinical grade *C. freundii* (12/244) and *S.* Typhi (16/126) have been developed and their intended use is to provide reference materials for manufacturers and NCLs to standardize Vi PS quantification. Separate aspects of the study dealt with the assignment of their mass unitages and the evaluation of their suitability to be used as standards in a variety of assays with samples representing plain Vi PS and Vi PS conjugate vaccines.

Determination of Vi 12/244 and 16/126 saccharide content

The establishment of a reference preparation containing a determined amount of Vi polysaccharide should facilitate calibration of in-house reference material to quantify the Vi content of the bulk saccharide, bulk conjugate and final fills of PS or PS-protein conjugate vaccines.

In line with WHO guidelines for a unitage assigned to an International Standard using Système International d'Unités the proposed mass unit assignment is derived from one physicochemical method, in this casse the qNMR, which is recognised as a potential primary method by Bureau International des Poids et Mesures. Results of CS574, showed that the Vi content by qNMR returned by participating laboratories had good agreement and a between-laboratory CV of 6.6% and 4.9% for Vi PS 12/244 and 16/126, respectively. Data from two laboratories were excluded and the results from the remaining six laboratories were given equal weighting to assign a final mass value to the content of the candidate ISs, Vi PS 12/244 and 16/126. Good agreement was also obtained between laboratories in their levels of *O*-acetylation. In addition, NIBSC evaluated their qNMR results based on ten vials of each Vi PS 12/244 and 16/126. For both candidate ISs, the relative standard uncertainty was extremely low, being within 0.8% of the estimate.

Suitability of the candidate international standards

In CS575 (2nd part), candidate ISs were assessed for their suitability in established methods to determine Vi saccharide content in vaccine samples by laboratories across the world. The results suggest that both candidate ISs can be used to estimate the amount of polysaccharide in final vaccines and vaccine bulk components. Both candidate ISs generated a dose response curve in the HPAEC-PAD, rocket immunoelectrophoreses, rate nephelometry and ELISA from which a standard curve could be derived to determine the polysaccharide content in samples representative of Vi PS vaccines. The study also showed that rocket immuno-electrophoreses may not be suitable for all bulk Vi PS conjugates. HPAEC-PAD, showed good inter laboratory comparability and the use of candidate ISs reduced the variability of measurements for *S.* Typhi Vi PS in test samples.

When in-house standards were used to measure the saccharide contents of an *S.* Typhi Vi PS sample, the Vi content was slightly higher when compared with using the two candidate ISs (Table 15 and Figure 3). A mass unitage assigned by qNMR improved accuracy and reduced uncertainty, however a laboratory may choose to use other physico-chemical methods or serological assays to determine the potency of Vi saccharide content in final products. In this case a slight reduction in Vi content may be observed with the use of the candidate ISs, depending on the product and current in-house standard. Thus careful consideration about introduction of a candidate IS in laboratories which test the the Vi saccharide content of licensed products, with a proven efficacy, is required, as product consistency is vital. Chemical- and serological-based methods will continue to be important in their testing. When the saccharide content was determined for bulk conjugates made with Vi PS from either *C. freundii* or *S.* Typhi, the in-house and the candidate ISs were in agreement for these methods, however a higher variability between laboratories and methods was observed (See Figure 3). Therefore our study shows that a mass unitage assignment for candidate ISs Vi PS by qNMR remains as the most appropriate method. The observation that use of the *C. freundii* Vi PS candidate IS in most methods and by most laboratories resulted in a lower saccharide content for samples of *S.* Typhi Vi PS, endorses the the use of a homologous Vi PS reference when assaying the content of Vi vaccines.

Stability and Storage of the candidate standards

Real time stability, reconstituted and accelerated thermal degradation studies are on-going. Data collected up to Month 6 on the saccharide content, molecular sizing, appearance and pH of the Vi PS candidate ISs indicates that both 12/244 and 16/126 are stable at elevated temperatures. However, it is recommended these Vi PS standards should be stored at -20°C. Sufficient time should be allowed for Vi PS 12/244 and 16/126 to be reconstituted due to their low solubility and high viscosity. The Instructions for Use will recommend thorough mixing and allowance for 2 hours at room temperature and overnight at +4 °C for complete dissolution.

Proposal

Based on the results obtained from CS 574 and CS 575, we propose that the Vi PS candidate IS 12/244 is established as the 1st International Standard for the *Citrobacter freundii* Vi polysaccharide with a content of 1.94 ± 0.12 mg Vi PS per ampoule (expanded uncertainty with coverage factor of k=2.11), and the Vi PS candidate IS 16/126 is established as the 1st International Standard for the *Salmonella enterica* subspecies *enterica* serovar Typhi Vi polysaccharide with a content of 2.03 ± 0.10 mg Vi PS per ampoule (expanded uncertainty with coverage factor of k=2.11), as determined by qNMR. The storage condition for both standards is -20^{o}C.

Both standards can be used in ELISA, HPAEC-PAD, rocket immuno-electrophoresis and rate nephelometry for the quantification of Vi PS in bulk Vi PS and bulk Vi conjugates and final lots of Vi vaccines. The standards have been assigned a mass unitage in SI units to adhere to the convention that the product specification of polysaccharide and polysaccharide conjugate vaccines is in SI units, that is, µg per dose. Indeed, WHO ISs for *Haemophilus influenzae* type b capsular PS, and for meningococcal serogroup A, C and X polysaccharides also have a unitage assigned in mg/ampoule (Mawas 2015; Vipond 2012; Vipond 2015).

Participants Comments

Participants were asked to correct and/or supply full names, addresses, method details, and to check that their data was correctly represented, which most laboratories did. In addition, participants were asked if they agreed with the overall recommendation of the draft report, and if they had any other comments to make. Responses were received from all laboratories except 1 and 6. All 16 who responded agreed with the proposal and the additional comments were made. Replies from the NIBSC authors are in italics.

Laboratory 7

Yes [we agree] as regards the overall conclusion of the fitness for purpose of both candidate standards. However, some clarifications are expected as regards the stability data presented (see section 4)

Page 12 – section on stability studies : the number of replicate analysis per time point should be specified as it is a critical information to ensure proper evaluation of trends. This relates as well on page 20 – Stability and Storage : the data in table 15 and 16 where some potential trends or inhomogeneity could be perceived instead of analytical variability. This should be discussed in the relevant section on page 20.

The number of replicate analysis per time point used for the stability testing has been listed in the Methods where samples were duplicate injected for HPAEC-PAD and HPLC-SEC, and a

sentence has been inserted into the Stability Results section acknowledging that further data points are required to confirm any trends.

As a side question, why was the stability monitoring not performed as well through Q-NMR ? Not stability indicating ?

Not being a high throughput method, only selected time-points will be selected for qNMR, which will be reported on in a future publication.

Page 13 – section on Q-NMR. Should the data from lab 6 be also discussed alongside 1 and 3 as they present either unexpectedly high variability or low value ?

Yes, a comment has been added to the NMR Results section. Thank you.

Laboratory 12

In fact, the micro-Hestrin's method has not been established at our laboratory. Hestrin's method been validated by our laboratory is the (macro) Hestrin's which use cuvettes and UV-Vis spectrophotometer. The (macro) Hestrin's method needs large amount of either sample or standard, so we have planned to validate micro-Hestrin's method for sample and standard efficiency. We perform the micro-Hestrin's validation along with collaboration work due to the limited availibility of samples and standard candidate. Perhaps, if we could perform the more repetition, our results would be better. Thank you very much for this opportunity led us to participate in this collaborative study. By participating in this collaborative study, we could improve our performance in TCV testing.

Thank you for your comments and valuable participation.

Laboratory 17

We are pleased as the overall performance of the study has an excellent outcome. We would like to comment on the Rocket Immuno-electrophoresis, as mentioned in the Result section and suitability of the candidate international standards section, that the Rocket Immuno-electrophoresis may not be suitable for all the bulk Vi PS conjugate.

In general, results of sample A (S.typhi Vi PS) from HPAEC-PAD and Rocket Immuno-electrophoresis were comparible, but for conjugate samples B and D, Rocket Immuno-electrophoresis gave lower Vi content comparing to HPAEC-PAD, The under estimation was considered due to slower moving rate of larger conjugate molecule in the gel. Decision of using the method should be made case by case with optimisation based on different types of conjugate (carrier protein), to compensate lower moving rate of conjugate to PS standard.

However, our Rocket Immuno-electrophoresis data for the samples are comparable with the HPAEC-PAD results. As 4 laboratories performed the Vi content by Rocket Immuno-electrophoresis and results for the other three laboratories did not turn out good (results are towards lower side) hence, the overall mean values is not comparable to the HPAEC-PAD data. Please consider to include a comment that only one lab data (Lab 17) of Rocket Immuno-electrophoresis is comparable to the HPAEC-PAD in the final report.

The comment contradictated with results of conjugate samples B and D in the report (Table 15). Resules from Laboratory 17 were lowest for sample B and highest for sample D, among all results from Rocket Immuno-electrophoresis method. Those results from Laboratory 17 were also much lower (approximately 20-50%) than average values from HPAEC-PAD method for the same sample. The authers are not in agreement with the comment.

Laboratory 18

One suggestion would be to add a comment in the Discussion saying that the chemical methods are relevant to assign a content per ampoule to a given purified polysaccharide or to monovalent bulks.
However, there are 2 restrictions:
(i) They are not applicable for the determination of individual polysaccharidic in multi-component vaccines (i.e. multi serotype pneumococcal and meningococcal vaccines). In these cases, the controls rely on immunological methods which use specific antibodies.
(ii) These purified products are suitable to be used as standard for immunological methods with the restriction that they do not match the exact composition of the final product of the vaccine (possible matrix effect).
Lastly, it is important to stress out that chemical and biological methods give different information on a specific product which are complementary: composition and structure vs biological activity.

