Collaborative study on saccharide quantification of the *Haemophilus influenzae* type b component in liquid vaccine presentations

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ABSTRACT

Before release onto the market, it must be demonstrated that the total and free polysaccharide (poly ribosyl-ribitol-phosphate, PRP) content of Haemophilus influenzae *type b (Hib) vaccine complies with requirements. However, manufacturers use different methods to assay PRP content: a national control laboratory must establish and validate the relevant manufacturer methodology before using it to determine PRP content.*

An international study was organised by the World Health Organization (WHO), in collaboration with the Biological Standardisation Programme (BSP) of the Council of Europe/European Directorate for the Quality of Medicines & HealthCare (EDQM) and of the European Union Commission, to verify the suitability of a single method for determining PRP content in liquid pentavalent vaccines (DTwP-HepB-Hib) containing a whole-cell pertussis component. It consists of HCl hydrolysis followed by chromatographic separation and quantification of ribitol on a CarboPac MA1 column using high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The unconjugated, free, PRP is separated from the total PRP using C4 solid-phase extraction cartridges (SPE C4).

Ten quality control laboratories performed two independent analyses applying the proposed analytical test protocol to five vaccine samples, including a vaccine lot with sub-potent PRP content and very high free PRP content. Both WHO PRP standard and ribitol reference standard were included as calibrating standards. A significant bias between WHO PRP standard and ribitol reference standard was observed. Study results showed that the proposed analytical method is, in principle, suitable for the intended use provided that a validation is performed as usually expected from quality control laboratories.

KEYWORDS

Haemophilus influenzae type b (Hib), polysaccharide analysis, conjugate vaccine analysis, high performance anion exchange chromatography (HPAEC), pulsed amperometric detection (PAD), acid hydrolysis, collaborative study.

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1. INTRODUCTION

Current *Haemophilus influenzae* type b (Hib) vaccines are made from the capsular polysaccharide (PRP), which is conjugated to a carrier protein to induce a T-dependent B-cell response in infants and hence an immune memory effect [1, 2]. The native form of Hib polysaccharide is usually covalently linked to tetanus toxoid, while as an oligosaccharide it is linked to a non-toxic variant of the diphtheria toxin, the cross-reacting material CRM197 [3]. The Hib glycoconjugate component can be combined with different vaccine antigens such as diphtheria (D), tetanus (T), whole-cell pertussis (wP) or acellular pertussis, hepatitis B (HepB) and inactivated polio vaccine (IPV). Combination with any of these antigens, as well as the presence of adjuvants, preservatives and other excipients, can interfere with the analysis of the critical parameters indicative of Hib vaccine quality and efficacy: molecular size distribution and total and free (unconjugated) saccharide content [1].

HPAEC-PAD chromatography has been found to be suitable for sugar analysis and is applied to the analysis of Hib vaccines [4, 5]. The sugar content is obtained after a depolymerisation step, generally through alkaline hydrolysis [5, 6]. HPAEC-PAD methodology is listed as appropriate in the relevant monographs of the European Pharmacopoeia [7] and in the World Health Organization (WHO) guideline for production and control of Hib vaccines [8].

Pentavalent DTwP-HepB-Hib liquid vaccine is used globally and is, accordingly, a high-priority vaccine, as indicated by the WHO Prequalification Team (WHO/PQT). WHO-contracted laboratories (Labs; many of which are official medicines control laboratories) perform tests to support WHO prequalification. However, they must establish and validate the methodology used by the manufacturer to determine the PRP content of any product they are requested to test. This is very time-consuming and creates a bottleneck in the testing process. Therefore, the contracted Labs usually apply their own methods to perform independent re-testing. The protocol used generally depends on the product that the Lab uses for domestic lot release of a Hib vaccine.

A Lab obtained non-compliant results for the Hib content of a pentavalent vaccine that had been submitted for prequalification by WHO. These results were in conflict with those previously obtained by the manufacturer. An expert committee was convened to review the conflicting results. The inconsistency resulted from differences in the test protocol applied by the Labs and did not indicate deficient product quality. The expert committee recommended that a standardised test protocol for HPAEC-PAD methodology should be used to determine the PRP content in liquid vaccine combinations.

The WHO Technical Assistance and Laboratory Services Group (TAL) therefore initiated a small ‘Hib project’ to identify a test protocol that could be applied to quantify the total and free PRP content of the Hib conjugate component of various liquid vaccine combinations. The results of the study showed that a protocol using acidic hydrolysis was suitable for quantifying the total and free PRP content of DTwP-HepB-Hib vaccine combinations produced by four different manufacturers [4].

Based on this outcome, WHO/TAL organised, in co-operation with the EDQM, an international collaborative study with a higher number of participating laboratories to assess the suitability of an analytical test protocol for the quantification of the total and free (unconjugated) PRP content of the Hib component in different liquid pentavalent vaccine (DTwP-HepB-Hib) presentations.

2. PARTICIPANTS

Ten laboratories from 7 countries (Belgium, France, Germany, India, Italy, Republic of Korea and United Kingdom) participated in the study. Five of these Labs are the official control laboratories of their national regulatory authority; the others are the quality control laboratories of manufacturers. A list of participants in alphabetical order by country is given in section 8. Herein, they are referred to by an arbitrarily allocated code number (1 to 10), not necessarily related to the order of listing.

3. MATERIALS AND METHODS

3.1. Vaccine samples

Four different WHO-prequalified DTwP-HepB-Hib liquid formulated vaccines were included in the study as shown in Table 1. One vaccine lot was especially prepared for the study with a sub-potent PRP content and high free, unconjugated PRP content. The vaccine samples were kindly donated by different manufacturers. The samples were shipped under temperature-controlled conditions. Participants were asked to store the vaccines at + 5 ± 3 °C upon receipt, until use.

3.2. Reference standards, positive control, reagents

Two reference standards were provided for the calibration curve: specifically, 1 ampoule of the WHO 1st International Standard (IS) *Haemophilus influenzae* b polysaccharide poly ribosyl-ribitol phosphate (PRP), NIBSC code: 02/208, and 5 g of the ribitol reference standard (Fluka, cat. no. 02240, batch BCBJ6567V).

The 2 standards were shipped separately at controlled temperature. The ribitol reference standard, upon receipt and until use, was to be stored at room temperature, while the PRP standard was to be stored at − 20 °C.

Each participating Lab was requested to prepare the positive control using a batch of ribitol other than that provided for the calibration curve.

The shipment of the test vaccines and reference standards was performed by WHO/TAL group.

3.3. Critical materials

Critical materials were indicated in the protocol. Columns and cartridges were provided by WHO/TAL to those participating national control laboratories that were not equipped with the materials indicated:

- SPE C4 wide pore cartridges for volume of 3 mL (VydacBioSelect, code 214SPE3000)
- CarboPac MA1 analytical column, 4 × 250, (Dionex, Product no. 44066)
- CarboPac MA1 guard column (Dionex, Product no. 44067)
- NaOH 50 % w/w from J.T. Baker.

The use of degassed, deionised type I reagent water (for example, MilliQ Biocel A10) was recommended.

3.4. Method and study design

All vaccines contained 8-12 µg of conjugated polysaccharide in a single human dose (shd). The vaccines were all liquid with an aluminium phosphate adjuvant (Table 1). Participants were requested to quantify the total and free PRP content of each vaccine sample by following the study protocol that had been provided. Each participant was requested to investigate each vaccine sample twice by preparing fresh vaccine pools on separate days and calculating both polysaccharide contents *vs* two calibration curves, i.e. *vs* the WHO PRP IS [9, 10] and *vs* the ribitol reference standard. The use of the two calibration curves was requested since manufacturers use either one of these standards.

Determination of the total PRP did not require any particular treatment of the vaccine sample. To assay the free PRP, the vaccine sample was treated with 5 mM phosphate buffer, pH 6.8 [5], centrifuged to eliminate the adjuvant, and the supernatant applied to a SPE C4 wide pore cartridge. The cartridge permeate was collected to recover the free PRP. Hydrolysis was performed adding 50 µL of 6 M HCl to all samples (1 ml of each point of the calibration curve, of the positive control and of the system suitability test (SST)), and incubating them for 2 hours at 100 °C. Thereafter, the samples were cooled for 10 min at + 5 ± 3 °C and 400 µL of 1 M NaOH was added. Each sample was then appropriately diluted, filtered and analysed by HPAEC-PAD.

The study protocol defined the chromatographic conditions to comply with. The Labs were asked to complete a form regarding the characteristics of their HPAEC equipment, details of the ribitol used as a positive control (% purity, moisture content, diluents, time and temperature of storage), the SST in place, any deviations from the study protocol, any difficulties encountered and any observation(s) regarding the study protocol.

An electronic data reporting sheet was used to record the experimental data: total and free PRP content were reported in µg per single human dose (µg/shd), and to two decimal places. The free polysaccharide content was also recorded as a percentage, to two decimal places.

3.5. Statistical analysis

The statistical analysis of the data was performed at the EDQM using the CombiStats software [11]. Repeatability (intra-laboratory variation) and reproducibility (inter-laboratory variation) were calculated as defined in ISO-5725-1:1994. In brief, the calculation method involves an analysis of variance for each sample and for each measurand, to generate a mean square for intra-laboratory variation (MS_r) and a mean square for inter-laboratory variation (MS_R). These quantities are decomposed into a component for intra-laboratory variation $s_r^2 = MS_r$ and a component for inter-laboratory variation $s_L^2 = (MS_R - MS_r)/p$ where p is the number of runs per laboratory, in this case p = 2. If s_L^2 is negative, it is set to 0. The repeatability standard deviation is then obtained as the square root of s_r^2 and the reproducibility standard deviation is obtained as the square root of $s_L^2 + s_r^2$. Both quantities have to be multiplied by a factor of 2.8 to find the value below which the absolute difference between two measurements, carried out under repeatability and reproducibility conditions, is expected to fall with 95 % probability.

Inter-laboratory variation was also expressed as the geometric coefficient of variation (GCV) of the laboratory means. Two different definitions of GCV co-exist in the published literature. They are not equivalent and caution should be exercised when comparing the GCVs from different studies. The definition used in this report is GCV (%) = $(\exp(v) - 1)^{0.5} \cdot 100$ %, whereas some other publications use the definition GCV (%) = $(\exp(v^{0.5}) - 1) \cdot 100$ %. In both equations, v is the sample variance of the log-transformed activities.

