Evaluation of the Saccharide Content of the WHO 2nd International Standard for *Haemophilus Influenzae* Polysaccharide Polyribosyl Ribitol Phosphate (PRP) by HPAEC-PAD Analysis Following Acid Hydrolysis

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Abstract

With this report, we present the results of a collaborative study evaluating the content in mg per ampoule of the "WHO 2nd International Standard (IS) for *Haemophilus influenzae* polysaccharide Polyribosyl Ribitol Phosphate (PRP), NIBSC code: 12/306" (WHO 2nd IS for PRP), by applying High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) following acid hydrolysis of the PRP.

Seventeen laboratories from eleven countries participated in the collaborative study. The outcome of this study revealed a PRP content of the WHO 2nd IS for PRP of 4.338 ± 0.203 mg/ampoule. Results of a previous collaborative study that included eleven participating laboratories, yielded a content of 4.904 ± 0.185 mg/ampoule when applying the ribose assays (i.e., by orcinol method). Based on the outcome of this study, using HPAEC-PAD and acid hydrolysis of PRP, it is concluded that the determination of the PRP content is method dependent and therefore assigned values for the WHO 2nd IS for PRP should be tailored according to the applied method.

Keywords: *Haemophilus influenza* type b, Hib Polysaccharide International Standard, High Performance Anion Exchange Chromatography (HPAEC), Pulsed amperometric detection (PAD), acid hydrolysis.

1. Introduction

This study was organised and coordinated by the Technical Assistance & Laboratory Services (TAL) - Vaccines Group (subsequently renamed the Laboratory Networks and Services - LNS) within the Regulatory System Strengthening (RSS) Team, the Regulation of Medicines and Other Health Technologies (RHT) Unit, the Essential Medicines and Health Products (EMP) Department in the Health Systems and Innovation (HIS) Cluster of the World Health Organization (WHO), and was done in collaboration with the Unit of Biological and Biotechnological Products, Bacterial Vaccine Section of the Istituto Superiore di Sanità (ISS), Rome, Italy.

The aim of this study was to ascertain whether the PRP content of the WHO 2nd IS for PRP (NIBSC code: 12/306), when assessed by HPAEC-PAD following acid hydrolysis, was in line with the assigned value using the orcinol method [1].

2. Materials and Methods

2.1 Participants

A total number of 17 laboratories participated in the study; out of these, nine are National Control Laboratories and eight are quality control laboratories from vaccine manufacturers. Appendix A provides a list of participants and related institutions in alphabetical order by country. Herein, an arbitrarily allocated code number [1-17], which is not related to the order of listing, refers to the participating laboratories.

2.2 Study material

The study material, the WHO 2nd IS for PRP (NIBSC code: 12/306), was kindly donated by the National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire, EN6 3QG, WHO International Laboratory for Biological Standards, UK Official Medicines Control Laboratory.

Participants received one ampoule of the WHO 2nd IS for PRP. According to the instructions for use [2], each ampoule contains the freeze-dried powder of 2 ml of PRP in 0.56 mg/ml NaCl. When estimated by weighing after freeze-drying, each ampoule contains about 6.0 mg of dry material and has a moisture content of about 1.45%. The ampoule should be stored at or below -20°C.

2.3 Study design and reporting of results

Participants were requested to quantify the PRP content per ampoule in three independent runs (i.e., 3 separate testing days), according to the analytical protocol of a previously performed collaborative study [3]. The protocol method quantifies the *Haemophilus influenzae* type b (Hib) saccharide content by determining, using HPAEC-PAD, the ribitol obtained after acidic hydrolyses. Thus, the calculation was based on the calibration curve of the monosaccharide, D-ribitol (adonitol).

As ribitol accounts for 41.3% of the PRP dry weight, a conversion factor of 2.42 was indicated to convert ribitol to a PRP content on a per gram basis [1, 4].

Laboratories were asked to report the total content of PRP per ampoule in mg/ampoule and rounded to three decimals.

Participants were asked to record results from three independent test runs into a standardized electronic data reporting sheet that had been provided in advance by the coordinators of the study. The reported results were used for further calculations and statistical analyses performed at ISS. Test results were submitted between February 2019 and December 2019.