We agree. Additional emphasis on the usefulness of the chemical-based, and indeed serological-based quantitative methods has been givien in the Discussion section. While qNMR provides a more accurate unitage, standards with NMR-assigned unitages may not be suitable for products which have been licensed with potency determined using chemical- or serological-based methods. Decisions on the incorporation of the unitages must be made on a case-by-base basis; this study was not designed to determine unitage based on other commonly used methods, although the data presented from CS575 will allow laboratories to make individual assessments about the possibility of using the candidate IS.. It is anticipated that laboratories and manufacturers developing new products may be most easily be able to use these qNMR-assigned standards. Thank you for highlighting these important considerations.

Laboratory 20

I would like that more emphasis to be put on the high variability of results between the different methods and more important, between the use of the candidate ISs and the in-house reference standard. The use of the ISs for quantification of PS in established products with a proven efficacy should be considered carefully. Consistency of the products must be maintain and if not adequate for quantification, ISs standards could be used as a way to monitor in-house standard consistency and stability

The authors are grateful to you for your comments. That so many laboratories were willing to commit their resources to the study, with many participating in both studies and with multiple methods, has allowed these inter-method comparisons and observations to be made. We wholeheartedly agree with your concerns and thoughts about the need for careful consideration for ensuring product consistency. Also we realize the advantages of using a lyophilized well-characterized standard evaluated through collaborative study: traceability, decreased variability and improvement in stability and, possibly, supply.

As the method of calibration of the future first IS is RMN [Resonance Magnetic Nuclear], would it not be logical to include this type of methods as alternatives to immunochemical methods in the European Pharmacopeia monograph for Vi Polysaccharide vaccine ?

Thank you for this suggestion, which may be taken forward to other Pharmacopeias as well.

Responses were not received from Laboratory 1 and 6.

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Annex 2. Calculation of *C freundii* Vi PS (12/244) and *S*. Typhi Vi PS (16/126) standard residue weights

S. Typhi Vi PS (16/126) saccharide residue weight Sodium salt, fully *O*-acetylated

Atom	Mass[a]	Number	Total Mass
Carbon	12.011	10	120.11
Oxygen	15.999	7	111.993
Hydrogen	1.008	12	12.096
Nitrogen	14.007	1	14.007
Sodium	22.99	1	22.99
Total Mass			**281.196**

[a] IUPAC 2015 atomic mass values were used.

S. Typhi Vi (16/126) saccharide residue weight: 281.196 g/mol

The residue weight of the Vi PS repeating unit, non-*O*-acetylated, sodium form is 239.16 g/mol. To adjust for different levels of *O*-acetylation, add (x % *O*-acetylation * (43.045 g/mol for the acetate group - 1 hydrogen).

Citrobacter freundii Vi (12/244) saccharide residue weight: 278.675 g/mol

239.16 g/mol + (0.94 * 42.037 g/mol) = 278.675 g/mol

Table 1a. Summary of results from the manufacturer's analysis of *C. freundii* Vi purified bulk PS

Test Name	**Method**	**Result**
Vi content*	HPAEC-PAD	2.17 mg/ml (cv 2.5%)
Vi content*	Hestrin	1.71 mg/ml (cv 1.4%; 6.6 µmol/ml acetyl-esters)
*Vi content is expressed as equivalent of fully acetylated free acid form (repeating unit molecular weight, $C_{10}H_9NO_7$: 259 g/mol)		
Identity Test	^{1}H-NMR	Positive
O-Acetylation	^{1}H-NMR	99.5%
Molecular sizing	HPLC-SEC	K_D 0.357; peak width at 50% of height 3.03 min
		K_D 0.305; peak width at 50% of height 2.86 min
pH		6.42
Conductivity		464 µS/cm
Endotoxin	LAL	0.13 EU/µg Vi

Table 1b. Summary of results from the manufacturer's analysis of *S.* Typhi Vi purified bulk PS

Test Name	**Specification**	**Result**
Identity Test	Positive	Positive
Moisture Content	≤20%	4.8%
Molecular sizing	At least 67% of Vi PS elutes before Kd value of 0.25	80%
O-Acetyl content	≥2.0 µmol/mg of dry weight	3.6 µmol/mg
Protein content	<1.0% of dry weight	<0.3% dry weight
Nucleic Acid content	<2.0% of dry weight	0.6% dry weight
Endotoxin content	≤150.00 EU per vaccine dose (25 µg of PS)	< 6.25 EU/d

Table 2. Filling and Freeze-drying details for *C. freundii* and *S.* Typhi Vi PS

Lyophilization details	**Vi PS candidate IS**	
	12/244 (*C. freundii*)	**16/126 (*S.* Typhi)**
Amount H_2O added prior to fill	N/A	1.08 litre added to 2.5922 g
Date of fill	31 Jan 2013	13 May 2016
Temperature of fill	15-20°C	15-20°C
Freeze Drying run cycle	Freeze at -40°C for 4 h Lyophilize at -40°C and -25 °C for 37 h Desorp at 30°C for 44 h	
Date ampoules sealed	4 Feb 2013	19 May 2016
Mean Mass of fill and % CV (number of ampoules measured)	1.0099 g CV 0.28% (69 ampoules)	1.0076 g CV 0.24% (39 ampoules)
Mean dry weight and %CV (number of ampoules measured)	0.0029 g CV 20.0% (25 ampoules)	0.00170 g CV 9.51% (25 ampoules)
Mean residual moisture and %CV (number of ampoules measured)	0.1411% CV 38.1% (12 ampoules)	4.1172% CV 16.8% (12 ampoules)
Mean oxygen head space and % CV (number of ampoules measured)	0.36% CV 30.5% (12 ampoules)	0.35% CV 27.55% (12 ampoules)
Number of ampoules for stock	1849	881
Label Details	12/244 Vi Capsular Polysaccharide (*Citrobacter freundii*)	16/126 Vi Capsular Polysaccharide (*Salmonella* Typhi)

Table 3. Description of materials received by Collaborative Study participants

Study code	**Aim of study**	**Sample code**	**Sample description**
CS 574	Unitage assignment	A	*C. freundii* Vi PS, 12/244
		B	*S.* Typhi Vi PS, 16/126
CS 575	Fitness-for-purpose	Standard	Vi PS 12/244
		Standard	Vi PS 16/126
		A	S. Typhi Vi PS
		B	*C. freundii* Vi PS conjugate
		C	MenW PS
		D	*S.* Typhi Vi PS conjugate

Table 4. Details of the NMR parameters used by participants in the Vi polysaccharides Collaborative Study

Parameter	**Laboratory**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Brand	Bruker	Bruker	Bruker	Bruker	Bruker	Bruker	Bruker	Bruker
Model	AVANCE III	AVANCE III	AVANCE III	AVANCE III	AVANCE III	AVANCE III	AVANCE III	AVANCE III
Field (MHz)	500	600	600	400	600	500	500	500
Number of scans	80	80	128	80	64	A=256 B=184	80	A=16 B=32
D1 Value used	26	26	30	35	30	26	140	A=20 B=28
Temperature (°C)	50	50	50	50	50	50	50	50
Pulse width P1 (µsec)	12.97	13.28-14.09	8.1	8	14.24-15.36	10	11.42-11.68	A= 12.76 B= 12.75
Pulse sequence	zg	zg	Zg30	zg	zg	zg	zg	Noesy1d
Spectral reference (Name and cat #)	DSS	TSP-d4 Sigma Aldrich 269913	TSP-d4	Maleic acid Sigma Aldrich 92816	Maleic acid Sigma Aldrich 92816	Maleic acid Sigma Aldrich #BCBMB8127 V	Sodium formate Sigma Aldrich 456020	TSP-d4 Sigma Aldrich 269913
Spectral reference (Final conc in sample)	0.01% w/v	0.1723 mg/mL	1.65 mg/mL	0.99107 mg/tube	0.373 mg/mL	0.25 mg/mL	5.04 mmol/mL	0.1280 mg/mL
Sonicator Brand	Branson	NingBO Scientz Biotechnology Ltd	N/A	Bandelin	Branson	Elmasonic	Branson	VWE Ultrasonic Cleaner USC-TH
Model	1510	SB 25-12NDT	N/A	DT-52	1200	S180	Ultrasonic cleaner 2510	USC 300TH
Output power (Watts)	155W	500W	N/A	240W	-	-	100W	200W

Table 5. NIBSC ^{1}H parameters for qNMR of Vi used for both non-acetylated and De-*O* acetylated samples

Parameter	NOESY-1D
	Vi PS
Data File Name	C:Bruker /160816_Vi_12-244_(vial 1-10) /20/pdata/1/1r C:Bruker /160816_Vi_12-244_(vial A1-A4) /20/pdata/1/1r C:Bruker /160816_Vi_16-126_(vial 1-10) /20/pdata/1/1r C:Bruker /160816_Vi_16-126_(vial A1-A4) /20/pdata/1/1r
Origin	Bruker BioSpin GmbH
Solvent	D_2O
Temperature	323 K
Pulse Sequence	Noesy1d
Experiment	1D-NOESY
Probe	Z810701_0094 (PA BBI 500S2 H-BB-DD-05 Z)
Number of Scans	16 (12-244) 32 (16-126)
Receiver Gain	32
Relaxation Delay (sec)	20 (12-244) 28 (16-126)
Pulse Width (μsec)	12.76 (12-244) 12.75 (16-126)
Acquisition Time	8.7424
Acquisition Date	16th August 2016 (12-244) 17th August 2016 (16-126)
Spectrometer Frequency	499.78 MHz
Spectral Width	7496.3 Hz
Lowest Frequency	-2479.3
Nucleus	^{1}H
Acquired Size	65535
Spectral Size	131072

Table 6. Details of the Hestrin assay conditions used by participants in the Collaborative Study

