Vaccine 32 (2014) 2819–2828

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Quantitative high throughput analytics to support polysaccharide production process development

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ARTICLE INFO

Article history:
Available online 25 February 2014

Keywords:
Carbohydrates
Capsular polysaccharides
Phenol sulphuric acid assay
Sugar quantification
Endotoxin assays
High throughput process development

ABSTRACT

The rapid development of purification processes for polysaccharide vaccines is constrained by a lack of analytical tools current technologies for the measurement of polysaccharide recovery and process-related impurity clearance are complex, time-consuming, and generally not amenable to high throughput process development (HTPD). HTPD is envisioned to be central to the improvement of existing polysaccharide manufacturing processes through the identification of critical process parameters that potentially impact the quality attributes of the vaccine and to the development of *de novo* processes for clinical candidates, across the spectrum of downstream processing. The availability of a fast and automated analytics platform will expand the scope, robustness, and evolution of Design of Experiment (DOE) studies.

This paper details recent advances in improving the speed, throughput, and success of in-process analytics at the micro-scale. Two methods, based on modifications of existing procedures, are described for the rapid measurement of polysaccharide titre in microplates without the need for heating steps. A simplification of a commercial endotoxin assay is also described that features a single measurement at room temperature. These assays, along with existing assays for protein and nucleic acids are qualified for deployment in the high throughput screening of polysaccharide feedstreams. Assay accuracy, precision, robustness, interference, and ease of use are assessed and described. In combination, these assays are capable of measuring the product concentration and impurity profile of a microplate of 96 samples in less than one day. This body of work relies on the evaluation of a combination of commercially available and clinically relevant polysaccharides to ensure maximum versatility and reactivity of the final assay suite. Together, these advancements reduce overall process time by up to 30-fold and significantly reduce sample volume over current practices. The assays help build an analytical foundation to support the advent of HTPD technology for polysaccharide vaccines. It is envisaged that this will lead to an expanded use of Quality by Design (QbD) studies in vaccine process development.

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1. Introduction

The worldwide vaccine market is experiencing unprecedented growth. In 2009, the worldwide vaccine market was valued at $22.1 billion and was expected to grow to >$40 billion by 2015 [1,2]. The strength of the vaccines segment has revived investment in vaccine research and development and has led to numerous vaccine candidates entering the industrial development pipeline [3]. Multivalent polysaccharide vaccines will form an increasingly prominent share of future approved vaccines [3–5]. This class of vaccines incorporates several different polysaccharide serotypes in the drug product in order to confer broad protection against the diverse strains of infectious agents.

Manufacturing processes for multivalent polysaccharide vaccines are complex and expensive. Several different fermentation and purification processes must be developed and operated to produce material for a single product. Fortuitously, commonalities across a pathogen's polysaccharide serotypes reveal untapped potential for the creation of modular development and production approaches. A directed, modular approach to the rapid development of production processes for capsular polysaccharides at the micro-scale would greatly enhance productivity and speed the development of novel vaccines. This forms the motivation for the present study.

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http://dx.doi.org/10.1016/j.vaccine.2014.02.034
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1.1. Capsular polysaccharide biology

Capsular polysaccharides (CPS) form the outer layer of bacterial cell envelopes. These heterogeneous polymers exhibit vast structural diversity but are generally composed of monosaccharides joined through glycosidic and phosphodiester bonds into repeating oligosaccharide units [6]. Native capsular polysaccharides comprise tens to thousands of oligosaccharide 'monomers' linked together, ranging from kDa to MDa in molecular weight (MW). The underlying oligosaccharide repeat unit can be specific to particular bacterial species, to differentiated serotypes within a species, or even to structurally differentiated strains [7]. While the particular constitutional monosaccharide(s) are often conserved within a species, the oligosaccharide structure can differ markedly. In addition, due to the large number of hydroxyls on each oligosaccharide, covalent bonds can form at an array of locations, resulting in a highly complex and variable macromolecular structure.

1.2. High throughput processing impediments

Currently, high throughput processing development (HTPD) of polysaccharide vaccines is rarely practiced, primarily due to a lack of suitable high throughput analytics. Most of the pertinent published analytical literature encompasses methods assessing small molecules, proteins, or nucleic acids. Limited research has been presented on the high throughput quantitation of polysaccharides. Moreover, the compatibility of polysaccharides with various high throughput technology and the implicit methodological assumptions have not been tested. A major bottleneck is the identification of relevant product assays that can be performed in a highly automated fashion and that are resilient to the diverse conditions typically found in developmental studies.

1.3. Unique requirements for purification process development

Assays to support purification process development have contrasting demands compared to those for release testing. In purification development, feedstocks are usually in short supply so volume requirements for the assays must be minimal. Second, the assay should ideally be microplate-based so as to facilitate parallel processing. The assays should be simple, straightforward and rapid as multiple assays may be performed to support a single screen. Integration with robotic liquid handling systems and the typical room temperature environment of the robots is also desired. Another significant issue is assay interference because in-process samples typically have high levels of impurities that can interfere with assays. When combined with lower polysaccharide titres than are found in pure drug substance, this puts stringent demands on assay robustness. Fortunately, the requirements for accuracy are less stringent than for a release assay. Moreover, as purification HTPD favours the screening of purification conditions in a 96-well microplate, the precision of an assay is often more important than the accuracy. The results from a single screen are compared only within the screen, and the best conditions are subsequently verified with a scaled up process.

1.4. Analytics

1.4.1. Current practice

Most vaccine release assays are specified by the World Health Organization (WHO) or Pharmacopoeia organizations and have not changed much in decades. The relevant established assays and key drawbacks are highlighted in Table 1. While these assays are suitable and highly accurate for the release testing of highly concentrated, relatively pure formulations, they are poorly suited for integration in a high throughput purification context.

Table 1
Release assays for polysaccharide-containing vaccines [8–15,43].

Target	Assay	Limitations
Polysaccharide	Subunit	Highly specific, typically time-consuming, requires large amounts of polysaccharides, and subject to substantial interference
Protein	Lowry	Incompatible with most detergents
Endotoxin	Rabbit injections	Animal models, expensive, time-consuming
Endotoxin	LAL	Interference of enzyme cascade, fickleness of kinetic measurements, requirement of specialized equipment

Typical vaccine release specifications and in-process concentrations provide insight into analytical requirements. The European Pharmacopeia and WHO release specifications for protein and DNA levels in polysaccharide-containing vaccines do not require exhaustively sensitive analytics. With release specifications generally ≤1–3% (w/w CPS) protein or DNA and ≤100 IU/mg polysaccharide for endotoxin, detecting minute quantities of impurities is not necessary [8–15]. The conclusion is similar for titre measurements, where in-process polysaccharide concentrations typically range from 0.1 to 10 mg/mL. In this context, quantifying much less than 0.01 mg/mL holds diminishing value. This latter point is driven in part by the modest equilibrium purification factors that can be expected from a single stage purification experiment performed in a microwell.