2.4 Sample preparation

According to the instructions for use [2], the ampoule had to be reconstituted with 1 ml sterile distilled water and stored at -20°C in small aliquots until use. Further dilutions to yield a test solution in the correct concentration range for the assay were provided in the study protocol. Thus, a working solution containing 19.732 PRP ng/mL (based on ribose content) had to be prepared and then diluted 1:20 in MilliQ water. To 1 mL of this solution, 50 µL 6N HCl was added and then incubated for 2 hours at 100 °C to hydrolyse the PRP. Afterwards the sample was cooled for 10 min at 5 ± 3 °C and 400 µL of 1 M NaOH was added.

The monosaccharide, D-ribitol, was used for a five-point calibration curve ranging from 0.075 mcg/mL to 1.05 mcg/mL and treated as the PRP solution (i.e. hydrolysis and addition of NaOH).

2.5 Assay by HPAEC-PAD

Prior to the analysis by HPAEC-PAD, all samples were filtered. Ribitol was separated on a CarboPac MA1 analytical column (4 × 250, Dionex,) connected with a CarboPac MA1 guard column (Dionex). The analytical protocol defined the chromatographic conditions to be applied and these are shown in the Table 1.

Table 1. Chromatographic conditions.

Parameters	Setting
Column temperature	30°C
Detection	Pulsed amperometric detection (PAD)
Wave Form	Carbohydrate standard quadruple potential
Gold electrode	Not disposable
Reference electrode	AgCl
Elution conditions / eluent	Isocratic / 580 mM NaOH
Autosampler temperature	4°C
Injection volume	100 µL
Flow rate	0.4 mL/min
Run time	40 min
Software for data acquisition and processing	Chromeleon version 6 or 7

2.6 Statistical analysis

All data were analysed by ISS using the software IBM SPSS 26.0 and MS Excel 16.0.
Precision of the method was calculated as intra-laboratory precision, inter-laboratory precision and reproducibility (see Table 2 for details) [5].

Table 2. Precision analysis.

VAR Component	VAR Estimate	% of tot	SD	RSD (%)
Inter-Laboratories (between)	0.096	64	0.31	
Intra-Laboratory (within)	0.055	36	0.235	5.5
Reproducibility	0.152	100	0.39	9.1

VAR: Variance, in (mg/ampoule)²; SD: Standard Deviation, in mg/ampoule; RSD: Relative Standard Deviation, in %

S_{Run}: Intra-Laboratory SD; it represents the "Variability among Runs + Repeatability of the Method"

S_{Run} is obtained by the pooled SD within the 3 Runs results, per each of the N=17 Labs

S_{Lab}: Inter-Laboratories SD; it represents the variability among the participating Laboratories. It is obtained by $\sqrt{S_b^2 - \frac{S_{Run}^2}{Nrun}}$

With S_b: Standard Deviation between laboratories, and N_{run} = number of Runs (i.e., 3)
S_{Repr} is the Standard Deviation of Reproducibility; it is obtained by $= \sqrt{S_{Run}^2 + S_{Lab}^2}$

To establish the content of the PRP per ampoule, an analysis of the raw data was carried out by a pre-assessment of potential outliers; for this purpose, both Dixon and Grubb's tests were performed. The hypothesis of normality was assessed by means of the Shapiro-Wilk Test. This is considered the most powerful normality test when there is a small sample size, as in this case of 17 laboratories. If the p-value of the Shapiro-Wilk test is less than the classical threshold of 0.05, the results indicate a significant deviation from the assumption of normality, whereas Shapiro-Wilk p-values greater than 0.05 indicate no significant deviation from normality. However, for the purposes of this report, a more conservative approach was used. Specifically, a distribution was considered normal if normality was consistent with visual inspection of

exploratory graphs of the data and if the Shapiro-Wilk p-value was greater than 0.5. This conservative approach was adopted because the classical estimator of central tendency is very sensitive even to small deviations from normality. The content of the PRP in mg/ampoule was estimated after the above cited exploratory analysis of the data in order to choose a robust estimator instead of the classical calculation (i.e., which is based on the mean value).
The uncertainty of measurement [6] associated with the estimated PRP content value per ampoule was determined by considering the uncertainty sources that derived from the preparation of the IS stock solution (i.e., purity, weighing and moisture content of the standard), the uniformity of content of ampoules (i.e., homogeneity of filling weight), and the reproducibility of the test method (i.e., experimental part).