Parameter	Laboratory									
	4	**8**	**9**	**10**	**12**	**13**	**14**	**15**	**17**	**19**
Standard	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine	Acetylcholine
Catalogue #	A9101	A6625	A6625	A6625	A6625	A6625	A6625	A6625	A6625	A6625
Lot #	BCBR3675V	BCBM481V	Not given	BCBM4814V	BCBM4814V	123K2625	BCBK5708V	BCBM4814V	BCBM4814V	BCBK5708V
Purity	100%	≥99%	≥99%	≥99%	≥99%	≥99%	≥99%	≥99%	≥99%	≥99%
Standard curve	0.413-4.13 µmol/ml	0.055 - 4.125 µmol/ml	0.25 – 2.5 µmol/ml	6.87 – 412.6 nmol/ml	0.055 – 0.550 µmol/ml	6.87- 412.5 nmol/ml	17 – 242 µmol/ml	0.5 – 10 µmol/ml	0.0825 – 1.2375 µmol/ml	50 – 750 µg *O*-acetyl/tube
#levels	6	8	6	7	5	7	5	7	5	7
Diluent and blank	Water	Two fold dilution in water. Candidate ISs diluted to 200 µg/ml PS.	Two fold dilution in water.	"Blank and no blank" method	Sterile purified water	"Blank and no blank" method. 10 to 20 fold dilution in water.	"Blank and no blank" method	"Blank and no blank" method	"Blank and no blank" method	
Replicates	Duplicate	Triplicate	Duplicate		Triplicate	Single with two dilution points.	Duplicate	Duplicate	Duplicate and one correction tube per pair	Duplicate
Sample Volume	200 µl	50 µl	50 µl	750 µl	50 µl	750 µl	100 µl	100 µl	100 µl	
Optical density range	0.105-0.719	0.04 –0.561	-0.0003–0.3139	0.001 – 0.168	0.043-0.191	0.0005–0.1976	0.016-0.199	0.051–1.129	0.0130–0.2161	
Wavelength	540 nm	540 nm	540 nm	540 nm	540 nm	540 nm	540 nm	540 nm	540 nm	
Instrument	Perkin Elmer XLS+	Molecular Devices Microplate reader	Molecular Devices, SpectraMax i3		Xmark™ Microplate reader	Beckman Coulter, DU 800	UV-1800 SHIMADZU Spectrometer	BioTek Microplate reader	UV Vis Spectrometer	

Table 7. Details of the HPAEC-PAD conditions used by participants in the Collaborative Study

Parameter	**Laboratory**						
	4	**8**	**10**	**11**	**13**	**15**	**16**
In-house standard	Vi PS (*Citrobacter*)	Vi PS (*Citrobacter*)	Vi PS (*S.* Typhi)	-	Vi PS (*S.* Typhi)	Vi PS (*S.* Typhi)	Vi PS (*Citrobacter*)
Concentrations of in-house standard	200.9 µg/ml	1 mg/ml (qNMR)	1 mg/ml	-	1.42 mg/ml	1 mg/ml	209.6 µg/ml
Moisture content	-	-	-	-	-	9.685%	-
Standard curve	15-200 µg PS/ml	0.5-27 µg PS/ml	6-100 µg PS/ml or 12-200 µg PS/ml	0.5-27 µg PS/ml	10-100 µg PS/ml	2.5-100 µg PS/ml	16.9-209.6 µg PS/ml
#levels		5	7	5	4	6	6
Internal spike	-	NAcGlc-6-P	-	Glc-1-P	-	-	-
Hydrolysis	2M NaOH	2M NaOH	2M NaOH	2M NaOH	2M NaOH	4M NaOH	2M NaOH
Incubation	4h, 110°C	4h, 110°C	4h, 110°C	4h, 110°C	4h, 110°C	4h, 110°C	4h, 110°C
Make/Model	Dionex ICS3000	Dionex ICS5000	Dionex ICS3000	Dionex ICS5000	Dionex ICS5000	Dionex ICS5000	Dionex ICS5000
Electrode	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl	Au, Ag/AgCl
Waveform	Quadruple	Quadruple	Quadruple	Quadruple	Quadruple	Quadruple	Quadruple
Column (CarboPac)	PA1 (4x250 mm)	PA1 (2x250 mm)	PA1 (4x250 mm)	PA1 (4x250 mm)	PA1 (4x250 mm)	PA1 (4x250 mm)	PA1 (4x250 mm)
Guard column	PA1 Guard	Amino Trap	Amino Trap	PA1 Guard	PA1 Guard	PA1 Guard	PA1 Guard and Amino Trap
Column and Autosampler (AS) temperature	30°C (Column) 10°C (AS)	30°C (Column) 4°C (AS)		30°C (Column) 10°C (AS)	10°C (AS)	20°C (Column) 10°C (AS)	30°C (Column) 10°C (AS)
Eluting condition	0-2 min, 100mM NaOH, 40mM $NaNO_3$; 2-22 min, 100mM NaOH, 40-150mM $NaNO_3$ 22-31 min, 100mM NaOH, 40mM $NaNO_3$	0-2 min, 100mM NaOH, 40mM $NaNO_3$; 2-22 min, 100mM NaOH, 40-150mM $NaNO_3$ 22-31 min, 100mM NaOH, 40mM $NaNO_3$	0-2 min, 100mM NaOH, 40mM $NaNO_3$; 2-22 min, 100mM NaOH, 40-150mM $NaNO_3$ 22-31 min, 100mM NaOH, 40mM $NaNO_3$	0-2 min, 100mM NaOH, 40mM $NaNO_3$; 2-22 min, 100mM NaOH, 40-150mM $NaNO_3$ 22-31 min, 100mM NaOH, 40mM $NaNO_3$	0-22 min, 100mM NaOH, 40-150mM $NaNO_3$ 22-28 min, 100mM NaOH, 150 mM $NaNO_3$ 28-35 min, 100mM NaOH, 40mM $NaNO_3$	0-30 min, 100mM NaOH, 40-150mM $NaNO_3$ 30-35 min, 100mM NaOH, 40mM $NaNO_3$	0-22 min, 100mM NaOH, 40-150mM $NaNO_3$ 22-32 min, 100mM NaOH, 40mM $NaNO_3$
Flow rate Run time	1 ml/min 31 min	0.25 ml/min 31 min	1 ml/min 31 min	1 ml/min 31 min	1 ml/min 35 min	1 ml/min 35 min	1 ml/min 32 min
Eluting time	12.5 min	12.0-12.5 min	6.8-7.5 min	11.7 min	9.1-9.9 min	11.7-12.0 min	12.0-14.0 min

Table 8. Details of the Rate Nephelometry conditions used by participants in the Collaborative Study

Parameter	**Laboratory**	
	3	**18**
In-house standard (source)	Lyophilized *S.* Typhi Vi PS from manufacturer	Vi PS vaccine from manufacturer
Concentrations of in-house standard	22.32 µg/Vial, concd stock; 56 µg/ml working stock	46 µg/ml working stock
Moisture content	Not Provided	NA, Liquid
Standard curve	0.5 – 2.5 µg PS/ml	0.7 – 3.5 µg/ml
#levels	5	5
Response range	40-265 rate units	63-321 rate units
Sample buffer	0.85% NaCl and 0.02% w/v Tween-80	Phenol buffer solution
Sample preparation	Vortex, dilute with buffer, incubate at RT and vortex at 0, 30 and 60 min.	Gently stir, vortex, centrifuge to remove bubbles
Sample volume	~ 20 µl	21 µl
Vi antibody source	Manufacturer provided	Manufacturer provided, or other validated source
Diluent	Immage #447640	Immage #447640
Antibody dilution	10X provided by manufacturer	1/8
Equipment Make/Model	Beckman Coulter IMMAGE 800	Beckman Coulter IMMAGE 800
Wavelength (nm)	670	670
Temperature	22 °C	21 °C

Table 9. Details of the Rocket Immuno-electrophoresis conditions used by participants in the Collaborative Study

Parameter	**Laboratory**			
	9	**17**	**18**	**20**
In-house standard source	*S.* Typhi Vi PS from manufacturer	Internal working standard	Vi PS from manufacturer	Lyophilized *S.* Typhi Vi PS
Concentration of in-house standard	3.95 mg/ml (as Total Solids)	27.2 µg/dose (0.5 ml)	100 µg/ml	22,3 µg/flacon (reconstituted at 100 µg/ml)
Standard curve (µg/ml)	100, 50, 25, 12.5, 6.25	50, 40, 30, 20	65, 55, 45, 35	100, 50, 25, 12.5
Electrophoresis voltage and run time	12V/cm, until Bromophenol Blue reach the other edge of gel	100V, 2.5 hours	40V, 6 hours	3V/cm, 15-18 hours
Rinsing protocol	1-2 hours purified water	No washing step	3-48 hour saline followed by 10 mins purified water	Sodium chloride for 1 hour followed by 15 mins purified water
Stain and destain protocol	Stain for 15 mins, destain for 20 mins	Stain for minimal 5 mins, destain until clear peaks are observed	Stain for 5 mins, destain for 5 mins	Unspecified timings

Table 10. Quantitative NMR values for Vi PSs (μg PS per ampoule) from NIBSC only

Ampoule	Vi content (μg Vi saccharide/ampoule)	
	Vi PS 12/244	**Vi PS 16/126**
1	1.99	2.13
2	1.97	2.14
3	1.99	2.13
4	2.01	2.11
5	2.00	2.14
6	2.01	2.14
7	1.98	2.15
8	1.98	2.12
9	2.01	2.17
10	2.00	2.15
Mean	**1.99**	**2.14**
SD	**0.02**	**0.02**
CV	**0.8%**	**0.8%**

Table 11a. The mass unitage assignment in mg PS by qNMR for coded samples A and B

Sample A (C. *freundii* Vi PS, 12/244)

Lab	Day 1	Day 1	Day 2	Day 2	Mean	SD	CV		All	Excluding 1, 3
1	2.80	2.50	3.00	2.70	2.75	0.21	7.6%	**Overall mean**	1.98	1.94
2	1.82	1.84	1.82	1.83	1.83	0.01	0.4%	**SD**	0.36	0.13
3		1.42	1.36	1.70	1.49	0.18	12.3%	**CV**	18.3%	6.6%
4	2.01	2.04	2.08	1.98	2.03	0.04	2.1%	**SEM**	0.13	0.05
5	2.08	2.26	2.02	2.05	2.10	0.11	5.1%			
6	1.71	1.79	1.69	1.87	1.77	0.08	4.7%			
7	1.90	1.92	1.89	1.93	1.91	0.02	1.0%			
8	1.98	1.99	1.98	2.00	1.99	0.01	0.3%			

Sample B (S. Typhi Vi PS, 16/126)

Lab	Day 1	Day 1	Day 2	Day 2	Mean	SD	CV		All	Excluding 1, 3
1	2.40	3.20	3.20	3.50	3.08	0.47	15.3%	**Overall mean**	2.11	2.03
2	1.86	1.88	1.87	1.88	1.87	0.01	0.3%	**SD**	0.42	0.10
3	1.60	1.18	1.92	1.98	1.67	0.37	22.2%	**CV**	19.7%	4.9%
4	2.10	2.06	2.10	2.08	2.09	0.02	1.0%	**SEM**	0.15	0.04
5	2.11	2.08	2.08	2.04	2.08	0.03	1.4%			
6	2.30	1.90	2.11	1.97	2.07	0.18	8.5%			
7	1.95	1.95	1.93	1.90	1.93	0.02	1.2%			
8	2.11	2.13	2.11	2.12	2.12	0.01	0.6%			

NMR value from Lab 1 was excluded due to high variation.
NMR value from Lab 3 was excluded due to re-freeze drying of the materials.