1.4.2. Sugar assays

The consistent measurement of polysaccharide quantity may prove the most challenging measurement. The published assays available for capsular polysaccharides typically quantify a specific subunit of the repeating structure. Hence, each capsular polysaccharide or subset of serotypes tends to have a custom method for polysaccharide quantification. Many of these assays involve complex colorimetric procedures but research groups have found alternative approaches for measuring polysaccharide quantity [16–18].

Several authors have recognized the analytical bottleneck posed by sugar quantitation and devised high throughput methods. Methods based on anthrone have been developed and further scaled-down to microplates [17–19]. This assay's limitations include reagent instability, poor reactivity with pentoses and methylated sugars, interference by process substance such as phenol, and issues with consistency [20,21]. Refractive index has been used in conjunction with HPLC for many years to estimate sugar content. However, without the added purification and normalization provided by chromatography, this approach is exceedingly sensitive to background interference. Other methods involving phenol, 1-napthosulfonate, or aniline phthalate/trichloroacetic acid have been proposed but suffer from toxicity, interference, and limited reactivity with ketoses, respectively [20].

The phenol sulphuric acid method (PHS) is perhaps the most promising assay for integration with high throughput screening. This method is based on a colorimetric product formed when phenol, sulphuric acid, and sugar are reacted and was first described by Dubois et al. in 1951 [22]. This assay is broadly applicable and measures hexoses and pentoses in a variety of oligosaccharides, making it useful for quantifying neutral sugars [20,23]. The broad carbohydrate specificity of this assay underlies its attractiveness but the measurement may be confounded by the reaction of heterogeneous carbohydrate-containing substances, such as glycoproteins. In one modification on the original method, the PHS

procedure was refined by reversing the sequence of reagent addition to improve sensitivity for glycated proteins and uniformity with respect to sugar type [24]. Saha et al. removed the heating step and reduced volumes to 2.5 mL total per sample [25]. Subsequent efforts have focused on reducing the volume further and/or improving throughput but have required cumbersome heating and/or specialized pipetting not amenable to automation [25–28]. To further optimize and minimize interference, procedures for cleaning up protein interference have been described [29,30]. However, none of the described methods minimize sample utilization nor are microplate-based, while concurrently simplifying the heating procedures sufficiently for transfer to a robot for automation.

1.4.3. Impurity assays

Rapid impurity measurements are critical for the development of purification processes from biological feedstreams. Assays for impurities such as protein, DNA, and endotoxin are typically laborious and lack adequate robustness when used in complex backgrounds.

Endotoxin assays have historically been enzymatic, time-consuming, and rarely automated. A recent addition to the panel of commercially available assays offers promise for rapid detection [31]. The PyroGene^{TM} assay utilizes a recombinant protease zymogen, Factor C that is activated upon endotoxin binding. The activated enzyme then cleaves a fluorogenic substrate, which emits light at 440 nm when excited at 380 nm. As opposed to kinetic assays based on *Limulus* amebocyte lysate (LAL), the PyroGene^{TM} assay is an endpoint assay.

For protein quantitation, bicinchoninic acid (BCA) and Coomassie Blue assays for protein concentration can be readily performed in a microplate format [32,33]. In the BCA assay, proteins reduce Cu^{+2} to Cu^{+1} in alkaline conditions. A proprietary BCA-containing reagent then reacts with the cuprous ion to form a purple colour, absorbing at 562 nm [33]. The extent of reaction depends on the macromolecular structure, number of peptide bonds, and the amount of C, Y, and W residues in the protein [34]. The Bradford assay employs an acidic solution of Coomassie Brilliant Blue G-250 that absorbs at 595 nm when incubated with proteins containing basic and aromatic residues [35–37]. In this study, the Lowry assay was not tested due to its relative complexity, the multitude of substances (e.g. detergents) that interfere, and poor reagent stability [38].

Several high throughput methods exist for measuring DNA concentration. Simple methods based on either absorbance at 260 nm or the ratio of absorbance at 260 nm and 280 nm are excellent for relatively pure samples. Where a complex absorbance background precludes the use of absorbance measurements for DNA quantitation, fluorescent assays with Picogreen have proven exceptionally useful [39].

1.4.4. Interference

Central to the intelligent deployment of assays is an understanding of interference. The process streams created by unit operations occurring immediately downstream of a bacterial fermentor may have impurities with concentrations 10–100 fold higher than that of the product. Challenges also exist downstream of the first major purification unit operation where impurity loadings can still exceed the product concentration. Although the levels of interference ease further downstream, the potential presence of high concentrations of added excipients can impair assays. Therefore, a thorough investigation of the proposed assays for interference is critical to the success of high throughput process development.

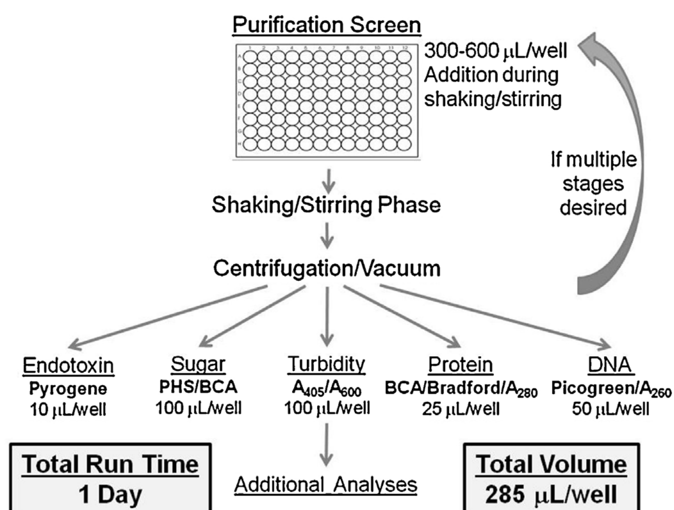

Fig. 1. Analytical workflow to support HTPD. The full suite of analytics can be completed in less than one day and requires less than 300 μL, the volume of a standard 96-well microplate.