The uncertainties associated with homogeneity of filling weight of the WHO 2nd IS for PRP has been derived from the data reported by Mawas and colleagues [7], as NIBSC is the custodian. The uncertainty associated with the purity of the ribitol standard is estimated by the declared contents on the Certificate of Analysis and under the assumption of a rectangular distribution from 99% to 100%.

The uncertainty associated with the moisture content of the ribitol standard was estimated under the assumption of a rectangular distribution from 0.0% to 0.4% (values provided by the participants).

The uncertainty of measurement due to weighing of ribitol standard was determined from calibration data for the balance used at ISS.

The uncertainty associated with the reproducibility of the test method is estimated by the standard error, i.e., $S_{Repr}/\sqrt{N}$, with S_{Repr} obtained by a one way Random ANOVA (see Table 3) and N equal to the number of the participating laboratories (i.e., 17).

Table 3. Robust estimators vs mean value.

		Robust Estimators						
	Shapiro Wilk p-value	Huber's M-Estimator [a]	Tukey's Biweight[b]	Hampel's M-Estimator [c]	Andrews Wave[d]	Alg_A[e]	Median	*Mean Value*
WHO 2nd IS	*0.088*	**4.338**	4.368	4.339	4.371	4.330	4.310	4.290
All results (except the p-value) are in mg/ampoule. In bold the adopted value for content								
a. The weighting constant is 1.339.								
b. The weighting constant is 4.685.								
c. The weighting constants are 1.700, 3.400, and 8.500								
d. The weighting constant is 1.340*pi.								
e. The weighting constant is 1.483.								

3. Results

All 17 laboratories reported their assay results for the WHO 2nd IS for PRP. All 17 laboratories that are included in the data analyses reported below carried out three assays (i.e., test runs) in accordance with the study protocol. With the exception of one laboratory, all other laboratories used the same standard material, namely D-ribitol (adonitol) supplied by Sigma-Aldrich for the calibration curve. This material had a purity greater than 99% (99.5% on average). Only laboratory 13 used the ribitol supplied by Acros Organic. Moisture content of the in-house ribitol standard ranged from 0.1% to 2.4%. When calculating the ribitol concentration, the moisture content was taken into account in case of the highest value.

Table 4 shows a complete listing of values reported by the laboratories. The table presents the results per individual run, the rounded geometric mean (GM) of the three runs, the geometric coefficient of variation (GCV, %) of the three runs, and the overall GM and GCV (%).

Table 4. Results from participating laboratories. Individual results received from the participating laboratories reported in milligram of PRP per ampoule.

Laboratory	Run 1	Run 2	Run 3	GM	GCV (%)
1	4.728	3.981	3.986	*4.22*	*9.92*
2	4.235	4.114	3.993	*4.11*	*2.94*
3	4.459	4.394	4.367	*4.41*	*1.07*
4	4.124	4.258	4.205	*4.20*	*1.61*
5	4.296	4.316	4.257	*4.29*	*0.70*
6	3.893	3.681	3.237	*3.59*	*9.48*
7	4.431	4.848	4.543	*4.60*	*4.65*
8	4.088	4.132	4.213	*4.14*	*1.53*
9	4.446	4.486	4.495	*4.48*	*0.58*
10	4.312	4.322	4.298	*4.31*	*0.28*
11	4.085	4.127	4.062	*4.09*	*0.80*
12	3.476	3.625	3.525	*3.54*	*2.14*
13	4.595	4.238	4.426	*4.42*	*4.05*
14	4.544	4.628	4.670	*4.61*	*1.39*
15	4.622	4.570	4.505	*4.57*	*1.29*
16	5.467	4.041	4.911	*4.77*	*15.41*
17	4.524	4.668	4.569	*4.59*	*1.60*
			GM	4.28	
	Overall		GCV (%)	8.21	