Table 11b. Summary of *O*-acetyl content in % by qNMR for coded samples A and B

	Sample A (*C. freundii* Vi PS, 12/244)							Sample B (*S.* Typhi Vi PS, 16/126)						
Lab	**(1)**	**(2)**	**(3)**	**(4)**	**Avg.**	**Mean**	**CV (%)**	**(1)**	**(2)**	**(3)**	**(4)**	**Avg.**	**Mean**	**CV (%)**
1	108.0	107.0	106.0	107.0	107.0			95.0	100.0	111.0	9700	100.8		
2	94.7	94.1	93.2	93.8	94.0			104.2	107.0	102.8	106.4	105.1		
3		88.0	88.0	100.0	92.0			100.0	97.0	102.0	102.0	100.3		
4	90.8	90.1	89.7	93.6	91.1	94.3	5.9	99.6	102.7	95.7	101.1	99.8	103.0	3.2
5	96.0	94.0	96.0	96.0	95.5			105.0	106.0	107.0	106.0	106.0		
6	88.3	88.5	88.7	86.6	88.0			99.4	99.1	99.5	98.3	99.1		
7	94.0	93.0	94.0	93.0	93.5			106.0	107.0	107.0	107.0	106.8		
8	93.3	93.3	93.4	94.2	93.6			105.8	106.4	106.9	106.2	106.3		

Table 12. Uncertainty determination of Vi PS content determination of candidate IS 12/244

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Weight of the TSP-d_4 reference material	From calibration data (±0.012mg, assuming triangular distribution)	12.700mg	0.005mg	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 (±0.019mg, assuming triangular distribution)	1000mg	<0.1mg	<0.01%
Random error and between-ampoule homogeneity	SD (Table 10)	1.99%	0.02	0.78%
Between-laboratory variability	SEM (Table 11a)	1.94mg	0.05	2.69%
Combined relative standard uncertainty				**2.86%**

Table 13. Uncertainty determination of Vi PS content determination of candidate IS 16/126

Source	How Assessed	Value	Standard Uncertainty	Relative Standard Uncertainty
Weight of the TSP-d_4 reference material	From calibration data (±0.012mg, assuming triangular distribution)	12.700mg	0.005mg	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 (±0.019mg, assuming triangular distribution)	1000mg	<0.1mg	<0.01%
Random error and between-ampoule homogeneity	SD (Table 10)	2.14%	0.02	0.81%
Between-laboratory variability	SEM (Table 11a)	2.03mg	0.04	2.00%
Combined relative standard uncertainty				**2.24%**

Table 14a. Mean *O*-acetyl content in µmole/mg saccharide of Vi PS candidates 12/244 and 16/126 by Hestrin assay

Lab	12/244 Vi PS					16/126 Vi PS				
	(1)	(2)	Mean	Mean	CV (%)	(1)	(2)	Mean	Mean	CV (%)
4	3.07	3.15	3.11			3.68	3.76	3.72		
8	2.80	3.30	3.05			3.00	3.30	3.15		
9	3.65	3.97	3.81			3.83	4.07	3.95		
10	3.31	3.09	3.20			3.40	3.38	3.39		
12	1.86	1.47	1.67	3.17	24.1	1.17	0.87	1.02	3.24	29.1
13	2.84	3.04	2.94			3.17	3.29	3.23		
14	4.00	3.88	3.94			4.31	4.10	4.21		
15	3.61	3.47	3.54			3.93	3.52	3.73		
17	2.19	2.32	2.26			2.13	2.43	2.28		
19	3.70	4.60	4.15			3.50	3.90	3.70		

Table 14b. Mean *O*-acetyl content in µumole/mg saccharide of coded samples A-D by Hestrin assay

Lab	Sample A (S.Typhi Vi PS)					Sample B (*C. freundii* Vi bulk conjugate)					Sample C (MenW PS)					Sample D (*S.* Typhi Vi bulk conjugate)				
	(1)	(2)	Mean	Mean	CV (%)	(1)	(2)	Mean	Mean	CV (%)	(1)	(2)	Mean	Mean	CV (%)	(1)	(2)	Mean	Mean	CV (%)
4	0.92	0.94	0.93			0.79	0.78	0.79			0.26	0.23	0.25			0.71	0.70	0.70		
8	0.85	0.84	0.85			0.89	0.84	0.87			0.25	0.20	0.23			0.65	0.72	0.69		
9	0.95	0.94	0.94			0.85	0.91	0.88			0.27	0.25	0.26			0.75	0.63	0.69		
10	0.88	0.90	0.89			0.66	0.71	0.68			0.22	0.22	0.22			0.68	0.76	0.72		
12	0.70	0.63	0.66	0.84	17.2	0.51	0.53	0.52	0.81	19.7	0.16	0.15	0.15	0.30	63.9	0.52	0.49	0.50	0.68	14.8
13	0.79	0.84	0.82			0.70	0.73	0.72			0.22	0.25	0.24			0.69	0.67	0.68		
14	0.64	0.65	0.65			1.06	0.99	1.03			0.83	0.78	0.80			0.70	0.76	0.73		
15	1.09	1.00	1.04			1.06	0.98	1.02			BDL	BDL				0.74	0.79	0.77		
17	0.59	0.73	0.66			0.69	0.71	0.70			0.27	0.27	0.27			0.55	0.46	0.50		
19	1.00	1.00	1.00			0.90	0.90	0.90			0.30	0.30	0.30			0.80	0.80	0.80		

Table 15. Laboratory mean estimates of Vi Saccharide contents in µg PS per ml for CS575 Samples coded A, B and D using A) candidate standard Vi PS 12/244 or B) candidate standard Vi PS 16/126 or C) in-house standards

Method	Lab	Vi Saccharide content, Sample A (*S.*Typhi Vi PS)									
		(1)	(2)	(3)	(4)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
A) Using candidate *C. freundii* Vi PS 12/244											
HPAEC-PAD	4	257.0	241.0	258.0	254.0	252.5	3.1	241.0	5.4	231.4	9.7
	8	248.9	239.5	224.7	213.1	231.6	6.8				
	10	231.0	258.4	247.7	245.7	245.7	4.6				
	11	256.5	235.9	243.4	209.3	236.3	8.4				
	13	253.0	253.3	270.6	251.6	257.1	3.5				
	15	230.0	218.6	226.9	216.2	222.9	2.9				
Rocket Immuno-electrophoresis	9	199.6	209.0	215.9	184.4	202.2	6.7	216.4	5.4		
	17	245.0	211.0	224.0	226.0	226.5	6.2				
	18	237.0	202.0	211.0	195.0	211.3	8.7				
	20	230.7	247.2	207.0	216.9	225.5	7.7				
Rate Nephelometry	3	216.6	242.7	227.5	230.0	229.2	4.7	211.0			
	18	191.0	169.3	215.3	195.7	192.8	9.8				
ELISA	7	274.0	273.0	273.0	277.0	274.3	0.7	274.3			
B) Using candidate *S.* Typhi Vi PS 16/126											
HPAEC-PAD	4	254.0	253.0	258.0	246.0	252.8	2.0	252.9	4.0	256.0	8.9
	8	249.1	255.8	239.6	246.4	247.7	2.7				
	10	242.3	249.6	260.4	258.9	252.8	3.4				
	11	236.1	252.9	287.3	264.4	260.2	8.3				
	13	259.4	263.4	279.0	264.7	266.6	3.2				
	15	248.3	239.5	233.7	227.8	237.3	3.7				
Rocket Immuno-electrophoresis	9	334.2	252.2	256.0	256.7	274.8	14.4	264.0	15.9		
	17	198.0	225.0	185.0	202.0	202.5	8.2				
	18	296.0	323.0	267.0	failed	295.3	9.5				
	20	285.6	285.0	279.9	283.2	283.4	0.9				
Rate Nephelometry	3	236.4	253.3	246.0	244.7	245.1	2.8	246.9			
	18	268.3	225.3	254.7	246.3	248.7	7.2				
ELISA	7	253.0	263.0	263.0	266.0	261.3	2.2	261.3			
C) Using In-house Vi PS standard											
HPAEC-PAD	4	247.0	256.0	255.0	253.0	252.8	1.6	276.7	14.4	284.0	12.6
	8	230.0	246.0	222.6	229.9	232.1	4.3				
	10	256.8	251.3	271.0	264.0	260.8	3.3				
	11	N/A	N/A	N/A	N/A						
	13	300.3	316.0	339.9	316.8	318.3	5.1				
	15	323.9	311.8	322.7	319.7	319.5	1.7				
Rocket Immuno-electrophoresis	9 {	312.8	364.6	370.2	281.7	} 342.6	9.2	282.3	15.6		
		368.2	362.6	347.5	333.2						
	17	266.0	224.0	232.0	252.0	243.5	7.8				
	18	270.0	257.0	241.0	261.0	257.3	4.7				
	20 {	278.1	299.7	281.4	293.1	} 286.0	2.5				
		284.7	282.3	287.1	281.4						
Rate Nephelometry	3	285.5	297.2	295.9	309.2	296.9	3.3	285.9			
	18	231.7	287.0	309.7	271.3	274.9	11.9				
ELISA	7	316.0	343.0	342.0	290.0	322.8	7.8	322.8			