1.5. Aims

This study describes the development of rapid and simple assays to enable the evolution of HTPD for the generation of novel purification processes. More specifically, we describe a set of analytical methods that will yield information on polysaccharide titre and impurity amount (i.e. endotoxin, nucleic acids, protein). Emphasis was given to developing methods that were versatile and would accommodate many different feedstreams, so as to facilitate future integration in a potential platform purification process. A schematic of the final analytical scheme is given in Fig. 1.

2. Materials and methods

2.1. Materials

All common chemicals were commercial analytical grade. Lysozyme from chicken egg white, albumin from bovine serum (BSA), L-arabinose, glycogen from oyster, chondroitin sulfate A sodium salt from bovine trachea, α-lactose monohydrate, glucose, N-acetyl neuraminic acid from *Escherichia coli*, Type II ι-carrageenan, 3-(N-morpholino)propanesulfonic acid (MOPS), and dextran were obtained from Sigma-Aldrich (United Kingdom). Deoxyribonucleic acid (DNA) sodium salt from salmon sperm was purchased from Fisher Bioreagents (United Kingdom). Sodium alginate from *Laminaria hyperborea* came from BDH (United Kingdom). Lonza Walkersville (Maryland, USA) provided endotoxin derived from *E. coli*. Gellan gum was produced by Applichem Biochemica (United Kingdom). High molecular weight hyaluronan (HA) was purchased from R&D Systems (United Kingdom). Endotoxin standards and stocks were purchased from Lonza Walkersville (Maryland, USA). All reagents were used as purchased. All sugars are D isomers except where noted otherwise. Solutions were filtered with 0.22 μm filters (GV PVDF, PES Express, Millipore, Massachusetts, USA) where appropriate. Polystyrene microtitre plates (Corning Costar #3596, Massachusetts, USA) were used with all assays, except where noted otherwise. All spectroscopic measurements were made with a Safire II spectrophotometer (Tecan, Switzerland). Standard abbreviations for L-arabinose (Ara), ribose (Rib), glucose (Glc), galactose (Gal), glucuronic acid (GlcA), guluronic acid (GulA), N-acetyl neuraminic acid (NeuNAc), mannose (Man) were selectively used to reduce graphical clutter.

2.2. Absorbance spectra

Absorbance spectra of pure carbohydrates and proteins were measured in solutions buffered with 20 mM MOPS, pH 7.2. Spectral measurements were also recorded following reaction in several colorimetric assays. Absorbance spectra were measured from 230 to 1000 nm in ≤3 nm increments using the microplate reader.

2.3. Bradford assay

The Bio-Rad protein assay (California, USA) based on Bradford's method was employed to measure protein concentration [36,37]. The instructions provided by the reagent manufacturer (version: Lit 33 Rev C) were followed. Samples were diluted in 20 mM MOPS, pH 7.0. Absorbance measurements were made at 595 nm and 990 nm. Blank-corrected standard curves were run in triplicate with absorbance at 990 nm subtracted from absorbance at 595 nm. Linear regression was used to fit the standard curve.

2.4. Bicinchoninic acid assay

The BCA assay kit (Pierce Thermo Scientific, Illinois, USA, version: 1296.7) was employed to measure protein concentration [40]. The microplate instructions provided by the assay kit manufacturer were followed. Samples were diluted into 20 mM MOPS, pH 7.0. Absorbance was measured at 562 nm and 990 nm. Blank-corrected standard curves were run in triplicate with a second order polynomial fit employed.

2.5. Phenol sulphuric acid assay

The procedures described by [20,25,26] were used as reference points to develop a modified PHS assay. The methods of Saha et al. formed the basis for the advent of a modified method as described in Table 2. In the modified method, 25 μL of 5% (m/v) phenol was added to 25 μL of sugar solution previously aliquoted into the microplate, followed by mixing with a pipettor. Next, 125 μL of H_2SO_4 was added to each well, followed by rapid mixing with a pipettor. Solutions were incubated for 30 min at room temperature (18–25 °C) before the absorbance was read at 485 nm in the microplate reader. Where applicable, samples were diluted in reverse osmosis-purified, distilled water. Except for the comparative study performed with glucose as a test sample, all PHS measurements were made with the modified method. All mixing was performed via 5 aspiration cycles with a pipette. Standard curves were run in triplicate with absorbance values corrected for the blank. The final yellow colour was found to be stable for 1 h, although slight development occurred with prolonged incubation following the reaction. Phenol solution was stored in the dark when not in use.

In certain circumstances with the modified PHS assay, a glass microplate (Zinsser, Germany) was evaluated.

2.6. PyroGene™ assay

A pyrogen assay (PyroGene™, Lonza, Maryland, USA) based on recombinant Factor C for endotoxin was qualified. The instructions provided by the assay kit manufacturer (version: 08299P50-658U/NV-612/07) were followed except where noted. Pyrogen-free consumables including reagent reservoirs, pipette tips, conical tubes, LAL Reagent Water, and serological pipettes were purchased from Lonza Walkersville. Samples were diluted into LAL Reagent Water. Standard curves were prepared and run in triplicate. For assay interference testing and positive product controls, 10 μl of a 10 EU/mL standard solution was added to 90 μL of sample, yielding a 1 EU/mL reference standard concentration. Endotoxin samples and standards were vortexed vigorously for the prescribed amount of time. Except where noted otherwise, microplates were incubated for 1 h at 37 °C inside the plate reader prior to reading. The measurement parameters were: excitation wavelength set to 380 ± 20 nm, emission wavelength set to 440 ± 20 nm, and an integration time of 40 μs. The log amount of endotoxin present was proportional to the log change in the relative fluorescent unit (RFU), with second order polynomial fits offering the most accuracy.

The methodology employed differed from the manufacturer's recommendations in two significant ways. A single measurement was taken approximately 60 min after the start of incubation at 37 °C instead of the recommended two-point measurement. In addition, incubations at 22 °C, 26 °C, and 37 °C were evaluated for varying durations during one experiment.