Visual techniques such as two-way plots and min-max plots were used to illustrate the distribution of results and the concordance between methods.

4. RESULTS

Figures 1A and 1B show the HPAEC-PAD chromatograms obtained by analysing the total and free PRP content of the vaccine sample HCS-3, according to the test protocol (Table 2). The ribitol elutes as a single peak at a retention time of about 15.9 min and can easily be assigned and integrated.

Table 2 presents the assay conditions of the test protocol, the participant Labs' deviations from the protocol, as well as further information regarding some of the chromatographic conditions. Hydrolysis was performed at 100 °C in dry oven/stove, heating blocks or in a water bath at 98 °C. Separation of the free from the total PRP was performed by all Labs using the SPE cartridge. Four Labs passed the samples through the SPE cartridges by gravity instead of applying vacuum.

All Labs had a Dionex chromatographic system and applied the quadruple potential waveform for carbohydrate analysis. CarboPac™ MA1 column and the relative guard column was used by all Labs. Lab 10 additionally used an amino trap column. The flow rate was kept by all at 0.4 ml/min. The column and auto-sampler temperatures were generally set at 30 °C and 4 °C, respectively.

Three participants used a disposable gold electrode. Labs 1 and 9 used a mobile phase with a different molarity from the test protocol, 640 and 175 mM, respectively (Figures 2 and 3). Lab 4 used the 580 mM NaOH mobile phase of the test protocol, but performed the analysis also using a multi-step gradient: 175 mM NaOH from 0 to 40 min, 600 mM NaOH from 40 to 55 min

and then again 175 mM NaOH up to 100 min. This approach improved the separation of ribitol (Figure 4). All Labs used a version of Chromeleon software to perform data acquisition, except for one Lab which used PeakNet.

Results for total and free PRP were provided by all participating Labs. All Labs carried out two independent runs *vs* the WHO IS and *vs* the ribitol reference standard. Lab 3 did not report results against the ribitol reference standard because these did not meet the test validity criteria. Lab 7 did not report results from the second run due to its column's poor performance.

A summary of the reported results is presented in Table 3 (*vs* WHO PRP IS) and in Table 4 (*vs* ribitol reference standard). The tables present the results per individual run (Ind.), the geometric mean of the two runs (GM), and the GM rounded to specification decimals (Round) for each test sample. Dark blue cells indicate instances where the result observed was out of specification (OOS). Light blue cells indicate instances where the result is compliant after rounding, but not before rounding. A graphical representation of the data is provided in Figures 5 and 6. The small dashes indicate the results from the individual runs; they are connected by a vertical line to show the range between the two runs; the crosses mark the geometric mean of the two runs.

Some results in Tables 3 and 4 are labelled with an asterisk for the following reasons:

- Lab 5 reported that results for free PRP in HCS-4 against the IS were provided for information only, because the values were outside the calibration range. In this report they are treated as valid results nonetheless.
- Lab 6 reported atypical results for free PRP content in HCS-4 and HCS-5 and was contacted to ascertain that these did not constitute a reporting mistake. The Lab confirmed that this was not a mistake but that they had encountered a problem and therefore replaced the column. These results are treated as outliers and excluded from all overall calculations.
- Lab 8 commented that the free PRP content in HCS-2 varied significantly between the duplicate injections of the first run. The result from the second injection was close to that of both injections in the second run, and also close to the results from other Labs. It is therefore reasonable to assume that an anomaly occurred with the first injection of the first run: further calculations were therefore based on the results from the second injection only.
- Lab 10 commented that the total PRP content in HCS-1 and HCS-3 should not be considered for the first run because data points did not fall within the range of the calibration curve due to a dilution error. However, in its calculation, the Lab corrected for this mistake. Since its results are in line with those of the other Labs they are treated as valid results nonetheless.

Table 3 (calculation against the WHO 1st PRP Standard) shows that HCS-4 was tested and found to be OOS for total PRP content by 9 of the 10 Labs. High free PRP content was confirmed by each of the 10 Labs, except for Lab 6. All other samples were found to be compliant except with respect to HCS-5 in Lab 6. A few cases deserve particular attention:

- Total PRP content in HCS-2 was borderline in both runs in Lab 6 and in the first run in Lab 10. However, after rounding to decimal places of the specification, the sample could be considered to have been found to be compliant by both Labs.
- Total PRP content in HCS-3 was borderline in the first runs in Lab 2 and Lab 6, and was OOS in the first run in Lab 8. However, the average of both runs was compliant in all cases, even without rounding to specification.
- Total PRP content in HCS-4 was OOS in all runs in all Labs except in the second run of Lab 10. The average of both runs in this Lab was also considered to be compliant. The sample can nonetheless be considered to have failed because the free PRP content was observed to be OOS.
- Free PRP content in HCS-4 was OOS in 5 of the 10 participating Labs, although most of them had one run in the compliant range. An interesting case occurred for Lab 9 which

obtained a compliant result in the first run, albeit borderline, but an OOS result in the second run. In practice, the sample would have passed testing because a second run would not be carried out routinely. However, in this report the average of both runs is considered and treated as OOS.

- Total PRP content in HCS-5 was OOS in the second run in Lab 8, and borderline in both runs in Lab 10. However, the average of both runs, after rounding, was compliant in both Labs. The OOS results from Lab 6 were probably due to a technical failure of the assay system and should be disregarded for the overall evaluation.

Table 4 (calculation against ribitol reference standard) shows that HCS-1 and HCS-2 were observed to be compliant by all Labs. HCS-3 was compliant in all Labs except in Lab 5, although Labs 2, 4, 8 and 9 also obtained OOS results or borderline results for individual runs. HCS-4 was found to be OOS in all Labs. HCS-5 was found to be compliant in all Labs, except Lab 8 (and disregarding the results obtained by Lab 6), although this sample would have passed in a routine situation because the first run was compliant after rounding. Again, several cases deserve particular attention:

- Total PRP content in HCS-2 was borderline in the first run in Lab 8. The result of the second run was considerably higher, so the average was well within specification.
- Total PRP content in HCS-3 was OOS in the first run in Labs 2, 5 and 8. The second run in these Labs was compliant. In the case of Lab 5, the average was OOS, but in the cases of Labs 2 and 8 the average was compliant, after rounding to specification.
- Total PRP content in HCS-4 was OOS in all runs in all Labs except in Lab 10's second run. The average of both runs in this laboratory was also OOS.
- Free PRP content in HCS-4 was OOS in 6 of the 10 participating Labs, although most of them had one run in the compliant range. An interesting case occurred for Lab 9, which had a compliant result in the first run, albeit borderline, but an OOS result in the second run. In practice, the sample would pass because the second run would not be carried out routinely. However, in this report the average of both runs is considered and treated as OOS.
- Total PRP content in HCS-5 was OOS in the second run in Labs 8 and 10. However, the average of both runs, after rounding, was compliant in Lab 10 but not in Lab 8. In a routine situation the sample would nonetheless have passed in Lab 8 because the second run would not have been carried out.
- Free PRP content in HCS-5 was OOS at Lab 6. These results were probably due to technical failure of the assay system and should be disregarded for the overall evaluation.

When comparing Table 3 with Table 4 it can be seen that the results tend to be lower when calculated against the ribitol reference standard. This effect is particularly relevant for HCS-3 where the results are frequently borderline or OOS when tested against the ribitol reference standard, whereas this is less frequently the case when tested against the IS. Two-way plots are shown in Figures 7A and 7B.

The plots show clearly that the large majority of dots are positioned below the diagonal line of equivalence. It can also be seen that the clouds in both plots are wedge-shaped, with smaller differences for low contents and larger differences for high contents. This indicates that the bias is proportional to the content.

The bias between WHO PRP IS and the ribitol reference standard is highly significant ($p << 0.001$ with two-sided paired t-test) and estimated to be − 10.3 % when based on results for total PRP content, and − 11.5 % when based on results for free PRP content expressed in µg/shd. The bias based on all results is − 10.9 %, with 95 % confidence limits of − 9.1 % and − 12.8 %. Of course, the bias does not exist for free PRP content when expressed as a percentage of total PRP content.

Another aim of the study was to investigate the method's repeatability and reproducibility. As a measure of reproducibility, the GCV of the laboratory means can be used. The GCV ranges

from 6.5 % to 14.6 % for total PRP content (both standards pooled), from 18.9 % to 30.9 % for free PRP content expressed in µg/shd, and from 10.2 % to 35.7 % for free PRP content expressed as percentage of total PRP content.

The resulting values for repeatability are summarised in Table 5. All values are expressed in the same units as the measurands. For example, a random laboratory carrying out two independent runs on HCS-1 is expected to find, at most, 1.50 µg/shd difference for total PRP content between both runs in 95 % of the cases, if the IS is used as reference.

The resulting values for reproducibility are summarised in Table 6. All values are expressed in the same units as the measurands. For example, two random Labs each carrying out one run on HCS-3 are expected to find at most 11.2 percentage points difference for free PRP content expressed as percentage of total PRP in 95 % of the cases if the IS is used as reference.

5. DISCUSSION

The study results show that the proposed test protocol is applicable. 4 of the 5 vaccine samples used in the study were proved to comply with the specification for their PRP content. One vaccine sample, HCS-4, was formulated *ad hoc* for the study with low total (below specification) and high free (but within specification) PRP content. All participating Labs found the total PRP content to be OOS, but discordant results were obtained for the free PRP. However, free PRP concordant results were obtained by using both reference standards.

Labs found that separation of the free PRP by SPE C4 was the most challenging step of the method. One Lab participant observed that training in this new method could have been beneficial and served to reduce the variability in the results of two test runs. In fact, WHO/TAL organised training in application of the method at the same time as it organised this collaborative study [12]. However, when organising the collaborative study, such training was considered unnecessary since each of the participating Labs was already experienced in Hib content determination by HPAEC-PAD, even if their experience was based on use of other protocols. That being said, providing the participants with an additional set of samples, to practice applying the test protocol before performing the analysis for the study, might have been worthwhile.