Method	Lab	Vi Saccharide content, Sample B (*C. freundii* Vi bulk conjugate) (1)	(2)	(3)	(4)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
A) Using candidate *C. freundii* Vi PS 12/244											
HPAEC-PAD	4	289.0	261.0	266.0	268.0	271.0	4.6	235.9	14.5	206.2	22.1
	8	269.1	242.7	249.7	249.5	252.8	4.5				
	10	174.6	215.5	184.0	183.3	189.3	9.5				
	11	268.1	248.4	279.8	231.4	256.9	8.3				
	13	245.3	249.5	246.8	253.5	248.8	1.5				
	15	200.7	190.8	199.5	194.4	196.4	2.3				
Rocket Immuno-electrophoresis	9	N/A	N/A	N/A	N/A			195.6	20.4		
	17	146.0	140.0	155.0	170.0	152.8	8.5				
	18	216.0	209.0	193.0	192.0	202.5	5.9				
	20	216.3	252.3	234.3	223.2	231.5	6.8				
Rate Nephelometry	3	158.1	155.0	155.3	152.5	155.2	1.5	146.0			
	18	132.7	118.3	149.7	146.3	136.8	10.5				
ELISA	7	170.0	177.0	178.0	195.0	180.0	5.9	180.0			
B) Using candidate *S.* Typhi Vi PS 16/126											
HPAEC-PAD	4	265.0	262.0	259.0	260.0	261.5	1.0	246.6	14.7	223.2	23.1
	8	269.2	259.4	266.1	288.6	270.8	4.6				
	10	185.8	207.8	191.1	194.7	194.9	4.8				
	11	246.9	267.1	332.5	295.6	285.5	13.0				
	13	251.4	259.5	253.8	266.6	257.8	2.6				
	15	216.8	207.8	205.4	205.4	208.8	2.6				
Rocket Immuno-electrophoresis	9	N/A	N/A	N/A	N/A			236.7	26.2		
	17	164.0	184.0	151.0	175.0	168.5	8.5				
	18	251.0	238.0	267.0	failed	252.0	5.8				
	20	288.3	294.0	272.7	303.9	289.7	4.5				
Rate Nephelometry	3	172.5	159.4	166.1	160.0	164.5	3.7	158.6			
	18	162.3	160.3	163.7	124.7	152.8	12.3				
ELISA	7	159.0	179.0	170.0	177.0	171.3	5.3	171.3			
C) Using In-house Vi PS standard											
HPAEC-PAD	4	255.0	239.0	252.0	255.0	250.3	3.0	259.8	14.8	235.9	18.8
	8	247.8	249.4	247.4	269.8	253.6	4.3				
	10	195.8	210.0	208.6	205.3	204.9	3.1				
	11	N/A	N/A	N/A	N/A						
	13	290.8	311.2	310.2	319.0	307.8	3.9				
	15	284.1	271.0	284.5	289.5	282.3	2.8				
Rocket Immuno-electrophoresis	9 {	N/A	N/A	N/A	N/A	}		234.9	23.8		
		N/A	N/A	N/A	N/A						
	17	179.0	161.0	186.0	195.0	180.3	8.0				
	18	262.0	232.0	204.0	232.0	232.5	10.2				
	20 {	262.5	304.8	312.3	299.7	} 291.9	5.4				
		286.8	290.4	279.6	299.4						
Rate Nephelometry	3	208.2	186.7	200.3	202.7	199.5	4.6	194.6			
	18	155.7	173.3	217.0	213.0	189.8	15.8				
ELISA	7	201.0	205.0	203.0	201.0	202.5	0.9	202.5			

Method	Lab	Vi Saccharide content, Sample D (*S.* Typhi Vi bulk conjugate) (1)	(2)	(3)	(4)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
A) Using candidate *C. freundii* Vi PS 12/244											
HPAEC-PAD	4	212.0	199.0	195.0	205.0	202.8	3.7	178.2	17.9	143.5	30.9
	8	197.1	180.7	184.9	163.4	181.5	7.7				
	10	128.2	143.8	142.7	141.2	139.0	5.2				
	11	213.7	222.4	228.6	185.1	212.5	9.1				
	13	206.4	179.8	193.4	198.1	194.4	5.7				
	15	138.2	136.8	144.6	137.3	139.2	2.6				
Rocket Immuno-electrophoresis	9	77.4	72.6	64.3	63.0	69.3	9.9	101.2	33.7		
	17	138.0	157.0	135.0	161.0	147.8	8.9				
	18	invalid	invalid	84.0	invalid	84.0					
	20	102.4	106.4	105.0	101.7	103.9	2.1				
Rate Nephelometry	3	138.1	138.2	139.5	122.8	134.7	5.9	127.3			
	18	119.7	105.3	125.0	130.0	120.0	8.9				
ELISA	7	132.0	140.0	137.0		136.3	3.0	136.3			
B) Using candidate *S.* Typhi Vi PS 16/126											
HPAEC-PAD	4	195.0	196.0	190.0	195.0	194.0	1.4	185.7	18.5	155.3	25.0
	8	197.2	191.9	197.5	188.9	193.9	2.2				
	10	139.2	137.8	146.3	151.5	143.7	4.4				
	11	196.3	237.8	268.9	230.7	233.4	12.8				
	13	211.0	188.0	197.2	210.3	201.6	5.5				
	15	149.6	146.1	148.6	146.5	147.7	1.1				
Rocket Immuno-electrophoresis	9	112.3	90.0	86.7	105.3	98.6	12.4	124.4	18.6		
	17	153.0	145.0	172.0	140.0	152.5	9.2				
	18	invalid	108.0	121.0	failed	114.5					
	20	131.2	128.2	126.6	141.3	131.8	5.0				
Rate Nephelometry	3	150.2	140.5	147.2	125.0	140.7	8.0	138.4			
	18	145.3	150.0	137.0	112.0	136.1	12.4				
ELISA	7	139.0	140.0	138.0	105.0	130.5	13.0	130.5			
C) Using In-house Vi PS standard											
HPAEC-PAD	4	193.0	170.0	181.0	184.0	182.0	5.2	192.3	16.5	166.6	21.5
	8	183.9	184.8	183.2	175.5	181.9	2.4				
	10	145.4	140.8	168.2	165.7	155.0	9.0				
	11	N/A	N/A	N/A	N/A						
	13	242.6	222.5	243.5	252.8	240.3	5.3				
	15	198.9	191.7	208.0	210.6	202.3	4.3				
Rocket Immuno-electrophoresis	9 {	122.6	122.1	106.2	97.6	} 119.5	10.8	134.7	22.3		
		127.6	127.5	114.4	137.9						
	17	184.0	170.0	167.0	194.0	178.8	7.0				
	18	109.0	108.0	119.0	114.0	112.5	4.5				
	20 {	121.7	133.1	127.9	126.4	} 128.0	2.9				
		128.5	125.5	128.1	132.5						
Rate Nephelometry	3	180.5	164.3	177.9	159.4	170.6	6.0	169.5			
	18	150.0	166.7	182.7	174.0	168.4	8.2				
ELISA	7	159.0	150.0	164.0	169.0	160.5	5.0	160.5			

Table 16. Vi saccharide content of real-time stability and accelerated thermal degradation samples by HPAEC-PAD.

Candidate ISs	Storage time (in Months)	Vi content (mg Vi PS/ampoule)		Vi content as % -20°C sample			
		-70°C	-20°C	+4°C	+20°C	+37°C	+56°C
Vi PS 12/244	1	2.33	2.30	108	103	114	99
	2	2.04	1.86	120	108	127	130
	4	ND	1.80	108	107	106	101
	6	1.87	1.81	99	103	101	100
Vi PS 16/126	1	1.71	1.92	110	102	102	109
	2	1.80	1.95	99	105	102	105
	4	ND	1.80	100	101	105	105
	6	1.76	1.87	100	96	97	93

Table 17. Vi content of reconstituted stability samples by HPAEC-PAD

Storage time (in Months)	Vi PS content (mg Vi PS/ampoule)			
	Vi PS 12/244		Vi PS 16/126	
	+4 °C	-20 °C	+4 °C	-20 °C
0.5	1.89		2.08	
1	2.50	2.78	2.07	2.35
2	2.05	2.21	2.13	2.20
6	1.73	1.86	1.80	1.80

Figure 1. ^{1}H proton spectrum of Vi PS (12/244) and (16/126) including peak assignment by NIBSC.

