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Quantitative assessment of C-polysaccharide in capsular polysaccharides of *Streptococcus pneumoniae* by ^{31}PNMR

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ABSTRACT

Capsular polysaccharides of *Streptococcus pneumoniae* are key components of commercially available anti-pneumococcal vaccines; meanwhile C-polysaccharide is considered an impurity. World Health Organization recommends a strict control over the presence of this biomolecule due to the possibility of introducing an undesired response. An alternative way for assessing this impurity is focused on detect the phosphocholine residues by means of quantitative ^{1}H-NMR. This could be tricky due to the amounts of this substituent may vary generating two C-polysaccharides forms. In this work we propose an improved quantitative NMR methodology based on ^{31}P-NMR for the quantification of C-polysaccharide on capsular polysaccharide preparations. The technique also focuses on phosphocholine but, conversely to above-mentioned methods, allows to discriminate between phosphocholine linked in different positions. The methodology was run on samples of eleven vaccine serotypes, including seven with phosphate groups. From a rational acceptance criterion of 10 wt%, the method allows to quantified from 30 μg of the impurity in 3 mg of total polysaccharide (1 wt%) with a signal/noise ratio of 16:1. Repeatability and intermediate precision evaluation showed a relative standard deviation of 3.33 % and 8.34 % respectively. Additionally, the method provides information about structural identity of phosphate contained in capsular polysaccharides and C-polysaccharide species. This constitutes a new contribution from the NMR that highlights the power of these techniques for assessing imperative parameters in carbohydrate-based vaccines.

1. Introduction

Streptococcus pneumoniae (*Sp*) is a wide bacteria group involved in several human infections like pneumonia, meningitis, otitis media, bacteraemia and sepsis representing an important morbidity and mortality worldwide [1]. Capsular polysaccharides of *Sp* (*Sp*-Ps) are biomolecules with molecular weights higher than 500 KDa [2]. They have been key antigens of different vaccine alternatives for around 40 years [3]. *Sp*-Ps antigens are produced by fermentation process of *Sp* strains. C-polysaccharide (C-Ps) is a common cell-wall-associated antigen present as a persistent impurity of most *Sp*-Ps preparations [4]. World Health Organization recommends a strict control over the presence of this biomolecule due to the possibility of introducing an undesired response together with the one induced by the main antigens [5,6].

The structure of C-Ps consists on a phosphodiester-linked linear pentasaccharide repeating unit (RU) with up to two phosphocholine (P-Cho) substituents (Fig. 1) [7]. Congruently, have been reported two C-Ps species depending of P-Cho content (C-Ps1 and C-Ps2). Those species differs among *Sp* serotypes [8].Thus, C-Ps is built up by current residues (eg. N-acetyl, 6-deoxy sugars, phosphate diester) present in most *Sp*-Ps. Moreover, molecular weights, between C-Ps and *Sp*-Ps, do not greatly differ. For these reasons, full elimination of this impurity from *Sp*-Ps preparations is a complex task. These factors become the assessment in a serious analytical challenge, aggravated by the existence of two possible C-Ps species.

A few methods have been reported to assess the content of C-Ps. Proton NMR spectrum of C-Ps shows a very sensitive signal from nine proton nuclei of P-Cho at δ 3.23 ppm. It uses to be an isolated signal from the very complex achieved matrix of signals for many *Sp*-Ps spectra [9]. These evidences have allowed to use quantitative proton NMR (qHNMR) for assessing C-Ps but limited. Some proton spectra (eg. *Sp*-Ps type 5) do not allow to use the method due to the presence of overlapped signals at δ 3.23 ppm. Furthermore, it is not able to discriminate between the two different species of C-Ps. Xu et al. reported two options focused on the separation between free C-Ps from the one linked to *Sp*-Ps [10]. The first one comprises a chromatographic assay based on High Performance Size Exclusion Chromatography (HPSEC) and the second is supported by a complex measuring self-diffusion rates using NMR. Other techniques