*GM: Geometric Mean; GCV: Geometric Coefficient of Variation (defined by sqrt (e^{ω} -1)*100), with ω = Sample Variance of the ln-transformed results). GCVs for each Lab are single measures of Intra-Lab Precision. These measures should be used assuming a Log-Normal distribution.*

The reported amount of PRP per ampoule ranged from 3.54 to 4.77 mg, with a geometric mean value of 4.28 and a GCV (%) equal to 8.21.

Figure 1 plots the GMs and the individual test results for each of the 17 laboratories, while in Figure 2, the frequency of all 51 determinations of PRP per ampoule is reported in a histogram plot format.

Figure 1. Descriptive plot of the three values of the PRP content in mg per ampoule obtained by the laboratories.

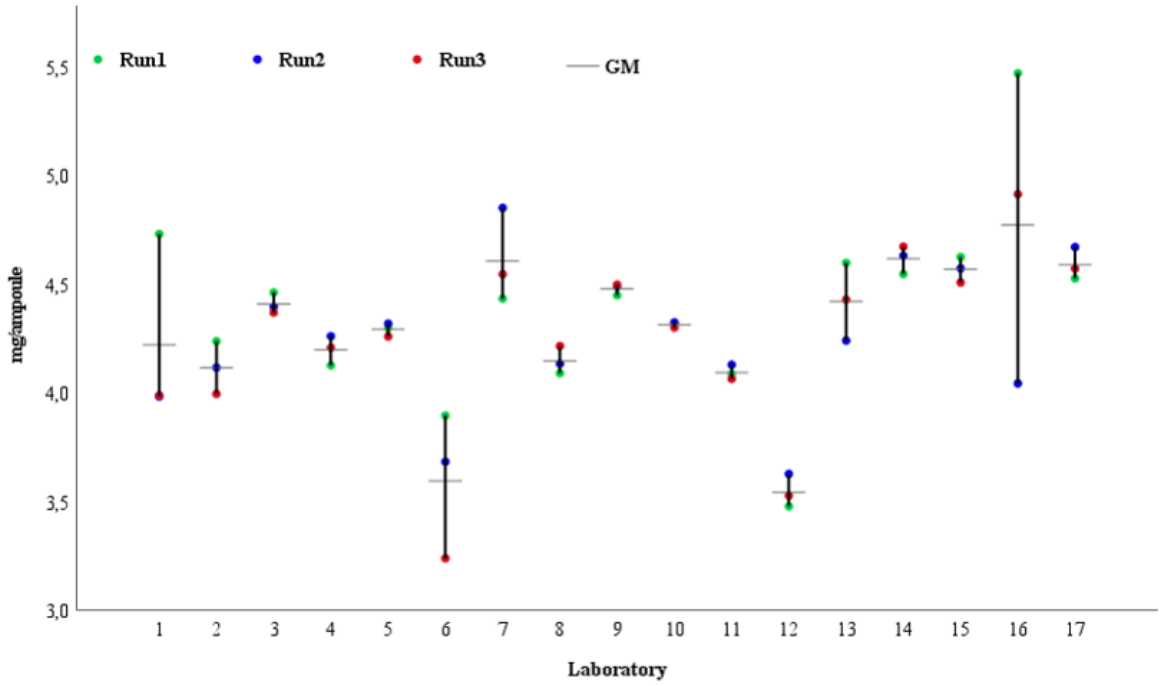


Figure 2 . *Individual results for the three determinations of the PRP content in mg per ampoule obtained by the laboratories. Numbers in the boxes represent the laboratory codes.*