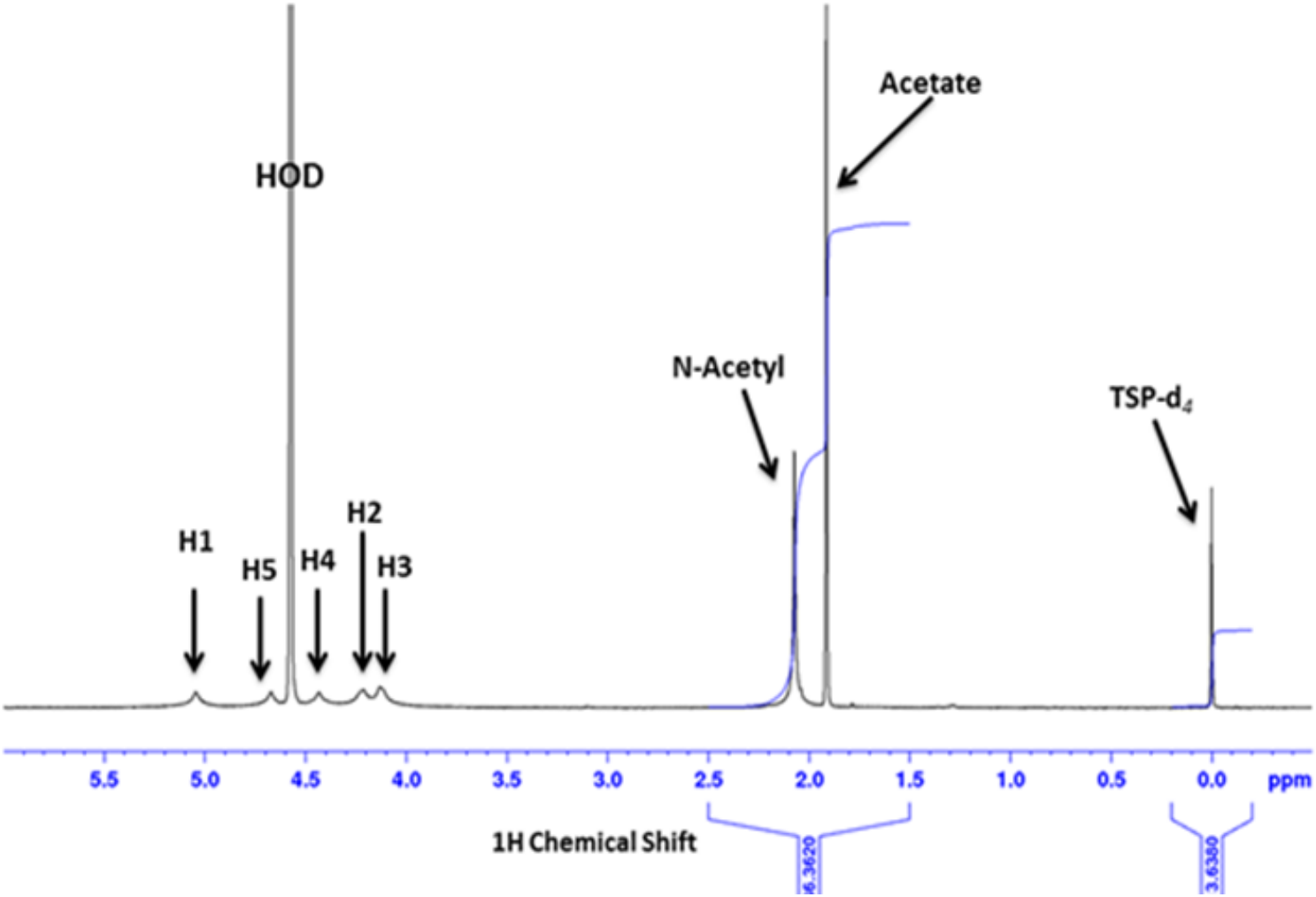
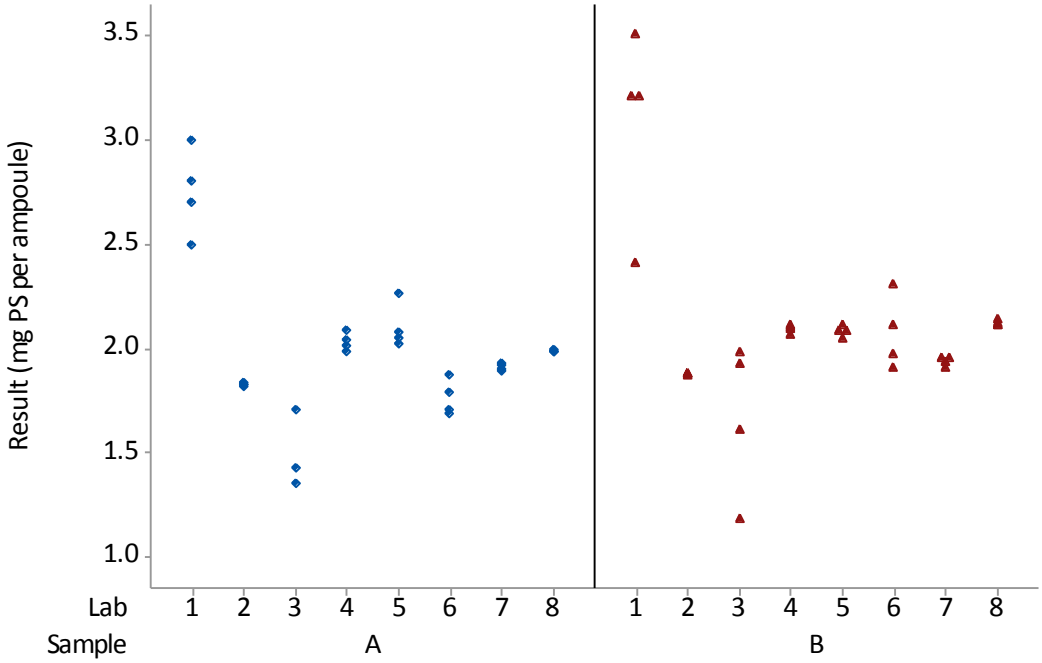


Figure 2. Summary of results for CS574 – a) mass unit assignment and b) *O*-acetyl content by qNMR

a)



b)

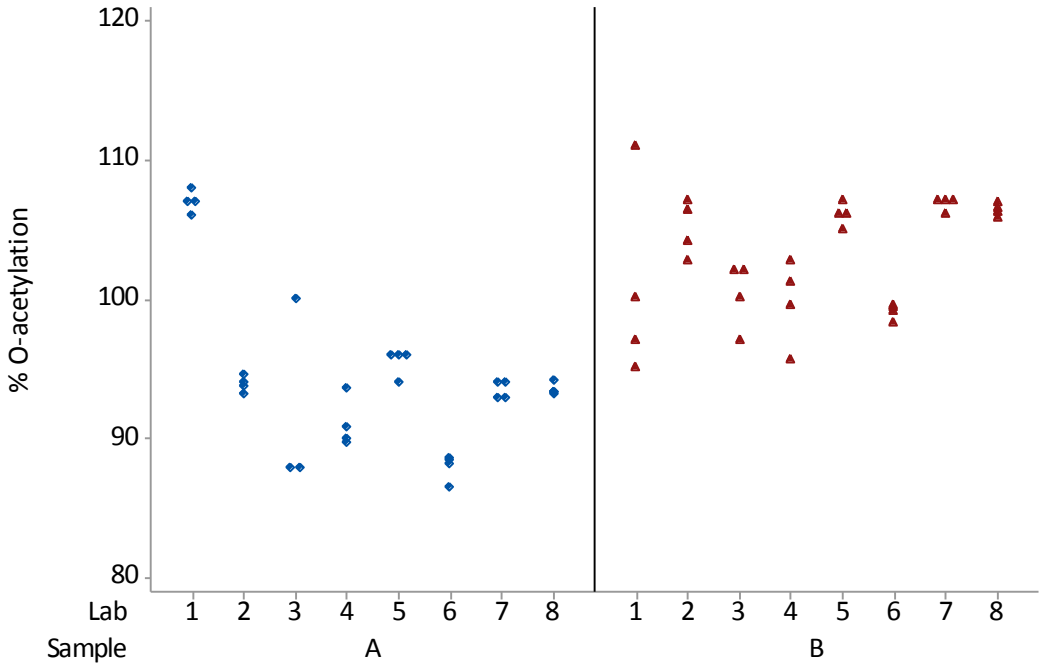
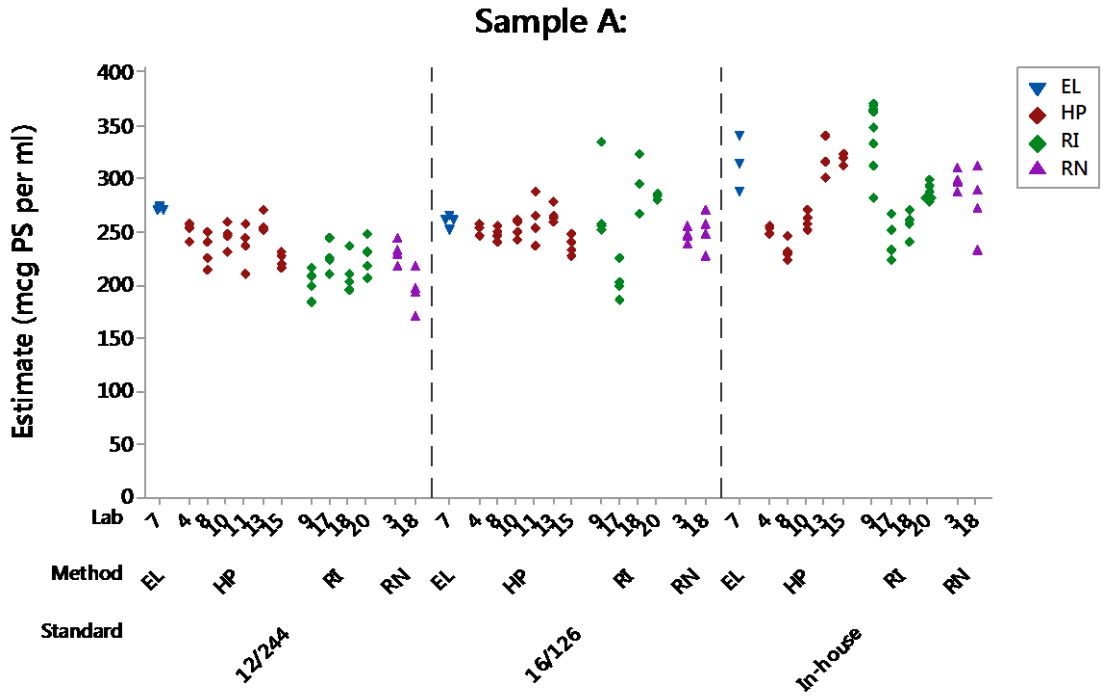
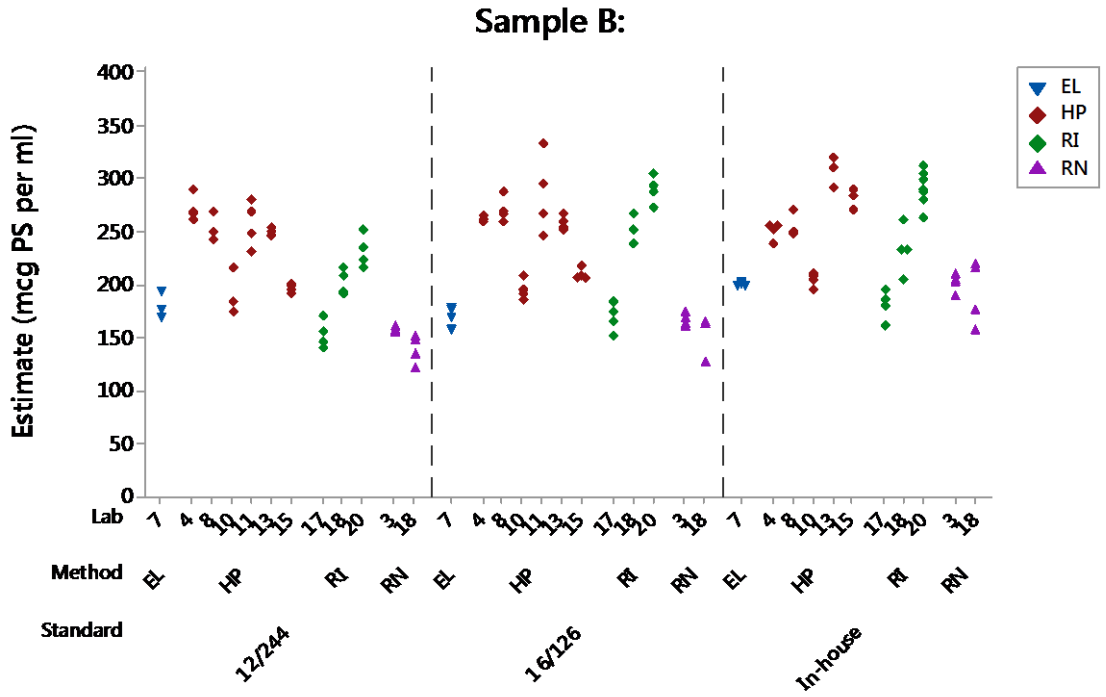