The deviations from the test protocol, such as molarity of the mobile phase, as well as separation of the free PRP by gravity, instead of under vacuum, are not considered to have had any relevant influence on the study results and give an insight on the robustness of the method. It was observed, though, that using a disposable gold electrode, depending on the type (i.e. PTFE or polyester), gave better results in terms of peak separations when used with a mobile phase of lower molarity (Lab 9) or a gradient (Lab 4). So the test protocol could be modified accordingly.

Some participating Labs observed a shift in the retention time of ribitol between the initial and the final injections. This phenomenon was most evident when many samples were analysed during one session. It was related to an insufficient regeneration of the column between injections. As carbonate ions tend to accumulate in the column, the performance of the ion exchange resin was reduced. The introduction of a wash step with a high NaOH molarity or with a sodium acetate/NaOH gradient between injections would regenerate the column and avoid this inconvenience. Additionally, with long runs, a change in signals can occur. An internal spike would then be needed to normalise the signals.

The participating Labs were asked to quantify the PRP content using two calibration curves based on the acid-hydrolysed ribitol reference standard and the WHO IS for PRP, as manufacturers use either one of them. The 1st WHO PRP IS was established in 2005 with a content of 4.933 ± 0.267 mg/ampoule on the basis of the ribose assay (colorimetric assay) carried out by seven participating Labs [9, 10]. The 1st PRP IS was considered potentially suitable for use in quantifying PRP content by other assays. The retesting of the 1st IS during the collaborative study for the calibration of the 2nd WHO PRP IS showed that the content of PRP per ampoule was 4.989 mg and 5.080 on the basis of ribose and HPAEC-PAD determination, respectively [13]. The HPAEC-PAD value was obtained by 5 out of 8 Labs using

the 1st IS for the calibration curve. In this collaborative study, in contrast to the preliminary study [4] on which this study was based, a significantly lower total and free quantity of Hib saccharide was determined by the majority of Labs using the ribitol reference standard. The repeatability of the test protocol was similar when using the two standards, while the reproducibility was slightly better when using the ribitol reference standard.

A better repeatability and reproducibility could be noted for vaccine sample HCS-2, a CRM conjugated Hib vaccine. An assumption is that this could be related to the higher absorption of the TT conjugate to the adjuvant than the CRM conjugate [14]. The repeatability and reproducibility of the method are expected to improve once the Labs have become accustomed to using the test protocol more frequently and/or routinely.

6. CONCLUSIONS

The overall results of the study show that the proposed test protocol is, in principle, suitable for the intended use, namely determining the PRP content in liquid pentavalent vaccines (DTwP-HepB-Hib) containing a wP component. Some adjustment may be needed if a disposable gold electrode is used and in instances where more than one test sample is to be analysed in one session. This would imply regeneration and equilibration steps between injections and the use of an internal standard. The statistical analysis observed a significant difference between the WHO 1st International Standard PRP and the ribitol reference standard. However, the data do not indicate that either one of the reference standards should be preferred. Evidently, a full validation of the test method, as usually expected from quality control laboratories, would need to be applied independent of the choice of reference standard.

7. ACKNOWLEDGEMENTS

The authors wish to sincerely thank all participating laboratories for their valuable contribution to this study. The vaccine manufacturers (in alphabetical order by name): Berna Biotech Korea Corp., Biological E Limited; Serum Institute of India Limited and Shantha Biotechnics PVT Limited are cordially thanked for their donation of vaccine samples and especially for the preparation of a pentavalent vaccine exclusively for the purpose of this study. The collaborative study was organised by WHO/TAL within the framework of WHO vaccines prequalification and with funding from the United States Agency for International Development, in collaboration with the Istituto Superiore di Sanità, Italy and the EDQM in the framework of the BSP of the Council of Europe and the European Commission (project run under code BSP135). The contribution of Dr D. Le Tallec (EDQM, DBO) is gratefully acknowledged.

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9. ABBREVIATIONS

BSP: Biological Standardisation Programme; CRM: cross-reacting material; DBO: Department of Biological Standardisation, OMCL Network & HealthCare; D: Diphtheria; EDQM: European Directorate for the Quality of Medicines & HealthCare; GCV: geometric coefficient of variation; GM: geometric mean; HepB: Hepatitis B; Hib: *Haemophilus influenzae* type b; HPAEC-PAD:

high-performance anion exchange chromatography coupled with pulsed amperometric detection; IPV: Inactivated Polio Virus; IS: International Standard; Lab: laboratory; OOS: out of specification; PRP: poly ribosyl-ribitol-phosphate; shd: single human dose; SPE: solid phase extraction; SST: system suitability test; T: Tetanus; TAL: Technical Assistance and Laboratory Services group; WHO: World Health Organization; wP: whole-cell Pertussis.

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Table 1 – *Test vaccines – related information*

Vaccine sample code	Components, carrier protein, adjuvant and relevant excipients	Specifications			Doses per container	Vials provided to participants
		Total PRP µg/shd*		Free PRP		
HCS-1	DTwPHepB-Hib, Hib-TT, Al phosphate, Thiomersal 0.01 %	≥ 8.8	Not less than 80 % of label claim (11 µg/shd)	≤ 35 %	10-dose	4
HCS-2	DTwPHepB-Hib, Hib-CRM 197, Al phosphate	8-12	/	≤ 25 %	1-dose	30
HCS-3	DTwPHepB-Hib, Hib-TT, Al phosphate, Thiomersal 0.005 %	8-12	80-120 % of label claim (10 µg/shd)	≤ 30 %	10-dose	4
HCS-4	DTwPHepB-Hib, sub-potent Hib-TT (total PRP below specification + high content of free PRP), Al phosphate, Thiomersal 0.01 %,	≥ 8.8	/	≤ 35 %	10-dose	4
HCS-5	DTwPHepB-Hib, Hib-TT, Al phosphate, Thiomersal 0.05 mg/shd*	8-12	/	≤ 20.0 %	10-dose	4

* shd: single human dose.

Table 2 – *Details of assay conditions used by participants in the collaborative study*

Parameter	Study protocol	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10
Hydrolysis performed in	dry oven	heating block	heating block	heating block	dry oven	dry oven	water bath, circulation at 98°C for 2 h	dry oven	/	hot air oven	stove
SPE cartridge use under	vacuum (peristaltic pump)	vacuum (peristaltic pump)	vacuum (peristaltic pump)	vacuum (peristaltic pump)	gravity	vacuum (peristaltic pump)	gravity	vacuum (peristaltic pump)	gravity	gravity	vacuum (peristaltic pump)
Chromatography Brand/ Model	Dionex DX500	Thermo-Fisher Dionex ICS 5000	Thermo-Fisher Scientific Co./ Dionex ICS 5000 RF-IC	Dionex ICS-3000	Dionex ICS 5000	Dionex ICS-3000	Dionex ICS 5000	Dionex DX500	Dionex ICS-3000	Dionex ICS-3000	Dionex ICS 2500
Gold electrode	conventional	disposable PTFE	conventional	conventional	disposable PTFE	conventional	conventional	conventional	conventional	disposable	conventional
Reference pH electrode	Ag/AgCl	Ag/AgCl	AgCl	Ag/AgCl	AgCl	Ag/AgCl	Ag/AgCl	Ag/AgCl		AgCl	AgCl
Potential PAD waveform	Carbo-hydrates Standard Quadruple Potential	Carbo-hydrates Standard Quadruple Potential	Carbo-hydrates Standard Quadruple Potential	Quad	Carbo-hydrates Standard Quadruple Potential	Carbo-hydrates Standard Quadruple Potential	Carbo-hydrates Standard Quadruple Potential	Carbo-hydrates Standard Quadruple Potential		Carbo-hydrates Standard Quadruple Potential	Carbo-hydrates Standard Quadruple Potential
Column	CarboPac™ MA1	CarboPac™ MA1	CarboPac™ MA1	CarboPac™ MA1	CarboPac™ MA1	CarboPac™ MA1	CarboPac™ MA1	CarboPac™ MA1		CarboPac™ MA1	CarboPac™ MA1
Guard column	CarboPac™ MA1 guard	CarboPac™ MA1 guard	CarboPac™ MA1 guard	CarboPac™ MA1guard	CarboPac™ MA1 guard	CarboPac™ MA1 guard	CarboPac™ MA1guard	CarboPac™ MA1guard		CarboPac™ MA1guard	CarboPac™ MA1 Guard
Trap column	no	no	no		no	no	no	no	no	no	Amino trap
Column/ auto sampler temperature	30 °C/4 °C	30 °C/4 °C	30 °C/4 °C	30 °C/4 °C	30 °C/4 °C	30 °C	30 °C/2-8 °C	30 °C/4 °C		30 °C/5 °C	30 °C/ 4°C
Mobile phase: NaOH	580 mM	640 mM	580 mM	580 mM	580 mM	580 mM	580 mM	580 mM	580 mM	175 mM	580 mM
Regeneration of column	/	none	1 N NaOH	580 mM NaOH 1 h prior runs		1 N NaOH for 1 h					1 M NaOH for 1 h
Column washing step	1 N NaOH for 1 h	Before and after analysis	580 mM NaOH	1 N NaOH between runs	1 N NaOH for 1 h	1 N NaOH for 1 h	1 N NaOH for 1 h	1 N NaOH for 1 h		600 mM NaOH for 20 min at end of the sequence	1 M NaOH for 1 h
Flow rate	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min	0.4 mL/min
Run time	40 min	40 min	40 min	40 min	40 min	40 min	40 min	40 min	40 min	70 min	40 min
No. of test samples per analytical session	1	all	1		all	all	2-3	2		1	2
Software	/	Chromeleon 7.2	Chromeleon 6.8	Chromeleon 6.8 and use of Excel	Chromeleon 7.2	Chromeleon 6.7	Chromeleon 6.8 SR11	PeakNet6	Chromeleon	Chromeleon	Chromeleon 6.60