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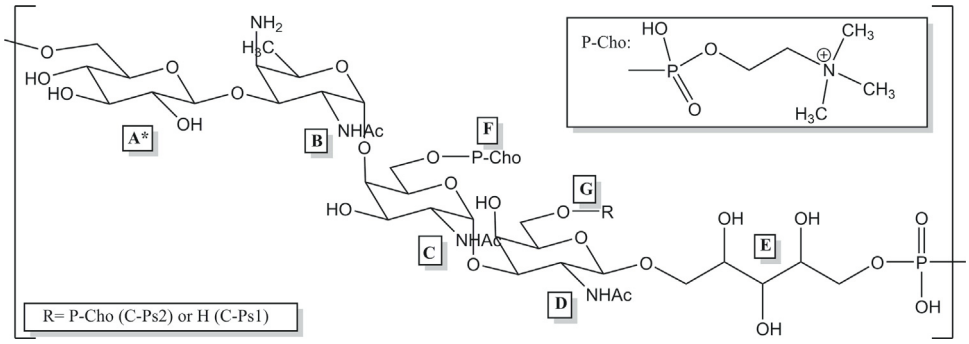

Fig. 1. Structure of C-Ps species. * residue A also has been reported changed by β-D-Gal*p* for *Sp*-Ps 5.

overcome the P-Cho species conflict by focusing the quantification in other chemical group. Talaga et al. have reported a method based on High Performance Anion Exchange Chromatography using Pulse Amperometric Detector (HPAEC-PAD) [11]. In this case, ribitol residue released from C-Ps by acid hydrolysis is quantified. But this implies that ribitol containing *Sp*-Ps (eg. serotypes 6A, 6B, 10A) cannot be assessed. It provides another chromatographic alternative to solve the conflict of C-Ps species.

We propose an improved and flexible alternative for assessing the absolute or relative concentration of this contaminant. The method is supported by quantitative ^{31}P-NMR (qPNMR). It also allows classification of C-Ps species by evaluating their structural identity. Previously some alternatives of qPNMR had been used for quantification of phosphocholine and glycerophosphocholine [12]. Moreover, similar methods have been proposed for phospholipids and many other phosphorus-based biomolecules [13].

2. Materials and methods

2.1. Samples preparation

Samples of eleven *Sp*-Ps were supplied by the Technological Development Direction, Finlay Institute of Vaccines, Havana. The samples analyzed comprise the most epidemiologically relevant *Sp* types (1, 5, 6A, 6B, 9 V, 14, 15B, 18C, 19A, 19 F, 23 F).

Each sample was prepared by dissolving 3 mg of *Sp*-Ps in distilled water (0.4 mL) for 5 mm NMR tubes and 15 mg of *Sp*-Ps in distilled water (2 mL) for 10 mm NMR tubes. Five additional samples were prepared in 10 mm NMR tubes with the serotype 14 *Sp*-Ps to verify the precision of the method.

2.2. NMR setup

NMR analyses were carried out at 25 °C on a Bruker Avance DPX250 at the ^{31}P frequency of 101.26 MHz. Two probes were used to verify the performance of the proposed method: most experiments were run on a 5 mm Quadruple Nuclear Probe (^{1}H/^{13}C/^{31}P/^{19}F) z-axis gradient probe (QNP) and a 10 mm Broad-Band direct Observation probe (BBO), for supporting intermediate precision experiments. The inversion–recovery experiments was run with a list of 30 values of mixing times between 0.2 and 15.0 s for relaxation time calculation and 90 s of recycled delay [14]. The proposed qPNMR were set with 3 s of relaxation delay, a recycled time of 6 s, and the pulse duration P0 was adjusted with a calculated Ernst angle of 84.8 degrees [15,16]. In addition, proton decoupling and 4096 scans were also set for ensuring an appropriated signal to noise ratio (s/n) [17]. Signal processing was made under TopSpin suite, ver. 1.3 and MestreNova software, ver. 9.0. Apodization process was carried out by an exponential function (line broadening =2.0 Hz). Integration analysis was based on the line-fitting procedure of up to three phosphorus nuclei from PPh_3 (external reference), *Sp*-Ps (main compound) and P-Cho residues from C-Ps (impurity).