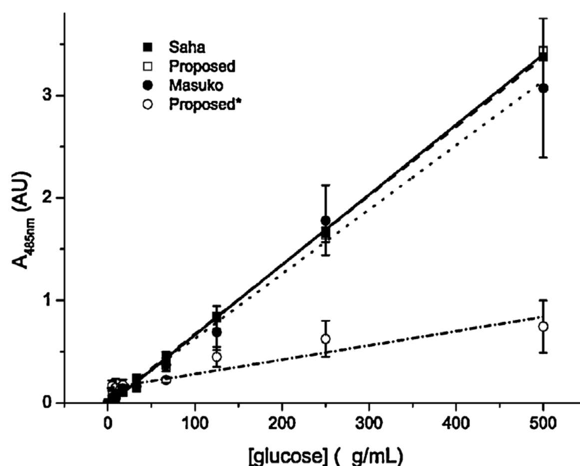

Fig. 2. Glucose standard curves generated with variants of PHS assay, prepared in triplicate. 'Masuko' refers to method described in Masuko et al. 'Saha' refers to method described in Saha et al. 'Current Work' refers to method as described in Section 2, and 'Current Work*' is equivalent to 'Current Work' but reacted in a glass instead of a polystyrene microplate [20,25,26]. The volumes prescribed in the original citations were followed. Absorbance was normalized to 300 μL per well. Correlation coefficients for each linear regression fit line were ≥0.99, except for Current Work*, where R^2 = 0.87.

3. Results and discussion

3.1. Modified phenol sulphuric acid assay

Several permutations of the original PHS method for sugar quantitation have been described. In order to establish a benchmark and compare the methods to the modified method described here, standard curves were generated with each method, using glucose as the standard carbohydrate (Fig. 2).

As shown in Fig. 2, the absorbance intensity attained for each method was very similar, irrespective of the specific PHS method employed. This observation suggests that the extent of reaction in each microwell was comparable. The Masuko method was expected to yield higher absorbance values due to a rearrangement of the reagent addition sequence but these signal increases were not realized [26]. Therefore it appears that previously observed sulphonated phenol-mediated attenuation was either consistent or insignificant.

The precision of the reported PHS procedures differed significantly. Across the 17–500 μg/mL, the mean relative standard deviation (RSD) for the Saha, optimized PHS, and Masuko assay were 6%, 10%, and 22%, respectively. While the Saha method exhibited the best precision, it required the most material (i.e. 0.5 mL). The decreased reproducibility of the Masuko method may be due to increased sensitivity to unintended variability in the time lapsed from sulphuric acid addition (i.e. the heat generation step) to the

Table 2
Comparison of PHS methods. The 'proposed' method is described in more detail in the accompanying text. 'RT' refers to room temperature, 18–25 °C operation.

Source	Dubois et al.	Saha et al.	Masuko et al.	Proposed
Year	1956	1994	2005	2012
Volume (μL)	7050	3500	230	175
[Sugar] (μg/mL)	5–35	10–100	4–585	10–1000
Method	Add 50 μL 80% phenol to 2 mL sugar	Add 0.5 mL 5% phenol to 0.5 mL sugar	Add 150 μL H_2SO_4 to 50 μL sugar	Add 25 μL 5% phenol to 25 μL sugar
	Add 5 mL H_2SO_4	Add 2.5 mL H_2SO_4	Add 30 μL 5% phenol	Add 125 μL H_2SO_4
	Incubate 10 min	Vortex	Incubate 5 min in 90 °C water bath	Incubate for 30 min at RT
	Shake	Incubate 30 min at RT	Cool to RT for 5 min in RT water bath	Read UV
	Cool in 25–30 °C water bath	Read UV	Wipe microplate dry	
	Read UV		Read UV	

addition of phenol. In this work, the order of addition was found to be important with better precision observed when the heat generation step was the final step, presumably leading to a more uniform reaction temperature.

A consistent reaction was generated by careful consideration of the factors affecting the temperature of reaction. In contrast to the method described here, which uses a polystyrene microtitre plate, a reduced signal was observed when the glass microplate was used (). This attenuated signal is likely due to the higher thermal conductivity and specific heat of borosilicate glass as well as the greater volume of material contained in the glass microplate relative to the polystyrene microplate. These factors presumably prevent the solution from attaining the high temperature required for robust reaction.

The testing with glucose established that the modified PHS assay had satisfactory accuracy and precision. This method was comparable to the method of Saha et al. and was characterized by superior precision to the method of Masuko et al. [25,26]. The reproducibility was particularly strong for higher polysaccharide concentrations, which is within the dynamic range most samples derived from typical purification HTPD will likely reside. The greater simplicity, speed, and ease of automation afforded by the elimination of the discrete heating steps warranted further development of the modified PHS method.

A diverse library of mono-, di-, and poly-saccharides were assayed with the modified PHS assay. The carbohydrates tested included glucose, α-lactose monohydrate, L-arabinose, maltose, hyaluronic acid, chondroitin sulfate, sodium alginate, gellan gum, dextran, ι-carrageenan, glycogen, DNA, endotoxin, and N-acetyl neuraminic acid. Initially, absorbance scans of the reacted sample were made to identify the wavelength at which maximum absorbance (λ_{max}) was measured. The λ_{max} fell in the range 477–487 nm which corroborates with the range of 480–490 nm published for more limited subsets of carbohydrates [20,25,26]. For hexose sugars ($n = 11$), the mean $\lambda_{max} = 485 \pm 3$ nm and for pentose sugars ($n = 2$), the mean $\lambda_{max} = 477 \pm 1$ nm. From this dataset, a fixed value of 485 nm was determined to provide robust measurement of diverse polysaccharides in the modified PHS assay.

Using a wavelength of 485 nm, standard curves were generated for the library of polysaccharides, with the corresponding gradient functions provided in Fig. 3. In the modified PHS assay, hexoses absorb more strongly than pentoses at 485 nm. This order is maintained even if the λ_{max} for pentoses is used for the absorbance measurement. The anionic polysaccharides absorb far less per unit mass than do the neutral carbohydrates. In large part, this is due to the presence of non-signalling anions such as sulfate.

It has been previously shown that for complex oligosaccharides containing different hexoses, the summed contribution of the reactive hexoses equates to the approximate reactivity of the polysaccharide [25]. Moreover, as N-acetyl galactosamine, N-acetyl glucosamine, and N-acetyl neuraminic acid have been demonstrated to insignificantly react in the PHS assay (data not shown), the contributions of certain structures can be discounted if other reactive pentoses and hexoses are present [26]. Similarly, the organic and inorganic anion groups do not signal and can also be disregarded. After applying these data transformations to oligosaccharides comprised of similar repeating sugar components, the absorbance response converges on a single line as a function of the concentration of particular reactive monosaccharide (Fig. 4).