Table 3 – *Results versus WHO 1st International Standard PRP*

3A. Total PRP in µg/shd

Lab	Run	HCS-1 (≥ 8.8)			HCS-2 (8 to 12)			HCS-3 (8 to 12)			HCS-4 (≥ 8.8)			HCS-5 (8 to 12)		
		Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round
1	1	10.68	11.16	11.2	10.66	10.57	11	8.91	9.02	9	7.22	7.24	7.2	10.44	10.68	11
	2	11.66			10.49			9.14			7.26			10.92		
2	1	10.07	9.65	9.7	10.06	9.85	10	7.76	8.17	8	5.44	6.32	6.3	8.85	9.92	10
	2	9.25			9.65			8.60			7.34			11.12		
3	1	12.85	12.99	13.0	11.28	11.32	11	10.11	10.32	10	7.85	8.01	8.0	11.34	11.49	11
	2	13.13			11.36			10.53			8.17			11.64		
4	1	10.23	10.24	10.2	8.94	9.10	9	8.06	8.06	8	6.30	6.25	6.2	9.06	9.14	9
	2	10.25			9.27			8.07			6.20			9.23		
5	1	12.06	11.69	11.7	10.28	10.27	10	8.91	8.79	9	7.21	7.09	7.1	10.10	10.30	10
	2	11.33			10.27			8.68			6.97			10.50		
6	1	14.07	14.15	14.2	12.21	12.14	12	12.19	11.40	11	8.52	8.57	8.6	13.00	13.17	13
	2	14.24			12.08			10.66			8.63			13.34		
7	1	11.67	11.67	11.7	10.12	10.12	10	9.74	9.74	10	7.31	7.31	7.3	10.63	10.63	11
	2	.			.			.			.			.		
8	1	10.44	10.92	10.9	9.43	9.78	10	7.24	8.15	8	7.89	8.12	8.1	8.01	7.61	8
	2	11.43			10.15			9.17			8.36			7.23		
9	1	11.92	11.88	11.9	10.58	10.20	10	8.96	8.56	9	5.88	6.29	6.3	10.05	9.86	10
	2	11.84			9.84			8.18			6.72			9.68		
10	1	11.87*	12.55	12.5	12.24	11.96	12	9.43*	9.95	10	8.45	9.65	9.6	12.32	9.84	10
	2	13.26			11.68			10.50			11.01			7.86		
GM			11.62			10.49			9.16			7.41			10.17	
GCV			11.27			9.20			11.69			14.58			14.38	

* See text for more details regarding values labelled with an asterisk. ^ Overall statistics for Free PRP in HCS-4 and HCS-5 are excluding Lab 6.
Ind. = Individual run. GM = Geometric Mean. GCV = Geometric Coefficient of Variation. Round = Rounded to specification.

Table 3 – *Results versus WHO 1st International Standard PRP*

3B. Free PRP in µg/shd

Lab	Run	HCS-1			HCS-2			HCS-3			HCS-4			HCS-5		
		Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round
1	1	1.13	1.12		0.33	0.31		1.06	1.03		2.29	2.21		0.62	0.57	
	2	1.11			0.30			1.01			2.13			0.53		
2	1	1.07	1.04		0.49	0.46		1.19	1.20		1.87	1.95		0.57	0.64	
	2	1.02			0.43			1.21			2.04			0.71		
3	1	1.40	1.44		0.45	0.44		1.37	1.22		2.25	2.29		0.77	0.76	
	2	1.49			0.44			1.09			2.33			0.75		
4	1	1.63	1.45		0.34	0.32		1.22	1.12		2.36	2.26		0.55	0.55	
	2	1.29			0.31			1.02			2.17			0.56		
5	1	1.56	1.63		0.51	0.49		1.42	1.40		2.56*	2.50		0.79	0.79	
	2	1.71			0.47			1.39			2.45*			0.80		
6	1	2.23	2.19		0.59	0.55		1.86	1.96		0.97*	1.00*		3.11*	3.12*	
	2	2.15			0.51			2.07			1.04*			3.14*		
7	1	1.69	1.69		0.45	0.45		1.59	1.59		2.88	2.88		0.92	0.92	
	2	.			.			.			.			.		
8	1	2.43	2.34		1.79*	0.81*		1.74	1.65		3.49	3.16		0.93	0.88	
	2	2.25			0.81			1.56			2.86			0.84		
9	1	2.18	2.07		0.52	0.48		1.55	1.51		2.07	2.40		0.83	1.03	
	2	1.96			0.44			1.47			2.78			1.28		
10	1	2.49	2.51		0.76	0.68		2.03	2.23		4.05	3.91		1.26	1.39	
	2	2.54			0.60			2.45			3.78			1.54		
GM			1.68			0.48			1.45			2.56^			0.81^	
GCV			30.90			24.04			25.15			21.62^			30.26^	

* See text for more details regarding values labelled with an asterisk. ^ Overall statistics for Free PRP in HCS-4 and HCS-5 are excluding Lab 6.
Ind. = Individual run. GM = Geometric Mean. GCV = Geometric Coefficient of Variation. Round = Rounded to specification.

Table 3 – *Results versus WHO 1st International Standard PRP*

3C. Free PRP in percentage of total PRP

Lab	Run	HCS-1 (≤ 35)			HCS-2 (≤ 25)			HCS-3 (≤ 30)			HCS-4 (≤ 35)			HCS-5 (≤ 20.0)		
		Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round
1	1	10.56	10.03	10	3.05	2.93	3	11.88	11.46	11	31.81	30.53	31	5.89	5.37	5.4
	2	9.52			2.82			11.06			29.30			4.89		
2	1	10.60	10.79	11	4.90	4.69	5	15.39	14.68	15	34.38	30.92	31	6.42	6.39	6.4
	2	10.98			4.48			14.01			27.80			6.36		
3	1	10.90	11.13	11	3.96	3.90	4	13.53	11.94	12	28.68	28.56	29	6.75	6.60	6.6
	2	11.36			3.85			10.53			28.45			6.45		
4	1	15.93	14.16	14	3.80	3.56	4	15.14	13.83	14	37.46	36.21	36	6.07	6.07	6.1
	2	12.59			3.34			12.64			35.00			6.07		
5	1	12.91	13.97	14	4.96	4.77	5	15.92	15.99	16	35.54*	35.39	35	7.77	7.70	7.7
	2	15.11			4.59			16.06			35.24*			7.63		
6	1	15.85	15.47	15	4.83	4.51	5	15.26	17.21	17	11.38*	11.71*	12*	23.92*	23.73*	23.7*
	2	15.10			4.22			19.42			12.05*			23.54*		
7	1	14.42	14.42	14	4.45	4.45	4	16.26	16.26	16	39.45	39.45	39	8.63	8.63	8.6
	2	.			.			.			.			.		
8	1	23.25	21.37	21	18.98*	7.95*	8*	24.05	20.23	20	44.28	38.91	39	11.63	11.60	11.6
	2	19.65			7.95			17.01			34.20			11.58		
9	1	18.25	17.38	17	4.91	4.69	5	17.27	17.61	18	35.16	38.15	38	8.27	10.44	10.4
	2	16.55			4.48			17.96			41.40			13.17		
10	1	20.94	20.04	20	6.18	5.62	6	21.57	22.41	22	48.00	40.58	41	10.26	14.16	14.2
	2	19.18			5.11			23.29			34.30			19.54		
GM			14.44			4.55			15.84			35.16^			8.14^		
GCV			25.98			19.28			21.53			12.86^			33.75^		

* See text for more details regarding values labelled with an asterisk. ^ Overall statistics for Free PRP in HCS-4 and HCS-5 are excluding Lab 6.
Ind. = Individual run. GM = Geometric Mean. GCV = Geometric Coefficient of Variation. Round = Rounded to specification.

Table 4 – *Results versus ribitol reference standard*

4A. Total PRP in µg/shd

Lab	Run	HCS-1 (≥ 8.8)			HCS-2 (8 to 12)			HCS-3 (8 to 12)			HCS-4 (≥ 8.8)			HCS-5 (8 to 12)		
		Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round
1	1	10.42	10.63	10.6	10.41	10.07	10	8.67	8.59	9	6.99	6.88	6.9	10.18	10.17	10
	2	10.84			9.75			8.51			6.77			10.16		
2	1	10.57	10.19	10.2	8.37	8.40	8	7.24	7.51	8	4.70	5.88	5.9	9.03	9.81	10
	2	9.83			8.44			7.79			7.35			10.65		
3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.			.			.			.			.		
4	1	9.85	9.85	9.9	8.60	8.75	9	7.75	7.74	8	6.06	5.99	6.0	8.72	8.80	9
	2	9.86			8.91			7.74			5.93			8.88		
5	1	10.05	9.97	10.0	8.53	8.75	9	7.36	7.47	7	5.90	5.99	6.0	8.38	8.77	9
	2	9.90			8.97			7.59			6.08			9.18		
6	1	11.56	11.35	11.3	10.03	9.74	10	10.41	9.60	10	7.71	7.45	7.5	10.84	10.83	11
	2	11.14			9.46			8.86			7.20			10.82		
7	1	11.88	11.88	11.9	10.30	10.30	10	9.13	9.13	9	6.79	6.79	6.8	9.42	9.42	9
	2	.			.			.			.			.		
8	1	9.55	10.05	10.1	7.96	8.94	9	6.58	7.51	8	7.25	7.83	7.8	7.65	7.09	7
	2	10.58			10.03			8.58			8.45			6.57		
9	1	10.01	10.36	10.4	8.95	8.95	9	7.99	7.78	8	7.42	6.72	6.7	8.95	8.48	8
	2	10.72			8.96			7.57			6.09			8.04		
10	1	11.33*	11.09	11.1	9.75	9.65	10	9.00*	8.80	9	6.71	7.78	7.8	9.82	7.93	8
	2	10.86			9.56			8.60			9.01			6.40		
GM			10.58			9.26			8.20			6.77			8.97		
GCV			6.48			7.16			9.62			11.19			13.10		

* See text for more details regarding values labelled with an asterisk. ^ Overall statistics for Free PRP in HCS-4 and HCS-5 are excluding Lab 6.
Ind. = Individual run. GM = Geometric Mean. GCV = Geometric Coefficient of Variation. Round = Rounded to specification.