Figure 3. Summary of results for CS575 – fitness for purpose a) Sample A – *S.* Typhi Vi PS, b) Sample B – *C. freundii* Vi bulk conjugate and c) Sample D – *S.* Typhi Vi bulk conjugate. Methods are abbreviated as EL, ELISA; HP, HPAEC-PAD; RI, Rocket Immuno-electrophoresis; and RN, Rate Nephelometry.

a)



b)



c)

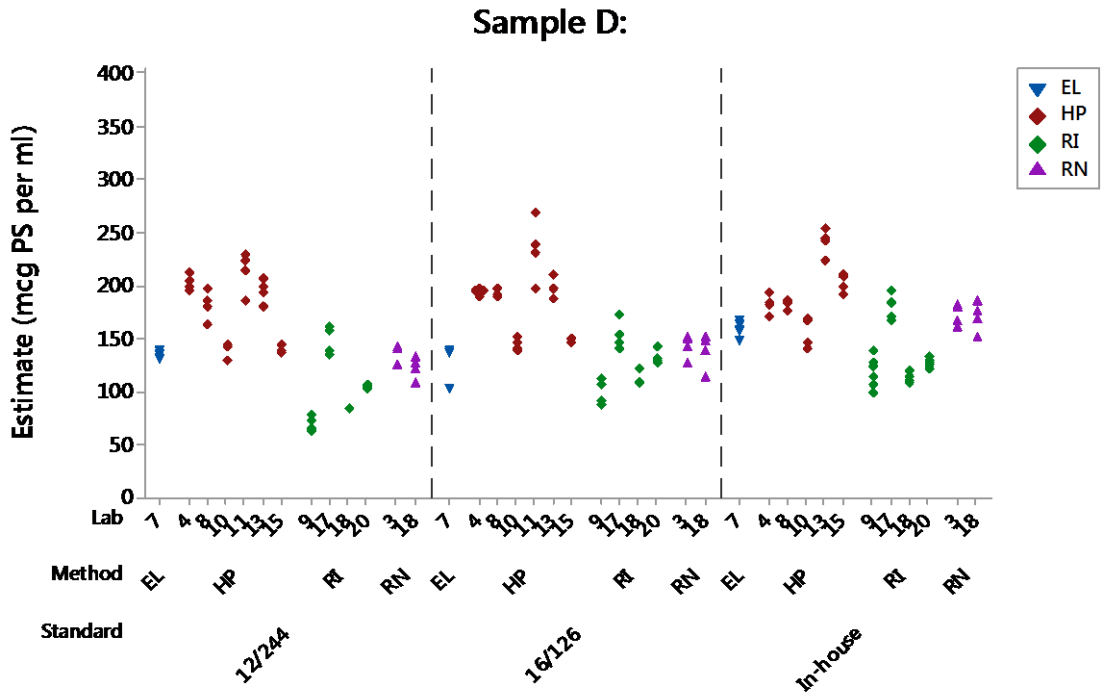
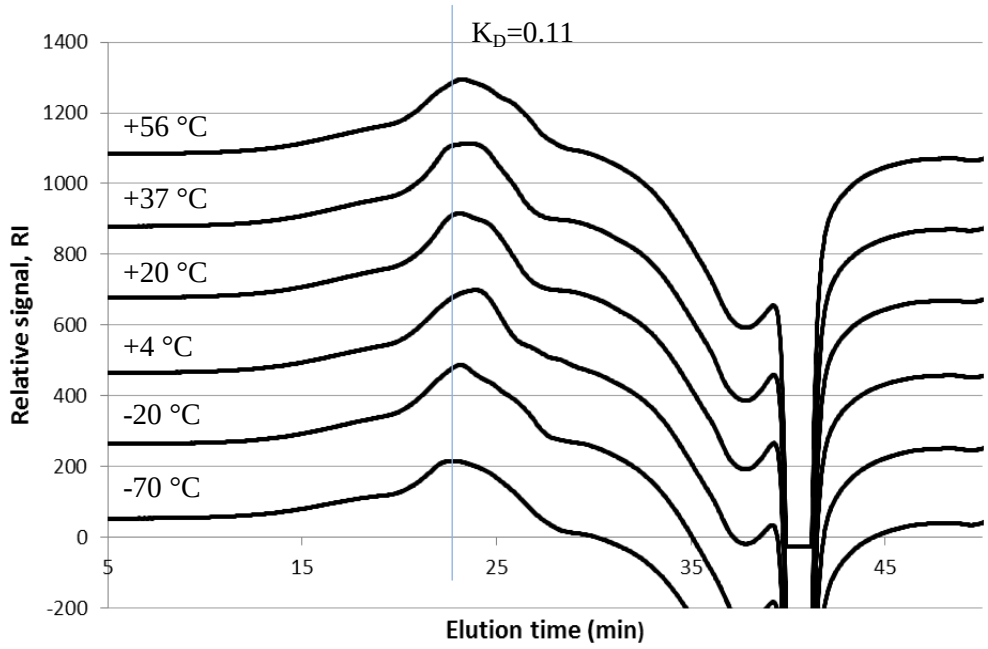


Figure 4. Molecular size evaluation of Vi PS 12/244 accelerated degradation samples**;** a) chromatogram of Vi samples following 6 months storage, with a vertical bar indicating the K_D

value used for integration, and b) percentage eluting before a K_D of 0.11 following storage at -70°C to +56°C for 1-6 months.

a)



b)