The data in Fig. 4 can be used to approximate the expected reactivity of diverse carbohydrates. Of the carbohydrate classes tested, the hexoses produced the highest absorptivity in the modified PHS assay. The absorbance of heteropolysaccharides can be approximated by the addition of the reactive components. However, it was noted that addition was imperfect when heteropolysaccharides composed of both glucuronic acid and glucose were summed, as the polysaccharide containing both units reacted slightly less than the sum of the independently generated glucose and glucuronic acid curves.

To facilitate appropriate comparisons, the molar absorptivities of the reactive units are displayed in Table 3. The absorptivity values in the modified PHS method are consistent with those described in the original PHS papers by DuBois et al. [20]. The absorptivities measured in the described PHS assay underpinned the spectrum of dynamic linear ranges depicted in Fig. 5.

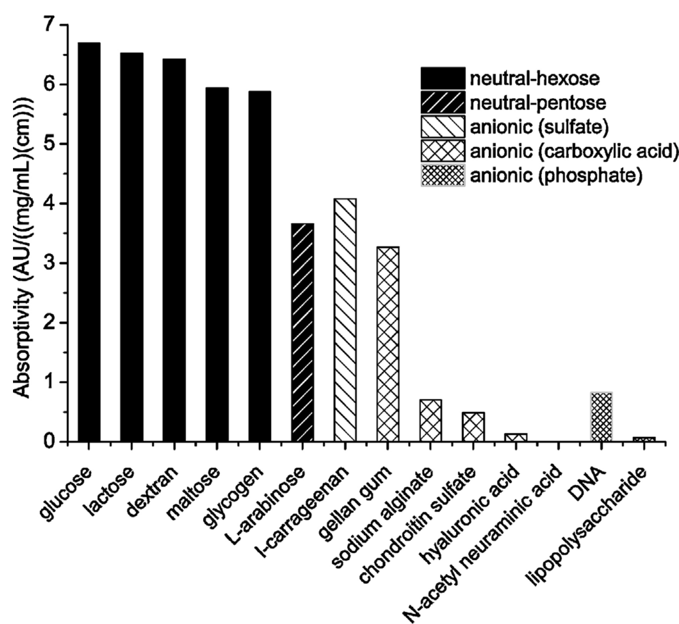

Fig. 3. Gradient functions of standard curves for neutral and anionic hexoses and pentoses in the modified PHS assay. The standard curves for the carbohydrate library were linear with a correlation coefficient >0.95 for each species tested. The legend describes the repeating monosaccharide for neutral sugars and the charged group for anionic sugars.

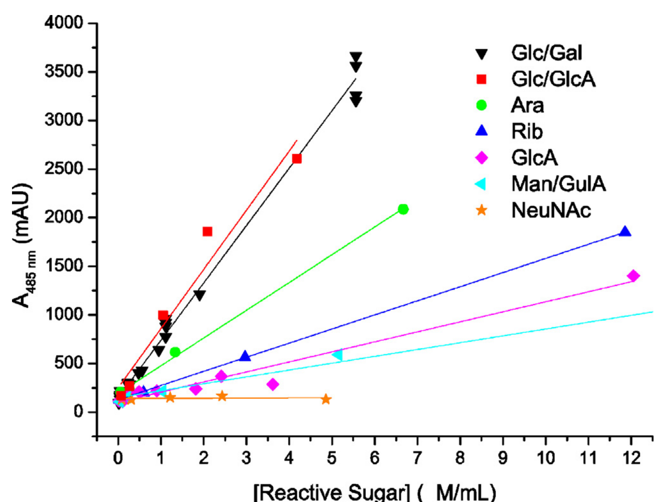

Fig. 4. Reaction of carbohydrates listed in Fig. 3, plotted as a function of common reactive sugars. Non-reactive structural components were ignored. $R^2 > 0.95$ for each fit line. Carbohydrates with glucose and galactose were co-plotted as the relative reactivity of these hexoses was found to be statistically similar when plotted independently (decoupled data not shown).

Table 3
Molar absorptivity of reactive sugars in proposed PHS assay. N-acetyl neuraminic acid is representative of a non-reactive carbohydrate.

Sugar	Molar absorptivity (AU((μmol/mL)(cm)))
Glc and Gal	1.135
Glc/GlcA	1.171
Ara	0.550
Rib	0.279
GlcA	0.198
ManA/GulA	0.135
NANA	0.002

Having an elevated lower limit of quantitation (LOQ) is advantageous when monitoring the array of concentrations across a microplate where the load material titre is 0.5–5 mg/mL. The loss of low-end sensitivity (i.e. <10 mg/L) is acceptable as detecting such minute concentrations is not practically relevant, particularly in purification HTPD, where concentration changes greater than 100-fold are rarely encountered.

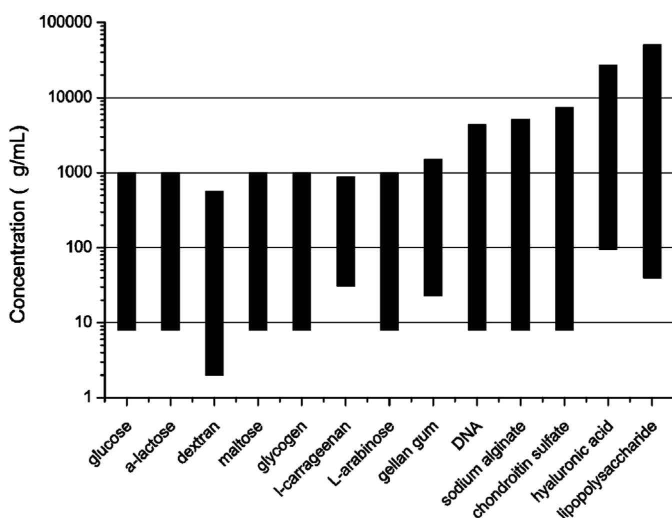

Fig. 5. Linear range of quantitation for carbohydrates in modified PHS assay. All $R^2 > 0.95$ when fit with linear regression. Range is linear up to $A_{485} = 2.0$ AU.