Table 4 – *Results versus ribitol reference standard*

4B. Free PRP in µg/shd

Lab	Run	HCS-1			HCS-2			HCS-3			HCS-4			HCS-5		
		Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round
1	1	1.10	1.06		0.31	0.29		1.03	0.98		2.26	2.11		0.59	0.54	
	2	1.03			0.28			0.94			1.97			0.50		
2	1	1.12	1.10		0.41	0.39		1.11	1.10		1.79	1.91		0.58	0.63	
	2	1.08			0.38			1.10			2.04			0.68		
3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.			.			.			.			.		
4	1	1.54	1.35		0.29	0.26		1.14	1.03		2.23	2.13		0.49	0.48	
	2	1.19			0.23			0.93			2.04			0.48		
5	1	1.31	1.40		0.41	0.41		1.19	1.20		2.17	2.16		0.65	0.67	
	2	1.50			0.41			1.22			2.15			0.70		
6	1	1.83	1.75		0.48	0.44		1.59	1.65		0.87*	0.86*		2.59*	2.57*	
	2	1.68			0.40			1.72			0.86*			2.55*		
7	1	1.72	1.72		0.46	0.46		1.50	1.50		2.75	2.75		0.81	0.81	
	2	.			.			.			.			.		
8	1	2.22	2.15		1.51*	0.80*		1.58	1.52		3.21	3.05		0.89	0.82	
	2	2.08			0.80			1.46			2.89			0.76		
9	1	1.81	1.81		0.44	0.42		1.38	1.36		2.61	2.55		0.74	0.73	
	2	1.81			0.40			1.35			2.49			0.73		
10	1	2.32	2.08		0.59	0.53		1.95	1.97		3.25	3.16		1.01	1.14	
	2	1.86			0.48			2.00			3.07			1.28		
GM			1.56			0.42			1.34			2.44^			0.71^	
GCV			26.16			24.06			23.68			18.91^			27.11^	

* See text for more details regarding values labelled with an asterisk. ^ Overall statistics for Free PRP in HCS-4 and HCS-5 are excluding Lab 6.
Ind. = Individual run. GM = Geometric Mean. GCV = Geometric Coefficient of Variation. Round = Rounded to specification.

Table 4 – *Results versus ribitol reference standard*

4C. Free PRP in percentage of total PRP

Lab	Run	HCS-1 (≤ 35)			HCS-2 (≤ 25)			HCS-3 (≤ 30)			HCS-4 (≤ 35)			HCS-5 (≤ 20.0)		
		Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round	Ind.	GM	Round
1	1	10.56	10.03	10	2.96	2.91	3	11.91	11.48	11	32.24	30.66	31	5.84	5.36	5.4
	2	9.52			2.86			11.06			29.16			4.92		
2	1	10.60	10.79	11	4.90	4.69	5	15.39	14.74	15	38.07	32.53	33	6.42	6.39	6.4
	2	10.98			4.48			14.11			27.80			6.36		
3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.			.			.			.			.		
4	1	15.63	13.74	14	3.37	2.95	3	14.71	13.30	13	36.80	35.58	36	5.62	5.51	5.5
	2	12.07			2.58			12.02			34.40			5.41		
5	1	12.98	14.00	14	4.80	4.69	5	16.13	16.09	16	36.67	35.96	36	7.70	7.66	7.7
	2	15.11			4.58			16.06			35.26			7.63		
6	1	15.83	15.45	15	4.79	4.50	5	15.27	17.22	17	11.28*	11.61*	12*	23.89*	23.73*	23.7*
	2	15.08			4.23			19.41			11.94*			23.57*		
7	1	14.46	14.46	14	4.39	4.39	4	16.42	16.42	16	40.43	40.43	40	8.62	8.62	8.6
	2	.			.			.			.			.		
8	1	23.25	21.37	21	18.98*	7.95*	8*	24.05	20.23	20	44.28	38.91	39	11.63	11.60	11.6
	2	19.65			7.95			17.01			34.20			11.58		
9	1	18.08	17.46	17	4.91	4.66	5	17.27	17.58	18	35.16	37.91	38	8.28	8.69	8.7
	2	16.86			4.43			17.89			40.87			9.11		
10	1	20.51	18.73	19	6.10	5.54	6	21.70	22.49	22	48.38	40.60	41	10.23	14.33	14.3
	2	17.11			5.03			23.31			34.07			20.06		
GM			14.72			4.51			16.31			36.41^			8.07^		
GCV			24.88			23.56			20.64			10.15^			35.72^		

* See text for more details regarding values labelled with an asterisk. ^ Overall statistics for Free PRP in HCS-4 and HCS-5 are excluding Lab 6.
Ind. = Individual run. GM = Geometric Mean. GCV = Geometric Coefficient of Variation. Round = Rounded to specification.

Table 5 – *Repeatability of the analytical test protocol*

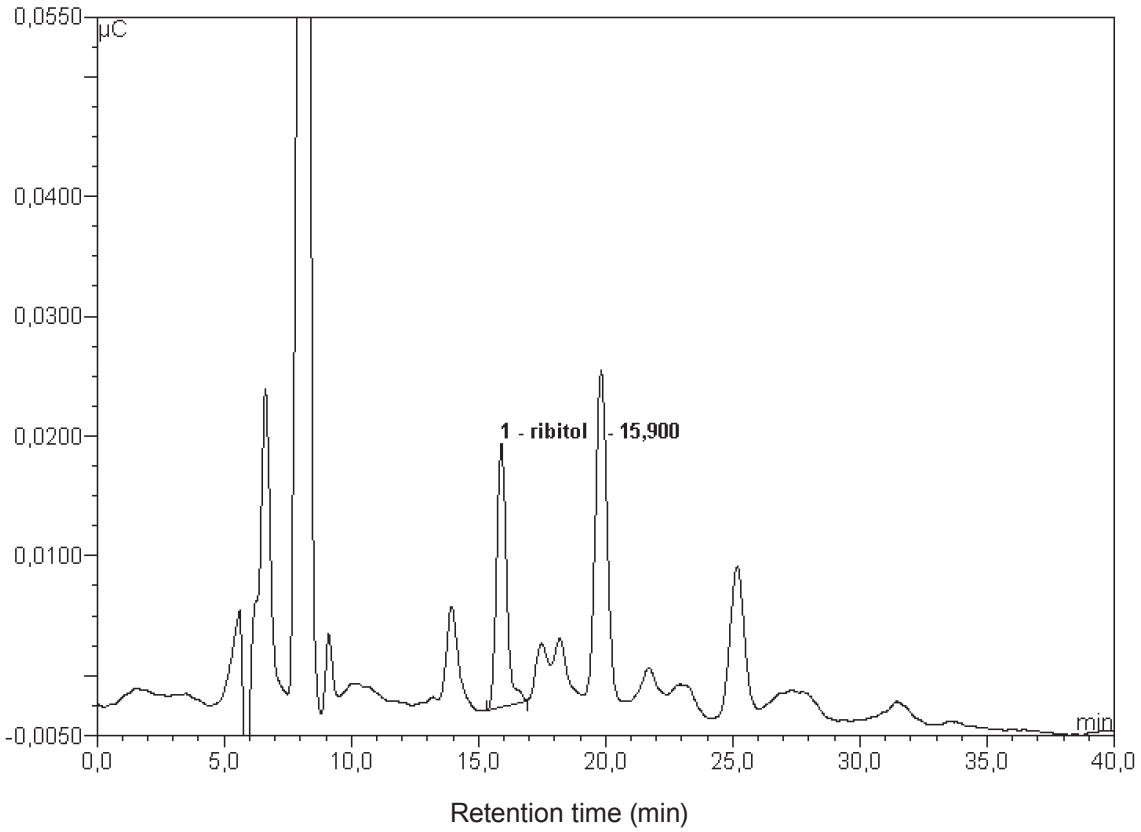
	Against WHO IS			Against Ribitol Reference Standard (Fluka)		
	Total (µg/shd)	Free (µg/shd)	Free (%)	Total (µg/shd)	Free (µg/shd)	Free (%)
HCS-1	1.50	0.32	4.01	1.15	0.45	4.70
HCS-2	0.86	0.15	1.06	1.62	0.12	1.19
HCS-3	1.96	0.41	6.20	1.87	0.21	6.23
HCS-4	2.22	0.73	13.72	2.79	0.43	16.08
HCS-5	3.39	0.39	7.38	2.88	0.25	7.42

Table 6 – *Reproducibility of the analytical test protocol*

	Against WHO IS			Against Ribitol Reference Standard (Fluka)		
	Total (µg/shd)	Free (µg/shd)	Free (%)	Total (µg/shd)	Free (µg/shd)	Free (%)
HCS-1	4.06	1.52	11.72	1.72	1.22	11.42
HCS-2	2.94	0.40	3.38	2.00	0.39	3.77
HCS-3	3.52	1.17	11.19	2.56	0.97	11.07
HCS-4	3.67	1.87	16.00	3.00	1.41	15.25
HCS-5	4.90	0.84	10.79	3.94	0.64	11.35

Figure 1 – *HPAEC-PAD chromatograms of the vaccine sample HCS-3 (CarboPac MA) following the test protocol and using a gold conventional electrode (Lab 7)*