2.3. Compounds and methods for referencing

The qHNMR experiments were set with 60 s of relaxation delay for calibrating external references [18]. The 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP-d_4) signal was referenced at δ 0.00 ppm to calibrate the proton spectrum. The triphenylphosphine (PPh_3) signal was referenced at δ -5.16 ppm for calibrating the chemical shift of ^{31}P spectra. One co-axial capillary tube with PPh_3 99.9 % in hexadeuterated dimethylsulfoxide (DMSO-d_6) was used as external reference (r) and to provide the deuterium signal for locking the system (Fig. 2a) [19]. For the 5 mm probe the reference was prepared with 2 mg of PPh_3 in DMSO-d_6 (0.15 mL). For the 10 mm probe a 5 mm NMR tube was inserted with 5 mg of PPh_3 in DMSO-d_6 (0.6 mL). The actual content of each reference was calibrated against TSP-d_4 (1 mg) by qHNMR (Fig. 2b).

3. Results and discussion

3.1. Scope and description of the method

The reported structure for the RU of C-Ps is composed by a backbone of tetrasaccharide-ribitol. It includes up to two residues of P-Cho (Fig. 1) [8]. C-Ps2 variant shows two P-Cho ("F" and "G") associated to positions 6 of N-acetyl galactoses "C" and "D". The other alternative, although less observed, links only one P-Cho "F" substituent to N-acetyl galactose "C" residue (Fig. 1) [10]. In addition, Vialle *et.al.* reported an interesting structural change of residue "A" from β-D-Glc*p* to β-D-Gal*p* for serotype 5. Fig. 1 shows the structure of P-Cho [12]. The identity of C-Ps species by ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR was studied deeply by Karlsson et al. [8]. Fig. 3a and ESI 1 illustrates the assignments for two species of C-Ps on ^{31}P-NMR spectrum(Fig. 3a, ESI 1). Signals from "F", "G" and the used reference provide quantitative information about the content for each C-Ps species.

The scope of this method is to provide an efficient alternative to face the assessment of C-Ps even in presence of a species able to change the weight of RU. It begins for acquiring the Free Induction Decay (FID) under quantitative conditions. This means that the experiment ensures recovering of more than 99.9 % of equilibrium magnetization before each pulse sequence [20]. Quantitative NMR is widely used as a primary ratio analytical method [20]. In addition, the parameters that become routine in quantitative NMR were validated by Malz and Jancke [16]. Several acquisition parameters were adjusted for achieving this condition. Firstly, longitudinal relaxation times (T1) were calculated for all involved nuclei by Inversion-recovery experiments [14]. Table 1 shows the obtained

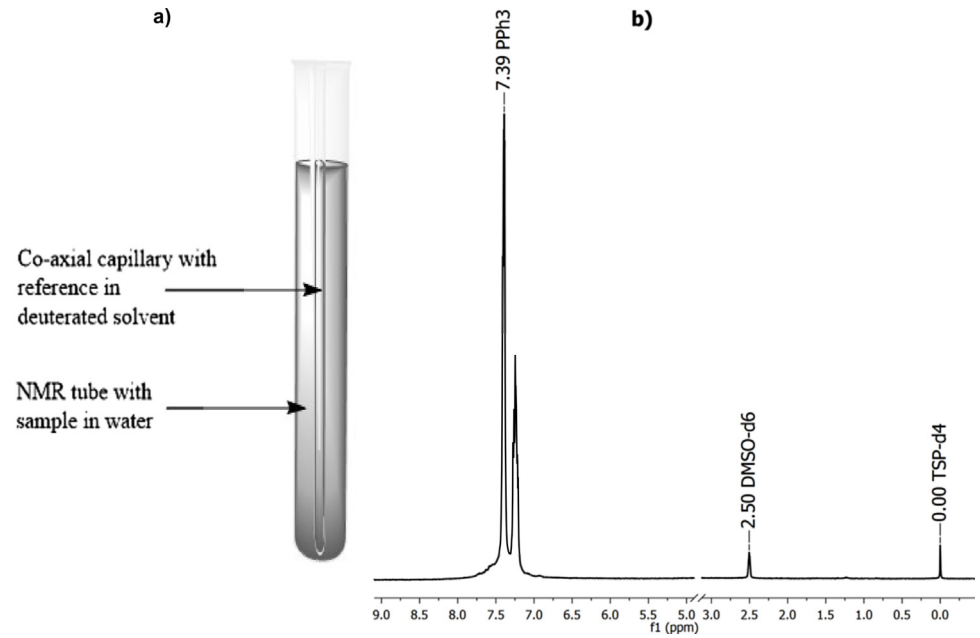

Fig. 2. Referencing. a) Scheme of the sample and reference into the NMR tube. b) proton qNMR spectrum for calibrating the references.