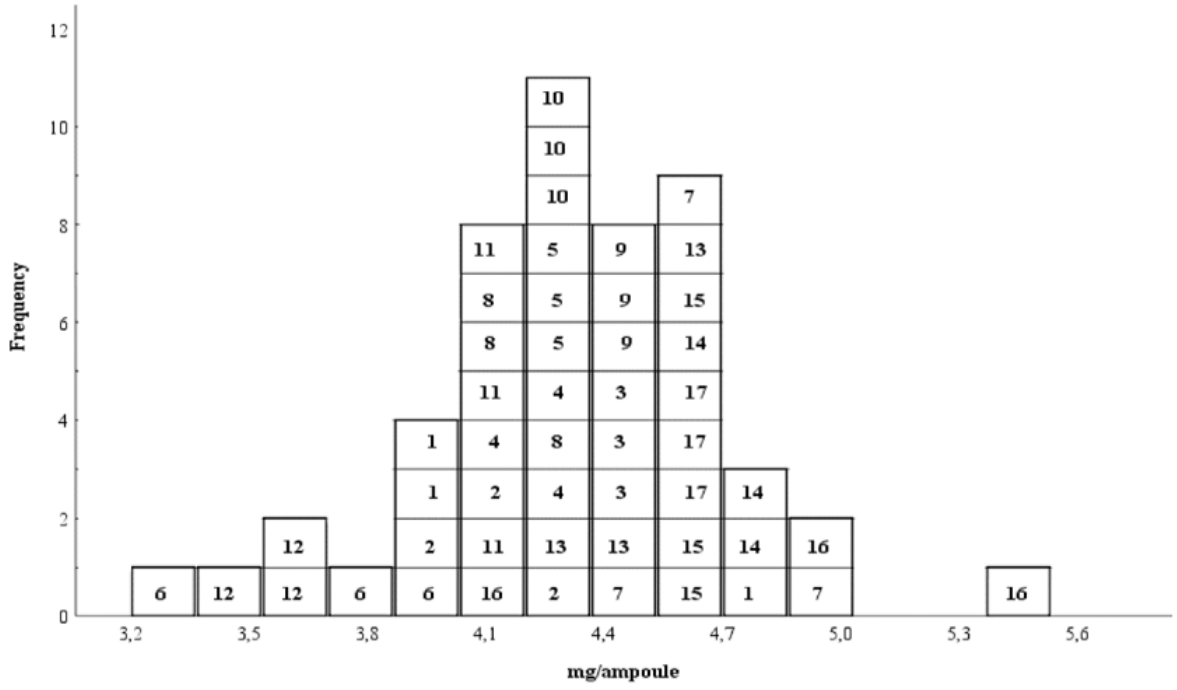
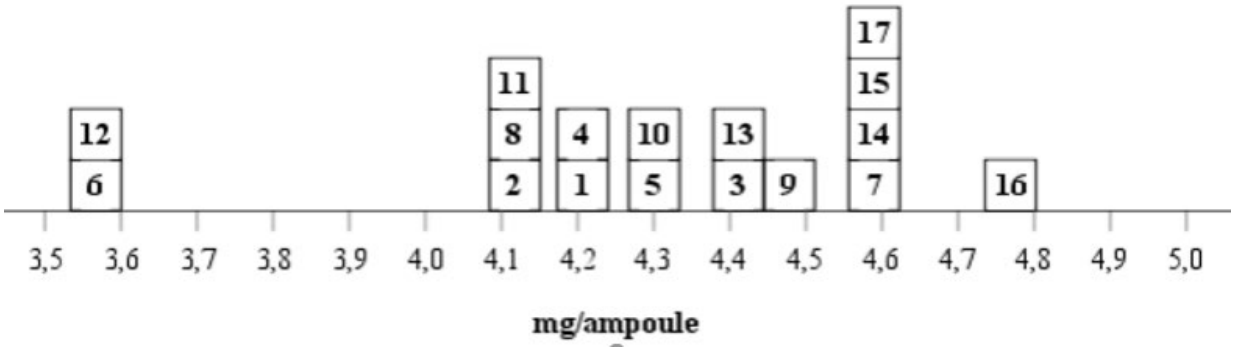


Figure 3 reports, in dot plot format, the distribution of the GMs of the 17 laboratories.