1A. Ribitol from total saccharide



1B. Ribitol from free saccharide

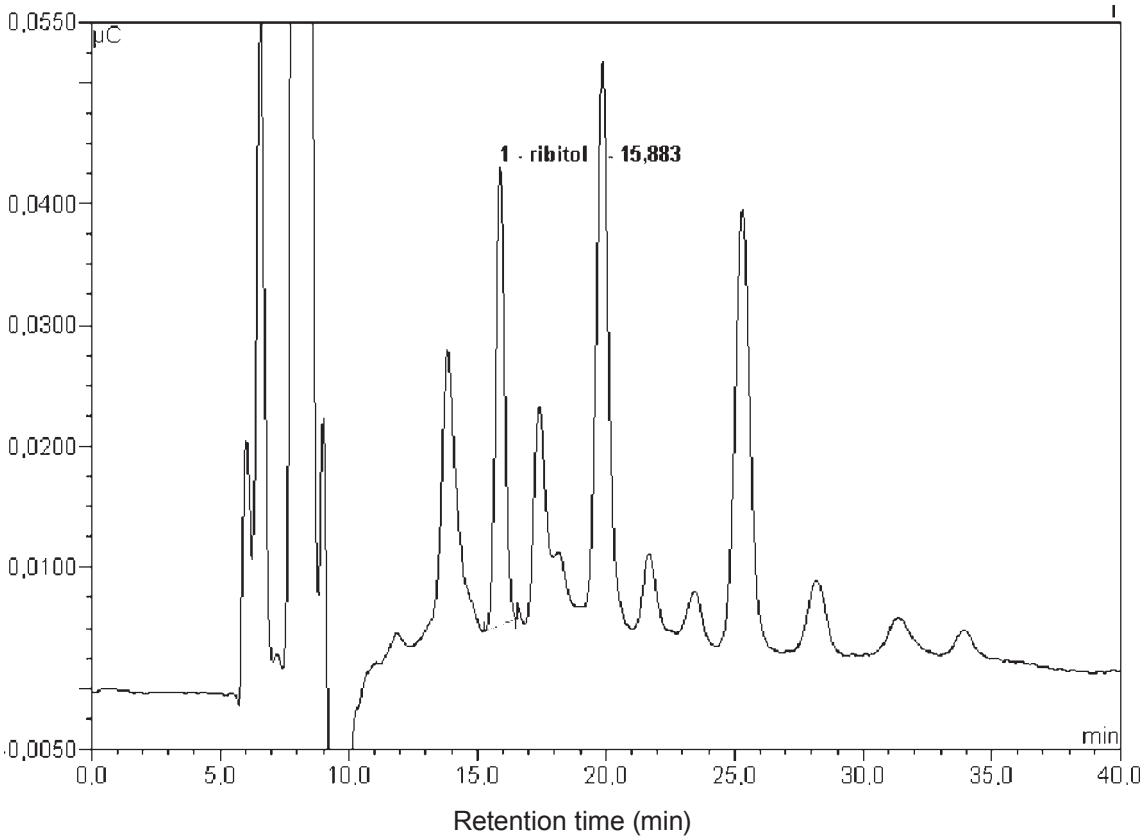
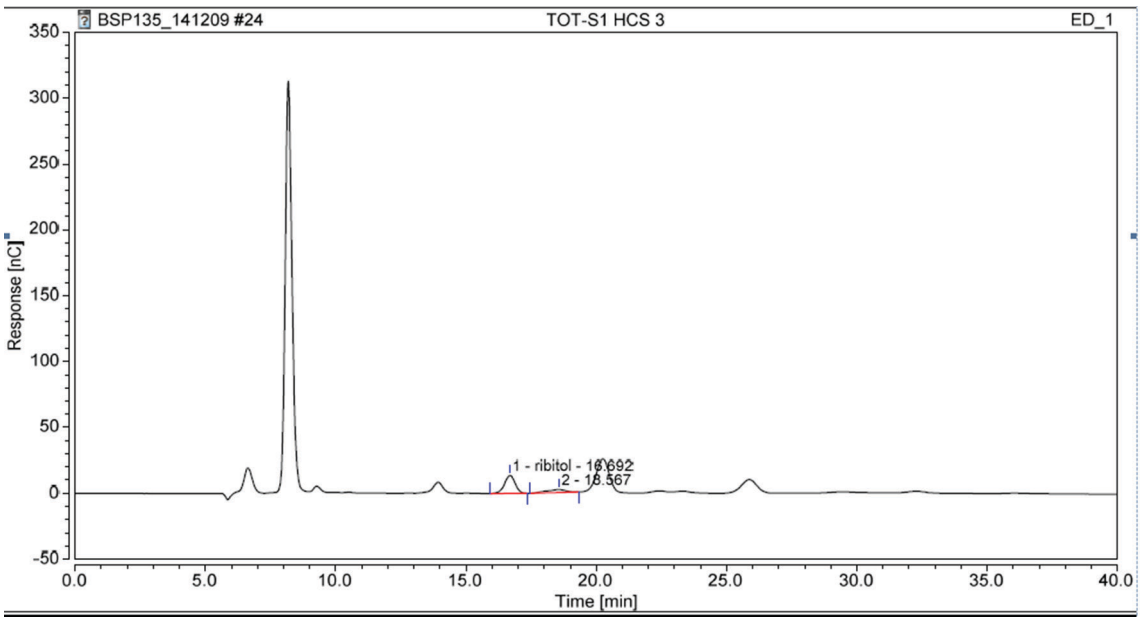
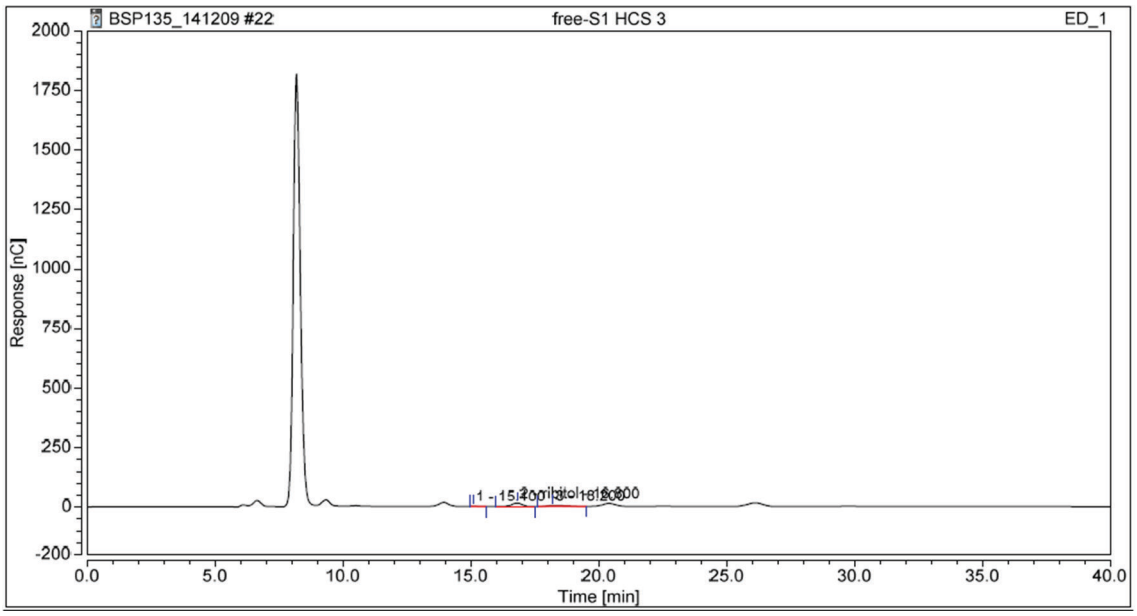


Figure 2 – *HPAEC-PAD chromatograms of the vaccine sample HCS-3 (CarboPac MA) using a 640 mM mobile phase and a disposable PTFE electrode (Lab 1)*

2A. Ribitol from total saccharide



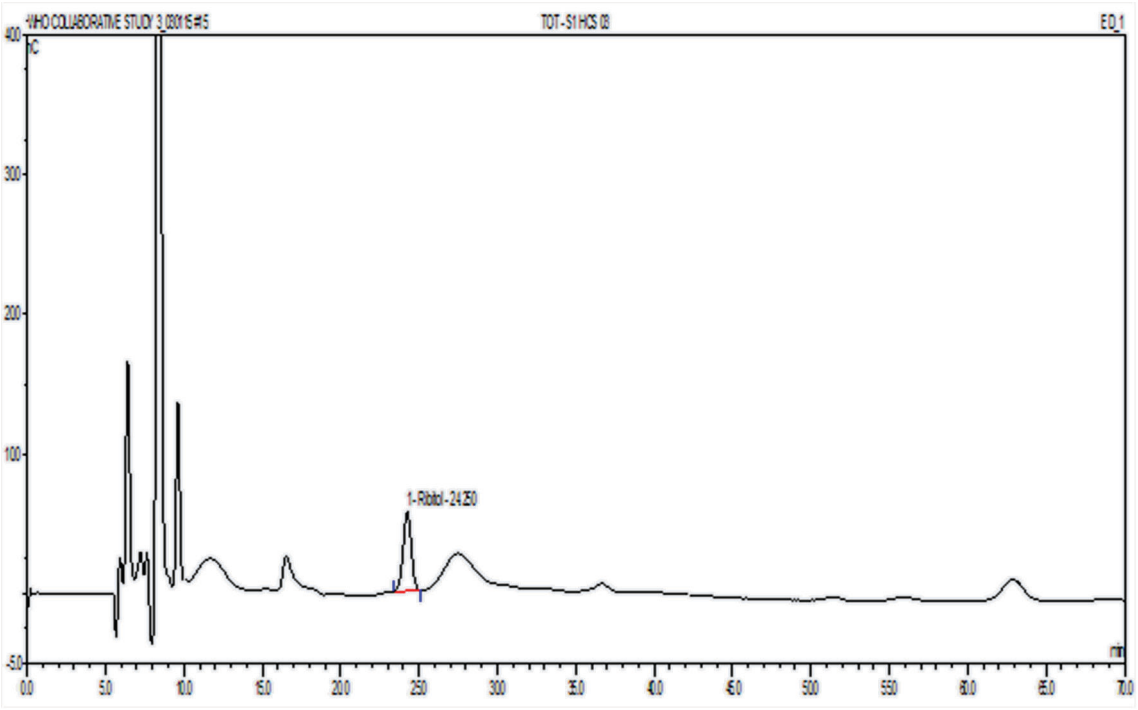
2B. Ribitol from free saccharide



Retention time (min)