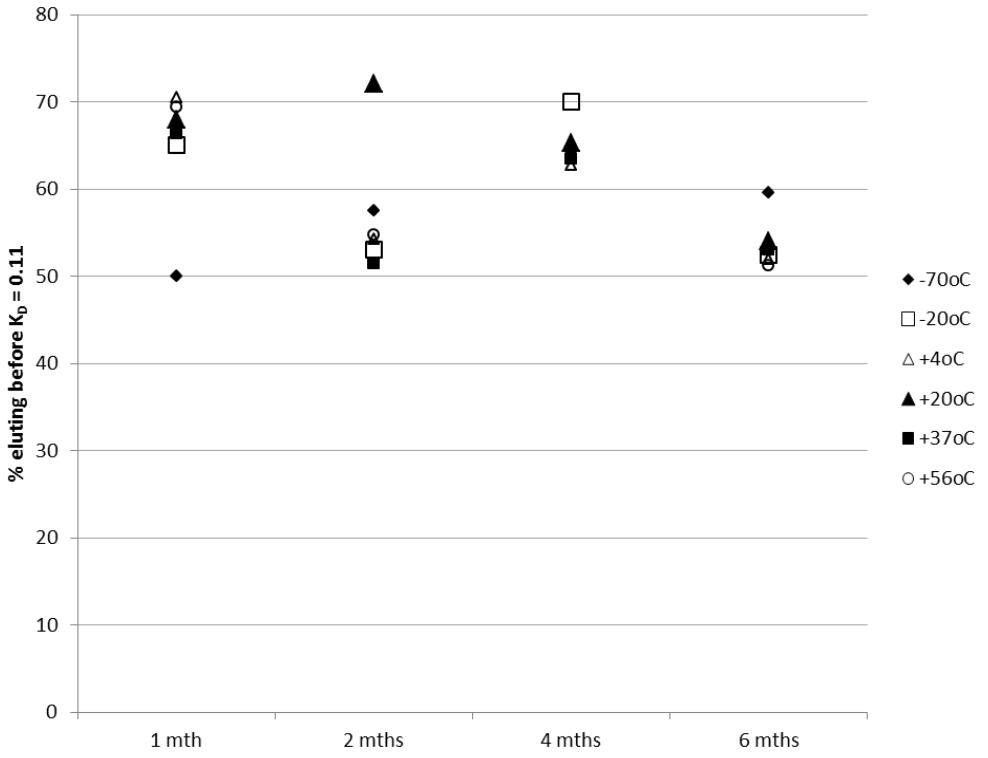
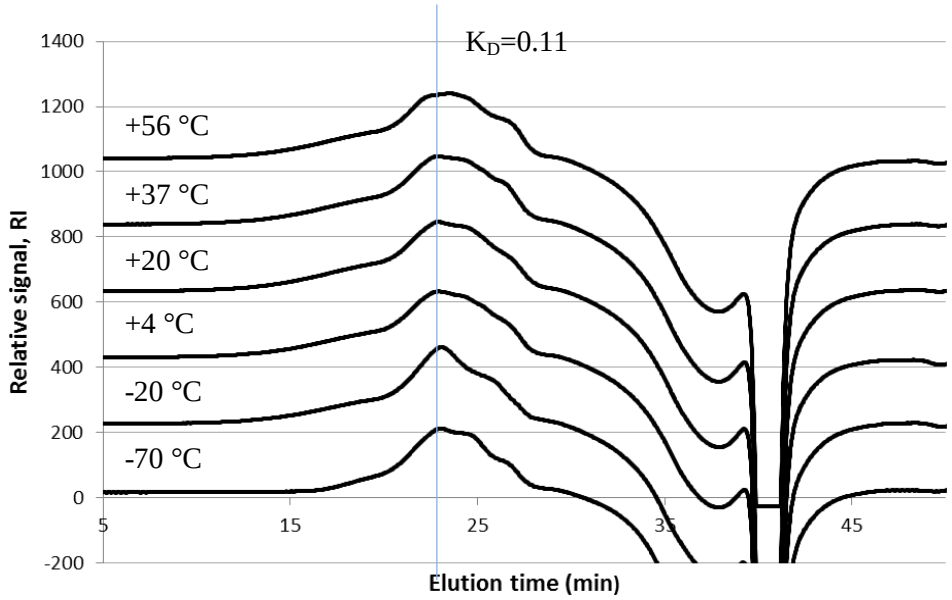


Figure 5. Molecular size evaluation of Vi PS 16/126 accelerated degradation samples**;** a) chromatogram of Vi samples following 6 months storage, with a vertical bar indicating the K_D value used for integration, and b) percentage eluting before a K_D of 0.11 following storage at -70°C to +56°C for 1-6 months.

a)



b)

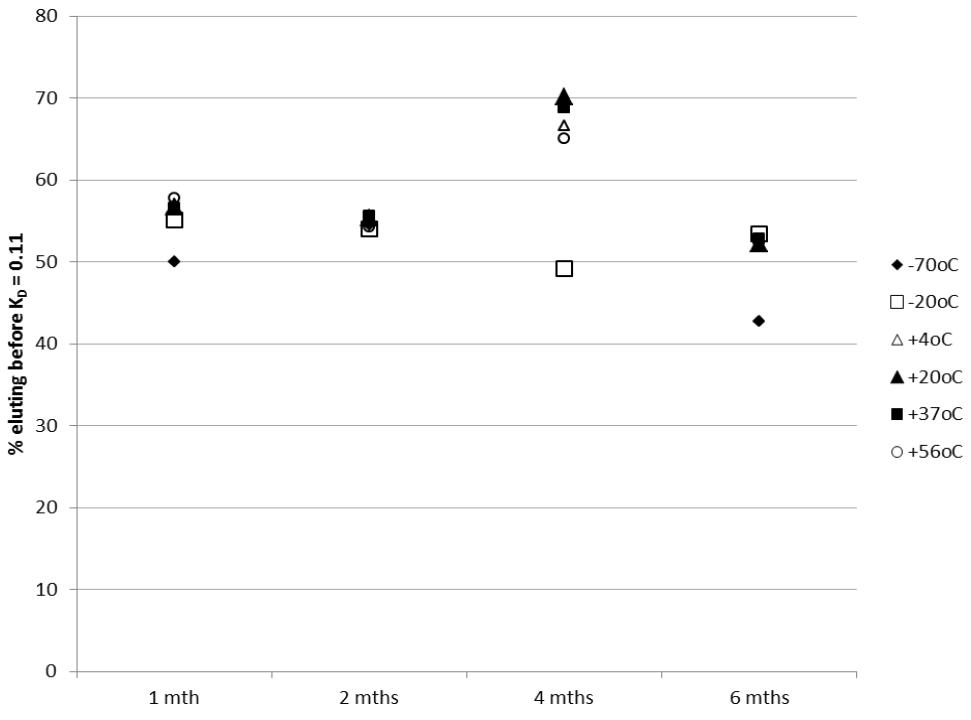
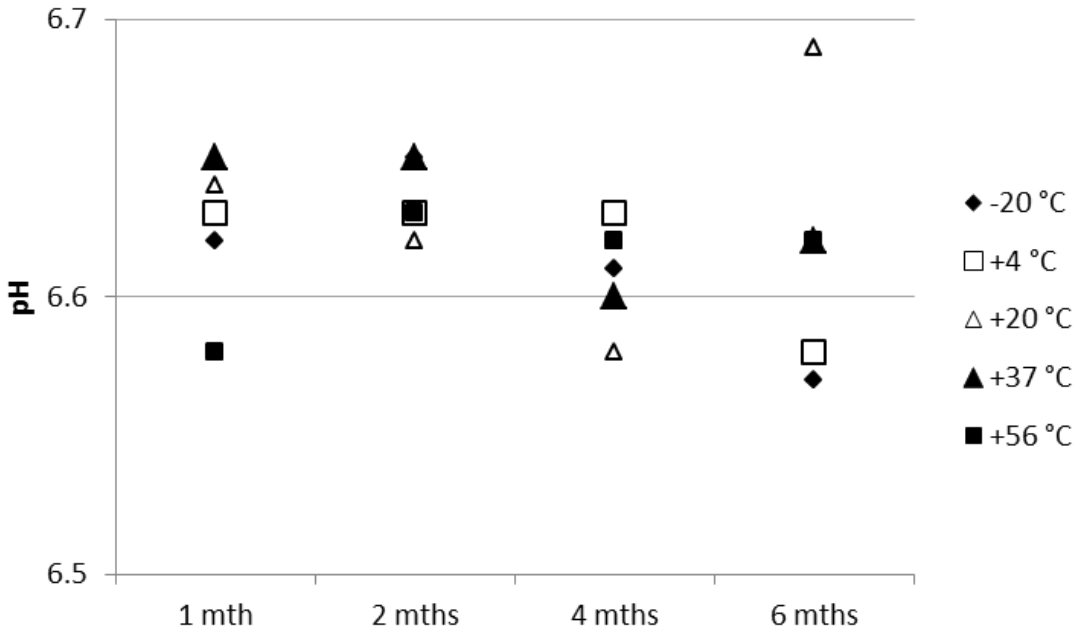
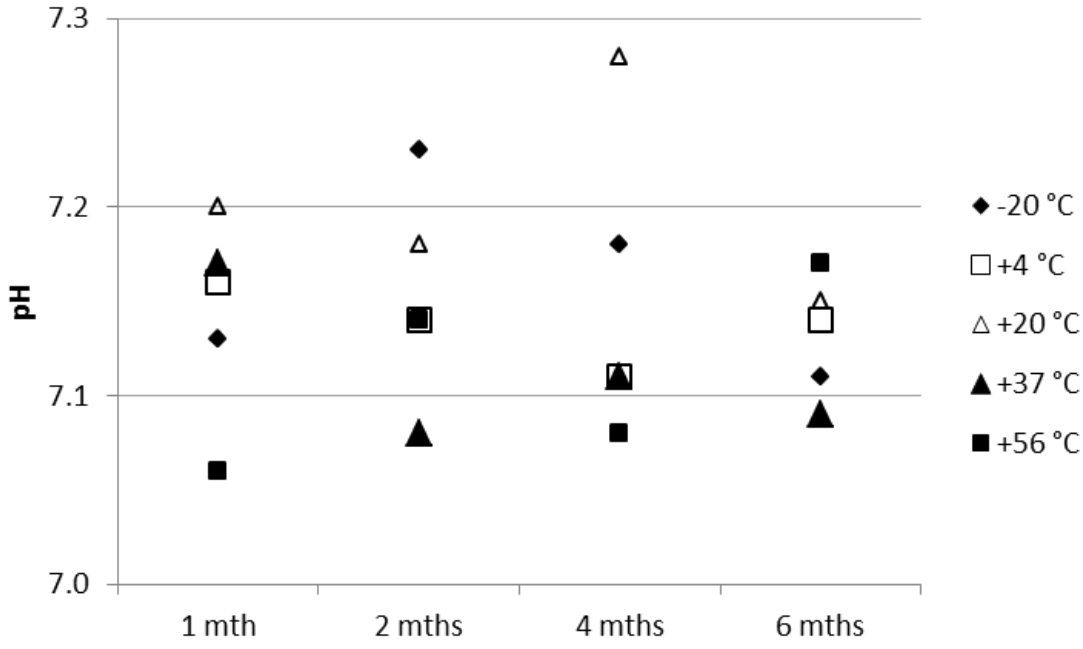


Figure 6. pH evaluation of Vi PS accelerated degradation samples; a) Vi PS 12/244, and b) Vi PS 16/126.

a)


6.7
6.6
6.5
pH
1 mth
2 mths
4 mths
6 mths
-20 °C
+4 °C
+20 °C
+37 °C
+56 °C

b)


7.3
7.2
7.1
7.0
pH
1 mth
2 mths
4 mths
6 mths
-20 °C
+4 °C
+20 °C
+37 °C
+56 °C

= = =