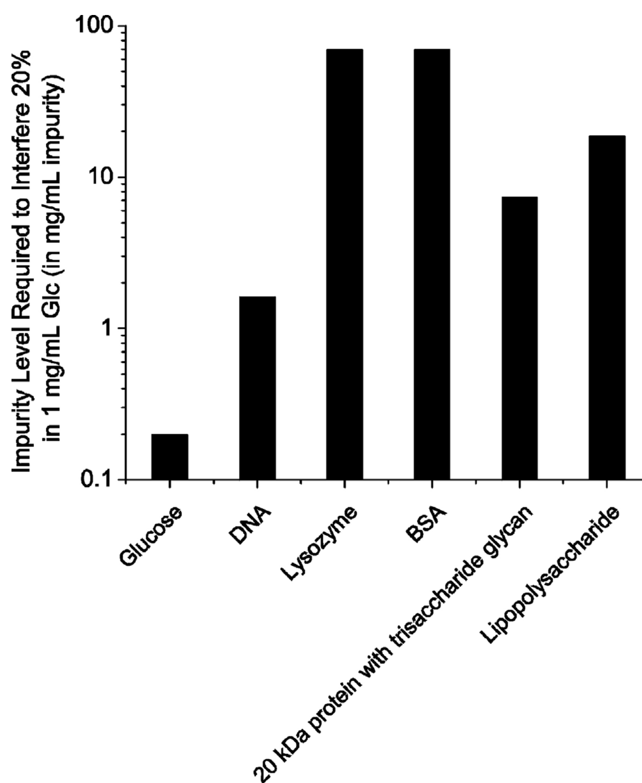

Fig. 6. Concentration of interfering species required to effect a 20% shift in the measured value. Y-axis values assume the desired analyte is 1 mg/mL glucose. 70 mg/mL impurity on the y-axis represents the maximum level that can be measured based on the limit-of-quantification for the assay. The hypothetical trisaccharide glycan is Glc-Glc-Glc to represent worst-case interference from the glycosylated protein.

Polysaccharide titre measurements will be required in impure samples possessing a complex background. DNA, protein, and endotoxin are impurities present in virtually all in-process samples. Therefore, a key element of the robustness of the any in-process sugar assay is the propensity of typical impurities to interfere Fig. 6.

Interference in the modified PHS assay was minor. As the assay is colorimetric and designed for in-process samples, a shift in measurements of ≥20% was deemed to represent significant interference. Every sample tested reacted substantially less strongly than did glucose. Although proteins did not react strongly, the tested proteins were not glycosylated. Therefore, based on the reactivity of the constituent glycan, an estimate was made of the interference posed by a glycosylated 20 kDa protein possessing one trisaccharide glycan per protein molecule. The theoretical degree of interference was slight for this composition, due to the low molarity of the pendant oligosaccharide. Based on Fig. 6, only far upstream in the purification process would samples be likely to contain concentrations of interfering species (i.e. simple sugars from broth/media, DNA) high enough relative to the target carbohydrate concentration to cause problematic interference. In such a case, a high throughput desalting step using a microtitre plate could be utilized to reduce interference.

3.2. Protein assays/differential assay for sugar

Two protein assays were screened for suitability for integration with polysaccharide HTPD: the BCA and Bradford assays. The standard curves generated with both protein assays exhibited good fit. For the BCA assay, a $R^2 > 0.99$ for the 0.025–2 mg/mL range was achieved with a relative standard deviation of 4%. Second-order

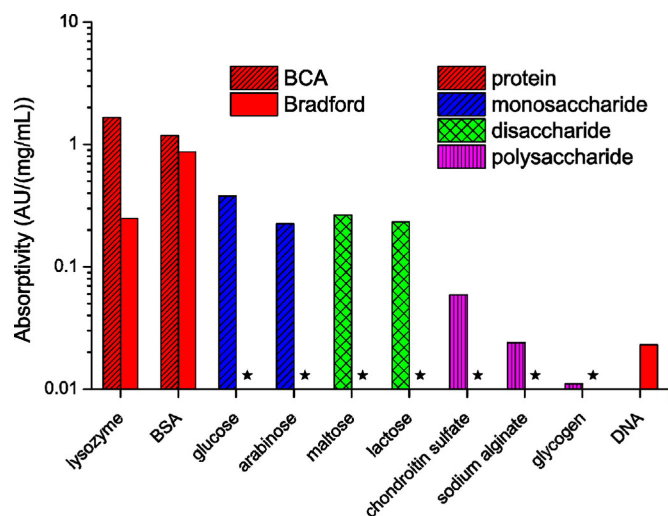

Fig. 7. Absorptivity of various carbohydrates in BCA and Bradford assay, with molecular classification provided for species reacting in the BCA assay. Standard curves in both assays were fit with linear regression although second order polynomial fitting produced slightly better fits for the BCA assay. For Bradford and BCA assays, all curves were characterized by $R^2 > 0.92$ and $R^2 > 0.98$, respectively. * denotes samples with absorptivities BLOQ or that did not signal.

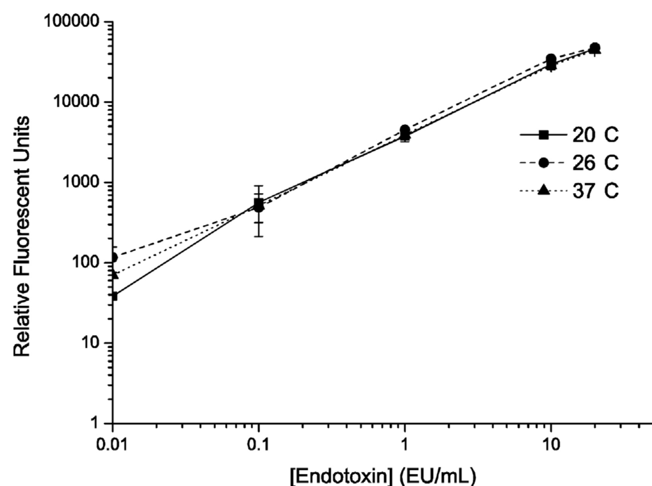

Fig. 8. Effect of incubation temperature on relative fluorescence measurements in the PyroGene^{TM} assay. The incubation duration was 1 h. No significant differences in measurements were noted when the incubation was extended to 2 h.

polynomial fitting improved the accuracy and the fit. Correcting for absorbance at 990 nm decreased the precision slightly and was not incorporated. With the Bradford assay, the correlation coefficient was found to be a function of the included range. Employing 0.025 mg/mL as the lowest non-zero concentration tested, linearly fit standard curves with an upper range of 0.5, 1.0, and 2.0 mg/mL were generated. The R^2 values for these curves were >0.99, >0.98, and >0.95, respectively, with curves based on the broader ranges overestimating the highest concentrations. Subtraction of the absorbance at 990 nm from the absorbance at 595 nm improved mean precision from 6% to 3% RSD.