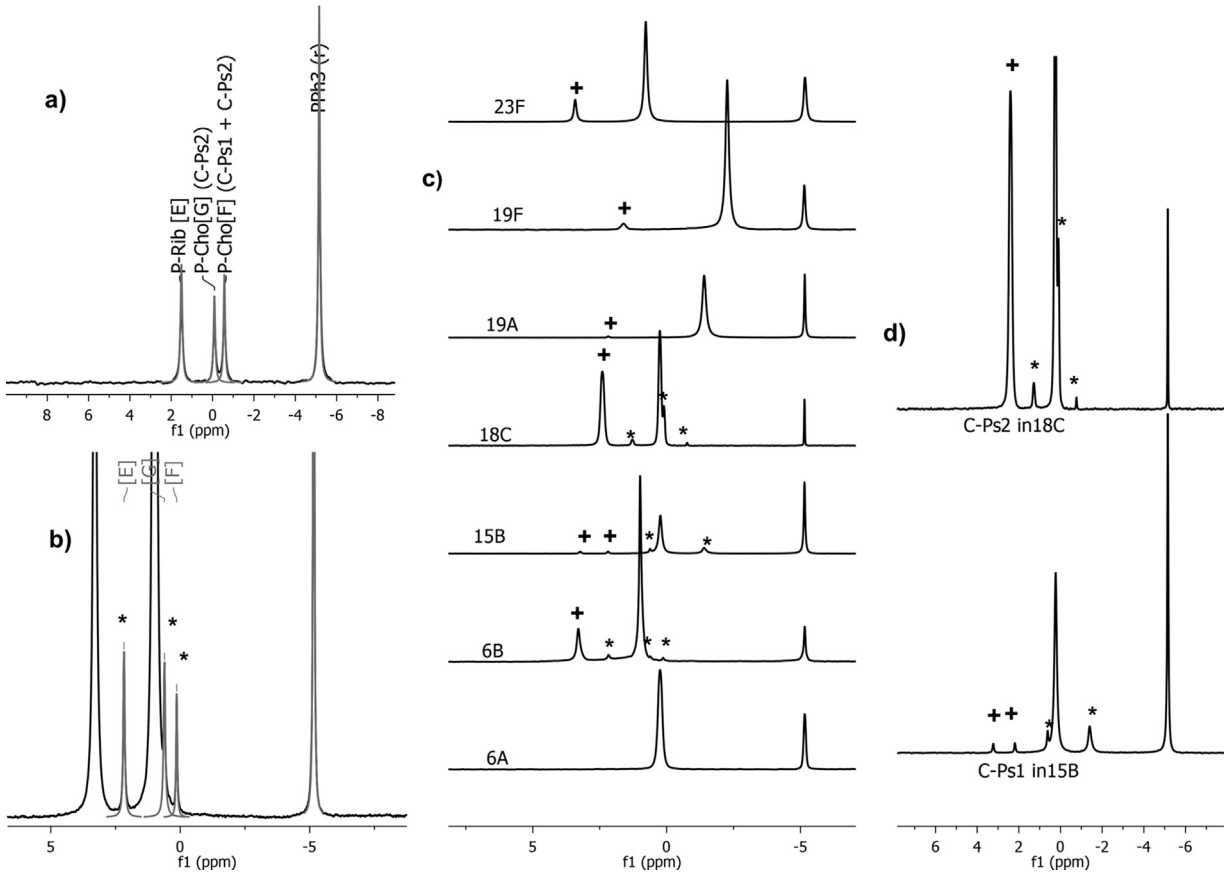

Fig. 3. qPNMR for assessing C-Ps. a) qPNMR for serotype 14. C-Ps signals assignment. b) qPNMR for serotype 6B. Line fitting of the C-Ps signals. c) qPNMR of phosphorylated *Sp*-Ps. d) Presence of both C-Ps species. Symbols: *[C-Ps signals], +[phosphate salts].

T1 values for all phosphorus signals. To calculate the Ernst angle [15] a recycled time of 6 s was set by fixing a moderate relaxation delay (d1) of 3 s and a value of 200 ppm of spectral width (sw). With the highest T1 found and the recycled time, the optimized Ernst angle was 84.8 degrees. This calculation was suggested by Ernst et al. [15]. It provides an optimized value for the pulse angle that ensure quantitative conditions while economize total experiment time.