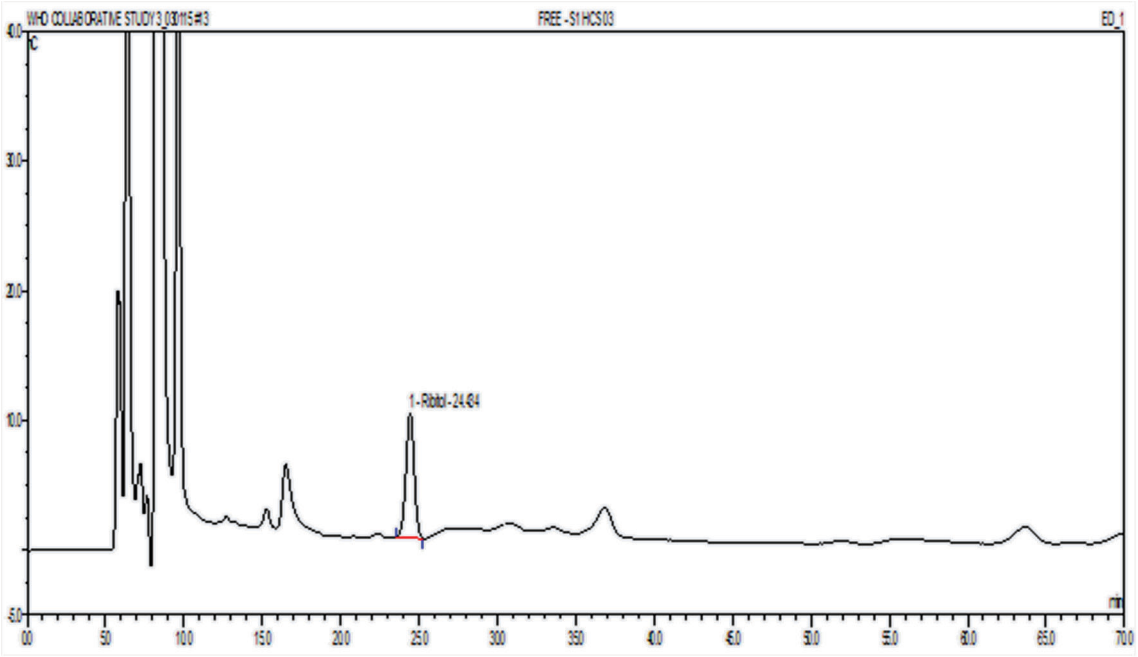
Figure 3 – *HPAEC-PAD chromatograms of the vaccine sample HCS-3 (CarboPac MA) using a 175 mM mobile phase and a disposable gold electrode (Lab 9)*

3A. Ribitol from total saccharide



Retention time (min)

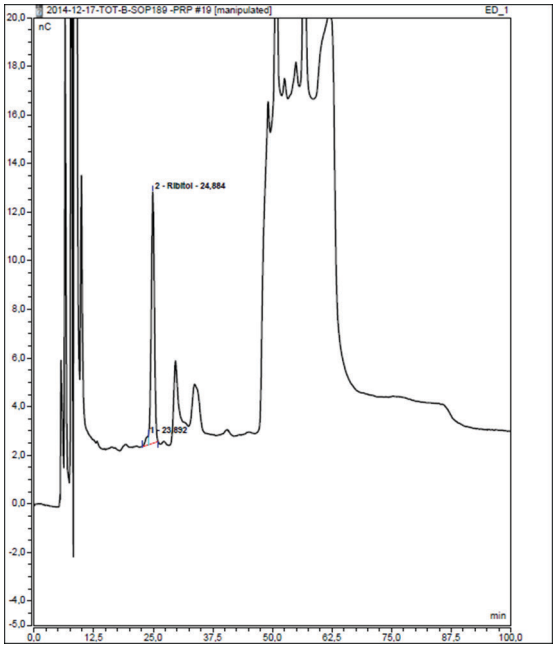
3B. Ribitol from free saccharide



Retention time (min)

Figure 4 – *HPAEC-PAD chromatograms of the vaccine sample HCS-3 (CarboPac MA) by performing the analysis using a disposable PTFE electrode (Lab 4)*

4A. Ribitol from total saccharide – Analysis applying a multi-step gradient and different run time



4B. Ribitol from total saccharide – Analysis according to the test protocol

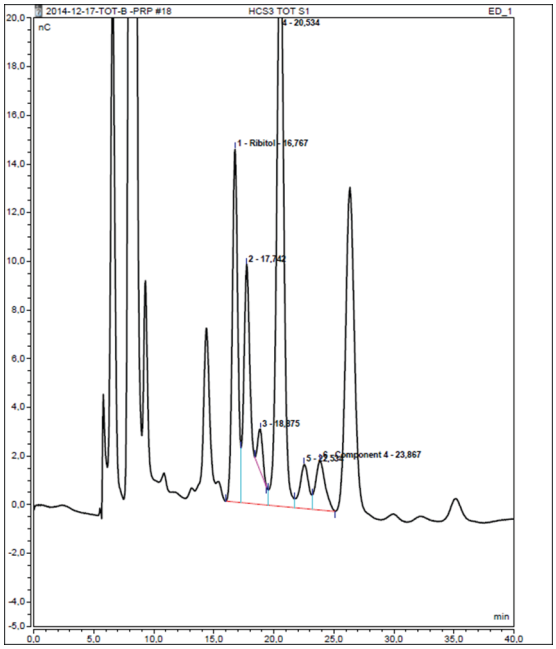
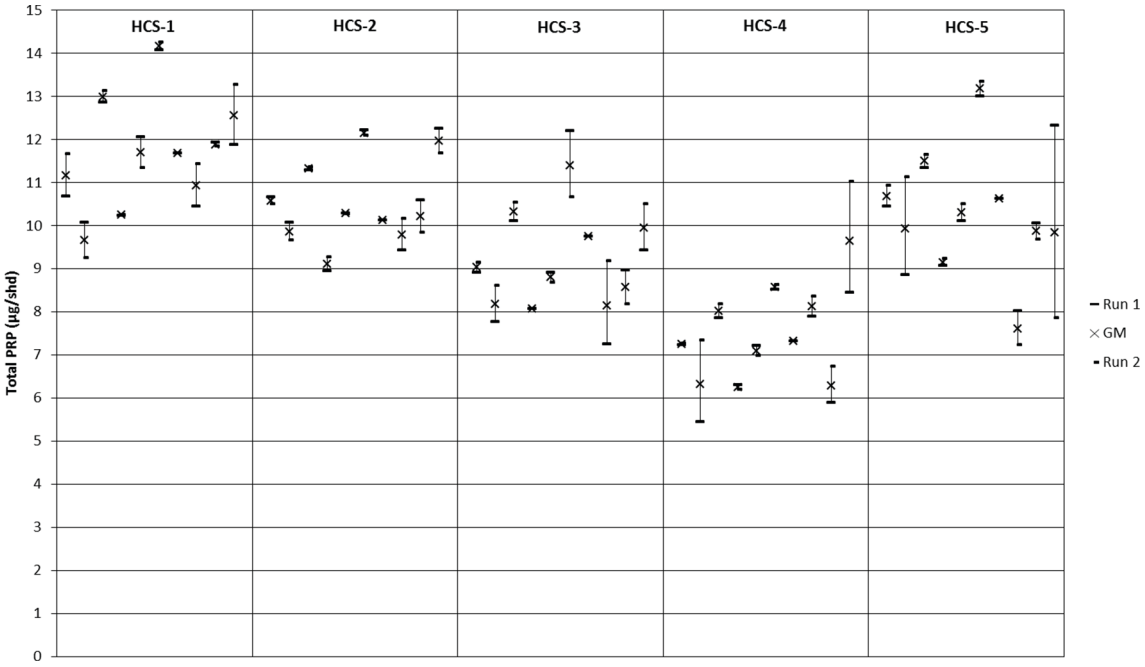
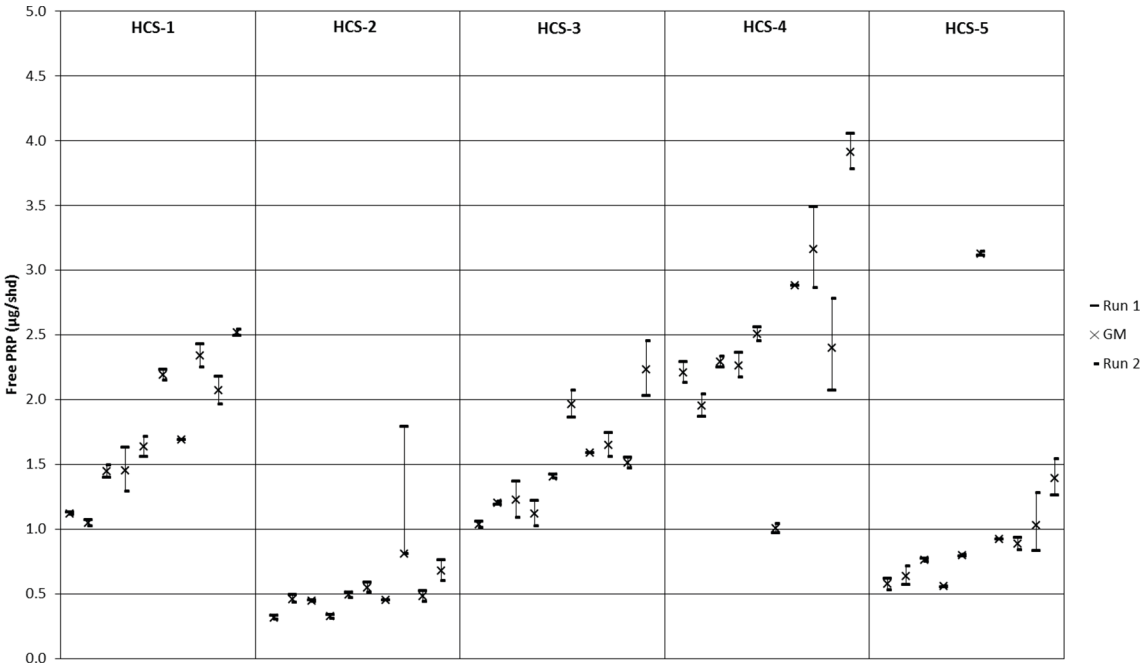