The impact of interfering species on the two assays was mixed (Fig. 7). Concentrated DNA (5 mg/mL) produced a significant response in the Bradford assay but did not react in the BCA assay. In the Bradford assay, the relative responses of lysozyme and BSA to DNA yielded a 28-fold and an 11-fold increase respectively, suggesting that any related interference would be manageable in a process development scheme. Endotoxin did not react in either assay. Similarly, sugars did not exhibit any reactivity in the Bradford assay.

Reducing sugars were oxidized in the BCA assay. Monosaccharide and disaccharide reducing sugars exhibited the highest absorptivity with no clear difference between hexoses or pentoses. Polysaccharides offered lower absorptivities, due to the localization of the reducing groups at the termini and the low relative number of reducing groups per polysaccharide. Indeed, dextran exhibited negligible reactivity due to the reducing groups being confined to a limited number of branched termini and representing a small portion of the total hexoses comprising the polysaccharide. Non-reducing carbohydrates including glycogen, HA, chondroitin sulfate, N-acetyl neuraminic acid, and sodium alginate did not react in the BCA assay (data not shown). In the Bradford assay, no carbohydrates except DNA formed absorbing species, although this was only substantial at >1 mg/mL, consistent with product literature [37]. An increase in the absorbance at 595 nm due to shifts in the charged dye equilibria may underlie this observation [35]. Depending on whether the carbohydrate or DNA concentration is known, the Bradford or BCA assay can both be used for measurement of protein contained in-process samples.

3.2.1. Differential use of two protein assays

Given the distinct responses of the two proteins assays to reducing sugars, an effort was made to use this differential signal to measure the titre of a reducing sugar. First, the capability to sum the reactive components of multi-component mixtures was examined. The slopes of the standard curves for glucose and BSA were independently measured, with the sum of the two slopes equalling 1.56 AU/(mg/mL). A standard curve for samples consisting of 50:50 BSA:glucose was generated and was characterized by a slope of 1.31 AU/(mg/mL), 18% below the expected value.

In a subsequent examination of the differential approach, glucose was spiked to a final concentration of 1 mg/mL in solutions containing from 0.020 to 0.50 mg/mL BSA. The amount of glucose was then calculated from the difference of the BCA and Bradford signal. This was achieved by using a calibration equation derived from the BSA standard curve (to measure glucose in units of mg/mL BSA) and normalizing by the ratio of the slopes of the glucose and BSA standard curves. The outcome of these experiments was an estimation of 0.72 ± 0.15 mg/mL of glucose. This result was imprecise and was significantly below the expected concentration of 1 mg/mL.

This trial indicated that the addition of the two assays was not accurate or robust enough to use for the purpose of estimating sugar concentrations. It is believed that the high observed variance and inaccuracy may be due to additive errors present when using multiple assay measurements for a single differential measurement. Equally problematic, since both protein assays react with an intensity that is dependent on the protein composition, if the relative reactivity of the two assays to a given sample varied sharply, it would hinder the utilization of this approach. This is particularly applicable for purification development where protein compositions could differ markedly across a microplate. The contrasting slopes for lysozyme and BSA standard curves when measured in both protein assays in Fig. 7 provide an indication of the noise that could be encountered. For these reasons, the differential method for reducing sugar quantification is better suited to samples purified to a greater extent, further downstream in the purification process.

3.3. Endotoxin assay

Due to its simplicity and ease of automation, particularly when compared to kinetic assays (e.g. kinetic QCL), the PyroGene^{TM} assay was qualified as the principal endotoxin assay [41]. As displayed in Fig. 8, the log–log standard curves were consistent and exhibited good fit with $R^2 > 0.99$ across a range of 0.01–20 endotoxin units

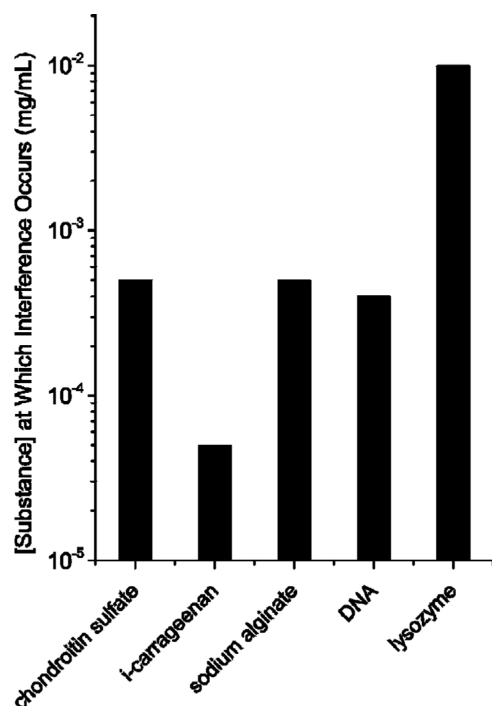

Fig. 9. Interference in the PyroGene^{TM} assay. Interfering substances were freshly prepared in depyrogenated LAL Reagent Water and spiked with endotoxin (20 mM MOPS, pH 7.0) to achieve a final endotoxin concentration of 1 EU/mL.

(EU)/mL. Precision was found to average 7% RSD across the tested range. Several incubation temperatures were evaluated in parallel with the standard incubation temperature of 37 °C (Fig. 8). Lowering the incubation temperature did not have a deleterious effect on the reproduction of the standard curve. Enabling the incubation period to occur at room temperature is helpful when automating assays with liquid-handling robots situated in room temperature environments.

The potential for various substances to interfere with the PyroGene^{TM} assay was evaluated through positive product control samples (Fig. 9). In these samples, endotoxin was spiked to a final concentration of 1 EU/mL in the presence of a concentration series of various impurities (i.e. proteins, sugars, and DNA). Chondroitin sulfate, DNA, sodium alginate, ι-carrageenan, and several anionic capsular polysaccharides (data not shown) inhibited the PyroGene^{TM} assay. The severity of the inhibition was high, with dilutions to <1 μg/mL required to abolish the effect. The inhibition was consistent across assays performed on multiple days with freshly made solutions, with multi-day variability of ~27% (data not shown). Each of the inhibitors was an anionic polysaccharide but other anionic polysaccharides such as HA, gellan gum, and N-acetyl neuraminic acid did not react, nor did the acidic protein, BSA. A common structural feature between the DNA, ι-carrageenan, and chondroitin sulfate is the presence of sulfates. Every species with a sulfate that was tested was found to inhibit the assay, but other anionic groups did not interfere consistently. For example, none of the uronic acid-containing polysaccharides reacted except for sodium alginate (and chondroitin sulfate, which also has sulfate groups). The mechanisms for inhibition are unknown but possibly due to electrostatic interactions with the zwitterionic endotoxin. However, inhibition of the recombinant Factor C through the active site is unlikely as the lipid domain of endotoxin serves as the substrate [41].