Figure 3. *Geometric means of the PRP content in mg per ampoule of the laboratories. Numbers in the boxes represent the laboratory codes.*



With respect to the precision of the analytical method (Table 2), the decomposition of the total variability (i.e., reproducibility) into the two sources (i.e., intra-laboratory and the inter-laboratory) was in line with what is expected in an inter-laboratory study since the inter-laboratory component, (which should likely be the major contributor but not the only relevant one) accounted for the 64% of the total variability, leaving the intra-laboratory component the remaining 36%.

With respect to detection of outliers, both Dixon and Grubb's tests were not significant whether performed on the 17 GM values (p-values 0.837 and 0.275, respectively) or on the 51 raw data values (p-values 0.056 and 0.071, respectively). Therefore, despite some anomalous values, all the results were included in the final dataset.

Based on the criteria in 2.6 (i.e., the Shapiro-Wilk p-value was <0.5), it was not possible to safely assume a normal distribution, and therefore the classical calculation of mean value and standard deviation was not used. Instead, a robust calculation based on Huber *et al.* [8] was preferred. A list of results from robust estimators compared to the mean value is provided in Table 3.

The standard uncertainty of measurement associated with the estimated content per ampoule of the WHO 2nd IS for PRP was calculated in relative terms, since the sources of variation to be pooled to obtain the combined uncertainty have different units.

Although the contributions from homogeneity of ampoule filling weight, purity, moisture content and weighing of the ribitol standard are definitely less than one fifth of the major contribution (i.e., standard error), and they would not change significantly the estimated measurement uncertainty [9, 10], they are all included in the combination of uncertainty sources (Table 5).

Table 5. *Relative standard uncertainties.*

Uncertainty sources (%)				
Experimental: Standard Error $\frac{u(Repr)}{Repr}$	Purity of ribitol standard $\frac{u(p)}{p}$	Moisture content of ribitol standard $\frac{u(m)}{m}$	Weighing (balance) $\frac{u(w)}{w}$	Homogeneity of filling weight $\frac{u(h)}{h}$
2.18	0.29	0.12	0.10	0.12

Thus, the combined relative standard uncertainty is derived as:

$$\frac{u_x}{x} = \sqrt{\left(\frac{u(Repr)}{Repr}\right)^2 + \left(\frac{u(w)}{w}\right)^2 + \left(\frac{u(p)}{p}\right)^2 + \left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(h)}{h}\right)^2} = 2.21\% = 0.096$$

mg/ampoule

Based on all the statistical analyses, the content of the WHO 2nd IS for PRP per ampoule was obtained by the Huber's robust estimators and corresponds to 4.338 with an expanded uncertainty of measurement equal to ± 0.203 mg/ampoule (relative U = 4.7%; k= 2.12, which corresponds to an approximate 95% level of confidence).

4. Discussion and conclusions

The quantification of the Hib conjugate saccharide in both forms (i.e., total and free saccharide) is a critical and mandatory release test for Hib containing vaccines. The determination of the total and free PRP content needs to be performed by both the manufacturers and the national control laboratories in charge of the regulatory oversight of the vaccine. When combination vaccines containing whole cell pertussis, for example, pentavalent vaccine (DTwP-HepB-Hib), are tested for the content of total and free Hib saccharide, an acidic hydrolysis step is necessary due to the interference by the whole cell pertussis component [3, 11].

The availability and appropriate use of reference preparations in independent testing is of critical importance for the interpretation of the results and allows for better comparison of biological measurements worldwide and the normalization of test results between different laboratories.

In 2013, an international collaborative study for the establishment of the WHO 2nd IS for PRP was carried out [1]. Based on the ribose assays performed by 11 participating laboratories, a PRP content of 4.904 ± 0.185 mg/ampoule (i.e., expanded uncertainty calculated using a coverage factor of 2.23 which corresponds to an approximate 95% level of confidence) was assigned to the WHO 2nd IS for PRP [7].