Figure 5 – *Min-max plot of PRP content against WHO IS*

5A. Total PRP content against WHO IS



5B. Free PRP content against WHO IS



5C. Free PRP content as percentage of total PRP against WHO IS

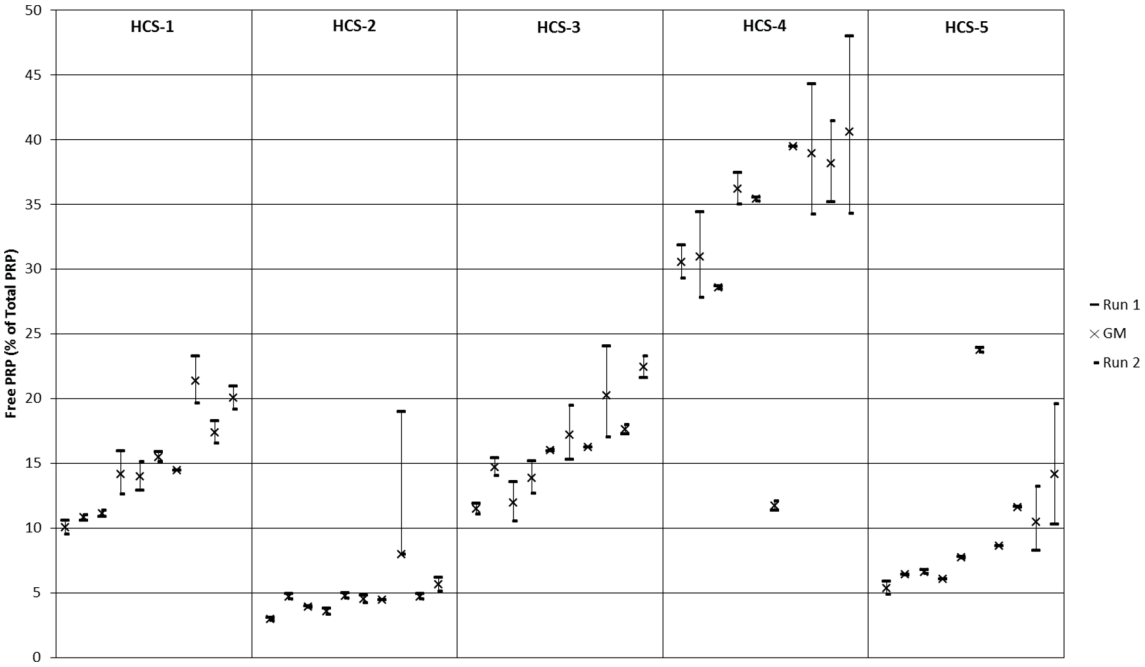
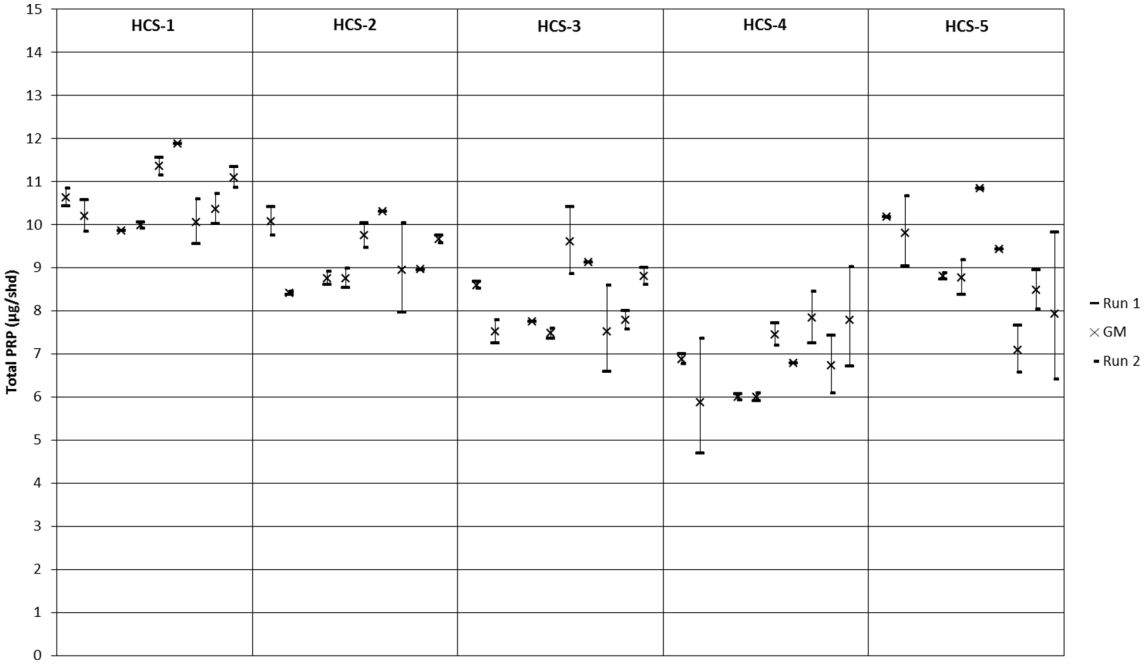
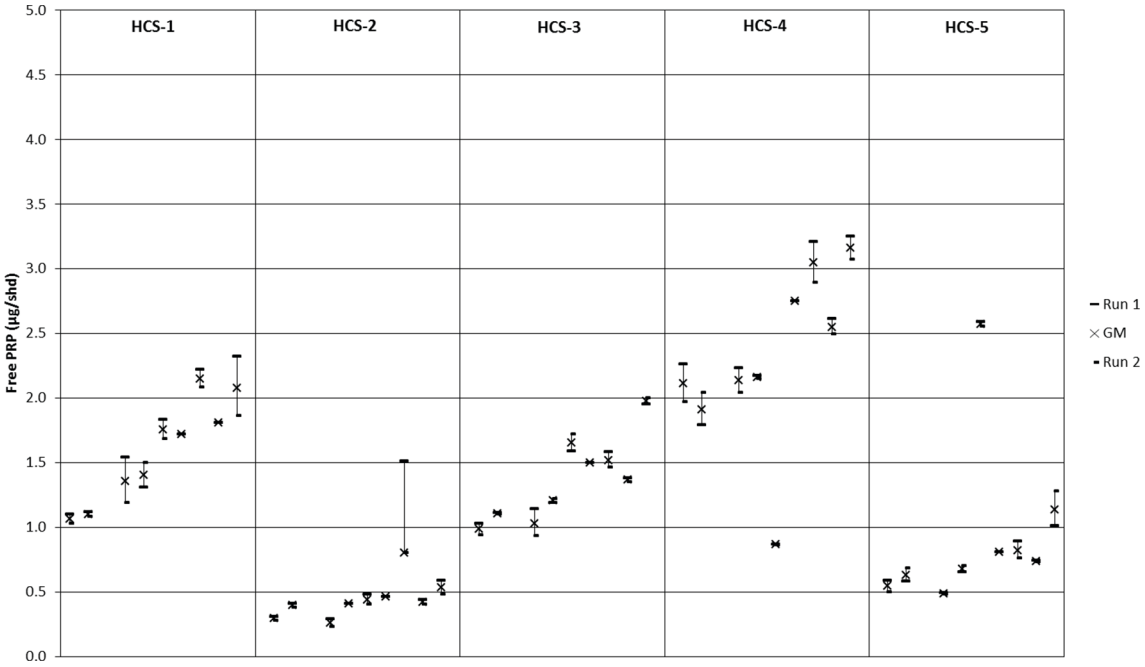


Figure 6 – *Min-max plot of PRP content against Ribitol reference standard*

6A. Total PRP content against Ribitol reference standard



6B. Free PRP content against Ribitol reference standard



6C. Free PRP content as percentage of total PRP against Ribitol reference standard

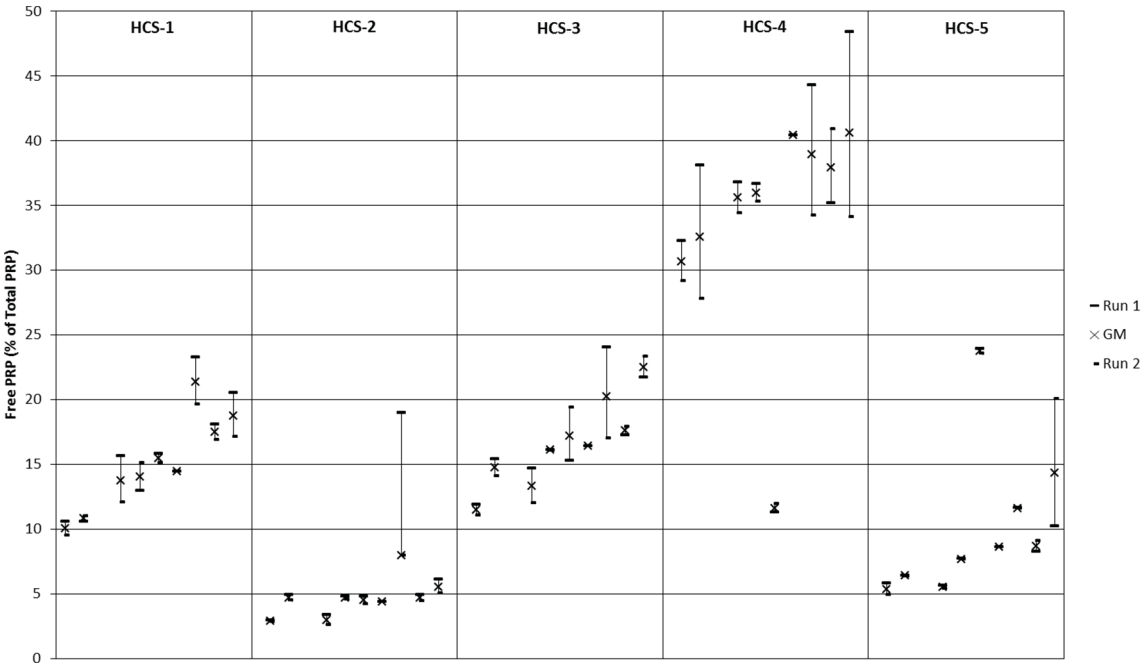
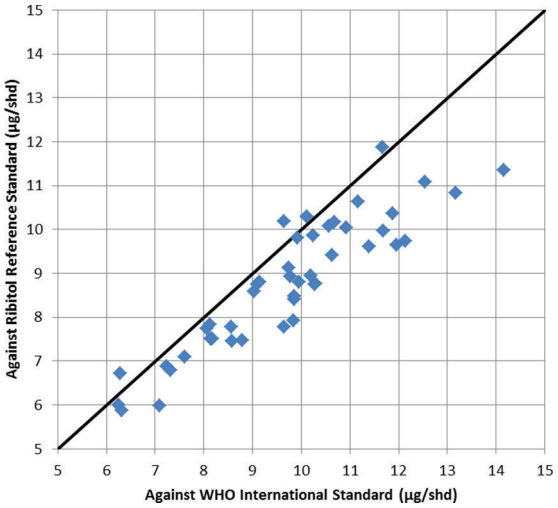


Figure 7 – *Two-way plot of PRP content using different standards*

7A. Total PRP content



7B. Free PRP content