Once the qPNMR FID are processed, the obtained spectra allow to assess the content of C-Ps and phosphorylated *Sp*-Ps (eg. 6A, 6B,

Table 1
Results of inversion-recovery experiment and signal assignments for qPNMR spectra.

	Signal	Shift (ppm)	T1 (s)
	6A	0.25	1.14
	6B	0.99	1.10
	15B	0.23	1.45
Sp-Ps (phosphorylated)	18C	0.26	1.50
	19A	−1.40	1.27
	19 F	−2.26	1.20
	23 F	0.79	1.60
	Rib E	1.41	1.60
C-Ps*	P-Cho F	−0.66	2.50
	P-Cho G	−0.17	1.90
Reference	PPh3	−5.16	2.00

* C-Ps results are based on *Sp*-Ps type 14

15B, 18C, 19A, 19 F, and 23 F). In addition, it provides an alternative criterion of the structural identity for each C-Ps (C-Ps1 or C-Ps2) and for phosphorylated *Sp*-Ps. The method for measuring the areas of the target signals from reference and analytes is focused on a procedure of line fitting. It was specially valuable for the C-Ps assessment of phosphorylated *Sp*-Ps where the lines of interest were localized too close (Fig. 3b, ESI 2).

3.2. Specificity and limit of quantification

The specificity assay was focused on demonstrating no interference of other signals. Some of the assessed samples of *Sp*-Ps arrived to our lab came accompanied with residual phosphate salts. Signals for these salts were localized above δ 1.60 ppm. The other possible interference came from phosphate that belong to the RU structure of the phosphorylated *Sp*-Ps used in this study (6A, 6B, 15B, 18C, 19A, 19 F and 23 F) (Fig. 3c, ESI 3). Table 1 shows the assignments for all the relevant nuclei (C-Ps, phosphorylated *Sp*-Ps and reference). The concentration of phosphate salts in the sample can affects the actual shift for the relevant signals of the spectrum. Karlsson et al. [8] studied the shielded effect caused by the increment of temperature and concentration of phosphate reference. Despite this effect, the shift difference between the signals from "E" and "F" remains always constant at the same temperature ($\Delta\,\delta$ 2.07 ppm at 25 °C) (ESI 1). It can be used as an additional criterion to detect C-Ps species in most *Sp*-Ps spectra. In the case of serotype 5, an expected unshielded of $\Delta\ \delta$ 0.24 ppm for signal of "E" was observed due to the change of β-D-Glc*p* by β-D-Gal*p* [21] (data not shown). However, the modification does not affect the results of this assay. Most *Sp*-Ps NMR lines do not match with the signals of P-Cho residues. Only 6B and 18C serotypes show a close overlap. However, the line fitting procedure can solve the area assessment for these signals (Fig. 3b, ESI 2). In the event of a high overlap, an alternative can be to change the selected C-Ps signal for the integration process.

The studied polysaccharides demand a quantitative assay able to assess C-Ps according to an acceptance criterion of up to 10 wt% of the impurity from the total polysaccharide content. The limit of quantification was verified by using the s/n of the smallest amount of C-Ps found. The sample of *Sp*-Ps serotype 5 (3 mg) yielded 30 μg of the impurity. It represented 1 wt% of C-Ps. The ICH (International Committee on Harmonization) guidelines for validation on analytical procedures suggests a minimum of 10:1 s/n for estimating the limit of quantification on methods that generate electronic noise [22]. The achieved s/n of the used C-Ps signal for aforementioned sample was 16:1.