In the 2013 collaborative study [7], a similar value for the content per ampoule of WHO 2nd IS for PRP (i.e., 4.940 mg/ampoule) was obtained by nine laboratories performing HPAEC-PAD after the alkaline depolymerization method and using the 1st WHO IS as a standard.
A correlation between the orcinol method (ribose) and HPAEC-PAD - alkaline depolymerization method was also verified by van der Put and colleagues [12] who stated that for the down-stream processing samples, *"it was found that the correlation between the results obtained by HPAEC-PAD specific quantification of the PRP monomeric repeat unit released by alkaline hydrolysis, and those from the orcinol method was high (R^2 = 0.8762)."*

The present collaborative study was specifically designed to evaluate the PRP content per ampoule by HPAEC-PAD based on the measurement of ribitol obtained after acid hydrolysis [3]. In recent years, various laboratories have obtained a lower value for the PRP content of the WHO 2nd IS for PRP when applying this test protocol, a method which employs a calibration curve based on D-ribitol. The outcome of the current collaborative study confirmed that the measured content of PRP per ampoule is lower when determined by HCl acid hydrolysis followed by HPAEC-PAD than when measured by the orcinol method.

Bardotti and colleagues [11] also found on average that a method using trifluoroacetic acid hydrolysis followed by HPAEC-PAD, led to a slightly lower value of PRP than was obtained using the orcinol (ribose) assay. The reason for this was not given. A similar result was observed when the WHO 1st IS was tested by HPAEC-PAD after trifluoroacetic acid hydrolysis [4].

The results of the current collaborative study demonstrate that a value of 4.338 ± 0.203 mg/ampoule was obtained for the WHO 2nd IS for PRP when quantifying the content according to the acid hydrolysis protocol reported in Rosskopf and colleagues [3].

Given that the acid hydrolysis method is needed when testing vaccines with a whole cell pertussis component, discussions should be initiated to evaluate whether method-dependent adjustments are required for the international standard to ensure accurate PRP quantitation when different methods are employed.

Authors Contributions:

Conceptualization, Christina von Hunolstein, Andrea Gaggioli and Ute Rosskopf; Formal analysis, Christina von Hunolstein and Andrea Gaggioli; Funding acquisition, Ute Rosskopf; Methodology, Andrea Gaggioli; Project administration, Ute Rosskopf; Supervision, Ute Rosskopf; Writing – original draft, Christina von Hunolstein, Andrea Gaggioli and Ute Rosskopf; Writing – review & editing, Christina von Hunolstein.

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Disclaimer: The authors alone are responsible for the views expressed in this article and they do not necessarily represent the views, decisions or policies of the institutions with which they are affiliated.

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Appendix A

List of participants, in alphabetical order by country.

Country	Name	Institution
Bangladesh	Dr Mohammad Mainul AHASAN	Incepta Vaccine Ltd.
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India	Dr Dipankar DAS	Bharat Biotech International Ltd.
India	Dr Srinivas KOSARAJU	Biological E. Limited
India	Mr Jaipal MEENA Mr Ade Ajay KUMAR	National Institute of Biologicals (NIB)
India	Dr Sunil GAIROLA	Serum Institute of India PVT LTD
India	Dr Radhakrishnam Raju MANTENA	Sanofi Healthcare India Private Limited (formerly: Shantha Biotechnics Limited (A Sanofi Company))
Indonesia	Dra Togi Junice HUTADJULU Ms Elisabeth ARISETININGSIH	National Quality Control Laboratory of Drug and Food (NQCLDF) National Agency of Drug and Food Control
Indonesia	Dr Dori UGIYADI Ms Lin SUSANTI	PT Bio Farma (Persero)
Italy	Dr Cristina PEZZELLA Dr Luisa RALLI	Istituto Superiore di Sanità (ISS)

Mexico	Ms Imelda Rocío GUZMÁN CERVANTES Ms Laura MUNGUÍA Dr Guillermo VEGA RODRIGUEZ	Commission for Analytical Control and Expansion of Coverage (CCAYAC) Federal Commission for Protection against Health Risks (COFEPRIS)
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South Africa	Dr Ruan ELLS	National Control Laboratory for Biological Products (NCLBP)
Thailand	Dr Supaporn PHUMIAMORN Mrs Wereyarmarst JAROENKUNATHUM	Institute of Biological Products (IBP), Department of Medical Sciences, Ministry of Public Health
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