3.3. Calculations from the NMR spectra

Eq. (1) is the main formula for absolute qNMR method. Variables A and N correspond to the areas and amount of nuclei involved respectively, for the evaluated signals, analyte (a) and external reference (r). In addition, M and m represents molar weight and actual mass, respectively. Due to the fact that all qPNMR signals involve only one phosphorus nucleus, Eq. (1) becomes Eq. (2).

$$m(a) = \frac{A(a)}{A(r)} \times \frac{N(r)}{N(a)} \times \frac{M(r)}{M(r)} \times m(r) \quad (1)$$

$$m(C-P_s) = \frac{A(C-P_s)}{A(PPh_3)} \times \frac{M(C-P_s)}{M(PPh_3)} \times m(PPh_3) \quad (2)$$

Either, integral A(E) or A(F) related to "E" or "F" signal respectively can be used in the assay of the C-Ps species present. We selected the former "A(E)" as first choice since it was an isolated line in most spectra. In addition, it is not a side chain that theoretically could be sliced out from the structure. Integral A(G) related to "G" signal only describes the content of C-Ps2. Thus, the difference between integrals A(F) and A(G) is a key alternative to use when relevant amounts of both C-Ps species are involved in the same *Sp*-Ps sample. Even thought, no relevant stoichiometric differences were observed in our work. Actually, we only found C-Ps1, in the sample of *Sp*-Ps 15B (Fig. 3d, ESI 1).Moreover, from the eleven evaluated samples only five (*Sp*-Ps 5, *Sp*-Ps 6B, *Sp*-Ps 14, *Sp*-Ps 15B and *Sp*-Ps 18C) reveal moderate amounts of C-Ps (1–7 wt%)(ESI 4). This may be related with the selected *Sp* strains for *Sp*-Ps fermentation process per serotype and the purification of the capsular polysaccharide preparations. Moreover, it is well known that all streins are not producers of high amounts of C-Ps [21].

Table 2
Summary of results for performance parameters verification.

Parameter assessed	Acceptance criteria	Results
Limit of quantification	s/n ≤ 10:1	16:1 s/n
Precision repeatability	RSDrepeteability ≤ 5 %	3.33 % RSD
Precision intermediate	RSDprecision ≤ 10 %	8.34 % RSD

3.4. Intermediate precision and repeatability

The precision at repeatability and intermediate precision levels were verified as final criteria of performance. Due to the nature of the deconvolution process, areas of the involved signals in this method show a moderate variability [23,24]. Precision assessment was based on Relative Standard Deviation (% RSD) calculation for the results of six replicates from the same *Sp*-Ps (type 14), measured under repeatability conditions (% RSD repeatability). In addition, six determinations were carried out with another probehead (10 mm BBO) as variation to assess intermediate precision [22]. The generated data were combined with the repeatability assay in order to calculate the % RSD under inter-instrument variations (intermediate precision) (% RSD precision) [22]. The resultant values of % RSD repeatability and % RSD precision were 3.33 % and 8.34 %, respectively (Table 2).

3.5. Final remarks for application to anti-pneumococcal vaccines

The C-Ps is an impurity that always will be involved in the anti-pneumococcal carbohydrate-based vaccines. The development of this method for assessing the content of C-Ps is strongly suggested for demonstrating that each vaccine lot includes only controlled amounts of this impurity. The proposed qPNMR method for this evaluation allows to cover at least the most relevant *Sp*-Ps but it can be easily extended to others serotypes.

To use this method, it is advised to take always similar amounts of total polysaccharide previously assessed by an appropriate analytical method (*eg.* Orcinol [25] or Biphenyl [26]). It will ensure that the possible shield effect comes only by the phosphate salts content (if the sample includes it). The method allows to assess the

content of C-Ps1, C-Ps2 or both. In addition, it allows to re-evaluate the content for phosphorylated *Sp*-Ps. These values of concentration for *Sp*-Ps and C-Ps can provide the required final percentage of C-Ps contained in by each polysaccharide lot. For *Sp*-Ps that do not include phosphorus in the structure the content should be the previously obtained value by other analytical method (eg. Orcinol [25] for total polysaccharide content).

Several NMR techniques had found purpose in polysaccharide evaluation, such as, structural identity confirmation [27,28], labile side chains determination [29] and impurities quantification, mainly based on ^{1}H nuclei [30]. Many of these have been even recommended by WHO for vaccines quality control [5]. New applications are still been developed. The inhere proposed ^{31}PNMR method increases the competence of NMR as an important tool for assessing imperative parameters in carbohydrate-based vaccines.

CRediT authorship contribution statement

Raine Garrido Arteaga: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Bárbara Baró Vicet:** Validation, Resourses, Formal analysis, Investigation. **Jean Pierre Soubal:** Writing - review & editing. **Darielys Santana Mederos:** Formal analysis.

Declaration of Competing Interest

There are no conflict of interests to declare

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113670.

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