Fresh lysozyme artificially increased the signal intensity of the PyroGene^{TM} assay. The dry chemical stock of lysozyme possibly harboured Gram-negative microbes or pyrogenic byproducts. Unlike with the LAL assay, high molecular weight carbohydrates such as carrageenan were not found to enhance the PyroGene^{TM} assay [42]. Several of the tested substances (i.e. BSA, HA, lysozyme, and dextran) exhibited apparent enhancement when initially tested. As these liquid samples had been stored non-sterile at 5 °C for two weeks, fresh stocks were prepared. Using the fresh stocks, no enhancement was observed, highlighting the importance of mitigating potential Gram-negative bacteria contamination. None of the tested species consistently interfered except for those shown in Fig. 9.

Utilization of the PyroGene^{TM} assay will necessitate extensive dilution (i.e. 10^{-3}–10^{-4}) to eliminate interference from bacterial feedstreams. The level of dilution will be predicated on the concentration and nature of components in the sample background, with samples upstream in the process requiring greater dilution than the more purified streams found further downstream in the process. Although the magnitude of the inhibition is significant, the PyroGene^{TM} assay is still suitable for measuring endotoxin in impure pools. In polysaccharide process streams derived from Gram negative bacteria, the starting concentrations of endotoxin are high. These values often exceed 20,000,000 EU/mL (personal communication from Dr. Bernie Violand; Pfizer R&D). However, the linear range of the PyroGene^{TM} assay is 0.01–10 EU/mL, necessitating multiple serial dilutions to fall within the standard curve. Because of the large difference between the range of the PyroGene^{TM} assay and typical endotoxin concentrations, it is possible to measure adequate LRV of endotoxin, even when factoring in dilution to eliminate interference (Table 4). With such high amounts of endotoxin present, dilution to 10^{-3}–10^{-4} should still enable the demonstration of 5–6 log removal value (LRV) of endotoxin clearance for harvest samples and 2–3 LRV of endotoxin clearance for polishing steps. Demonstration of adequate clearance may be hampered in samples taken downstream of polishing steps.

4. Automation

The capability to automate assays used to inform purification process development is clearly an important attribute. All of the described assays can be integrated into an automated analytical platform, enabling multi-faceted characterization of impurity clearance and product yield in less than one day by a single scientist. Automation requires an initial upfront investment of effort to refine but can be indispensable when repeat analyses are required. In purification process development, several high throughput screens can be run to evaluate different unit operations or distinct modes within a given unit operation. In addition, multivalent products such as most capsular polysaccharide vaccines, present the need for parallel screening between allied polysaccharide feedstreams. The

Table 4
Theoretical measurable endotoxin clearance values in the presence of interfering species. Assumes polysaccharide concentration equals 1 mg/mL and endotoxin assay range is 0.01–10 EU/mL.

	[Endotoxin] (EU/mL)	[Endotoxin] with Assay Dilution (EU/mL)	Available Log Removal Value (LRV)
Post-harvest	>20,000,000	2000–20,000	#9>7#6
Post-primary recovery	20,000	2–20	#9‘7#3

availability of a fast and automated analytics platform will expand the scope, robustness, and evolution of Design of Experiment (DOE) studies. It is envisaged that this will lead to expanded use of Quality by Design (QbD) approaches in vaccine process development.

5. Conclusion

Currently, the development of purification processes for vaccine polysaccharides is exceedingly complex, time-consuming, and laborious. HTPD of polysaccharides has lagged significantly behind current developmental archetypes for other biologicals such as monoclonal antibodies. The lack of simple, high throughput analytical tools has played a role in hindering the evolution of HTPD for polysaccharides.

Purification process development does not require the exquisite accuracy demanded of release assays. Instead, speed, simplicity, and precision are paramount. Especially in the context of high throughput process development, the desire to find the best conditions on a microplate, relative to the other wells, is critical. Excluding affinity separations, the maximum purification factor that can be achieved in a single-stage equilibrium experiment is typically 2 logs, obviating the need for extremely sensitive analytics. Accuracy is more important in the subsequent scale-up and demonstration of promising purification conditions.

Polysaccharides, endotoxin, proteins, and nucleic acids are the major components found in harvested bacterial fermentation broths employed in industrial polysaccharide vaccine manufacturing. Their critical importance is underscored by the inclusion of the respective assays in the batch release package for product characterization. In the current work, analytical techniques for quantifying polysaccharides, endotoxin, and proteins were qualified. In selecting methods, emphasis was given to procedural simplicity, amenability to automation, robustness, and precision over accuracy. In addition, the qualification process included evaluating the impact of impurities commonly encountered alongside the carbohydrate product as well as a diverse library of polysaccharides.

Novel procedures were described to simplify methods and facilitate automation. A phenol sulphuric acid assay was optimized for high throughput quantitation of mono-, di-, and poly-saccharides. The assay requires only 25 μL of sample and involves no heating steps that can stymie automation. The described procedure also reduces the quantities of hazardous chemicals such as phenol and sulphuric acid, requiring only 150 μL total per sample. A linear range of approximately 2 logs (e.g. glucose: 8–1000 μg/mL) was observed for every tested carbohydrate, with the actual range derived from the specific composition of reactive sugars present. The precision of the described assay was found to be 10%. The PyroGene^{TM} assay was simplified to a single measurement while removing a heated incubation step. This modified assay was demonstrated to be robust for the determination of the range of endotoxin concentrations present in samples drawn from harvest, clarification, and most polishing unit operations. For protein quantification, the BCA assay was shown to be superior for the determination of protein concentration while the Bradford assay offers an adequate assay when specific interferences exclude the BCA assay. Using the differential signal from these two protein assays, a method was conceived and demonstrated to be capable of estimating the amount of a reducing sugar present. When neither fine accuracy nor precision is required, this method may offer a less experimentally demanding and more streamlined approach for reducing sugar determination than the PHS assay. In conjunction with well-established methods for quantifying DNA, these methods comprise the core analytical techniques needed to support purification process development.

The described suite of analytics enables the rapid quantitation of key molecular classes in a microplate-based format that is amenable to automation. The deployment of these analytics will enable the development of high throughput processing platforms to speed the development of polysaccharide manufacturing processes.

Acknowledgements

Bernie Violand, Sa Ho, Khurram Sunasara, and Tom Emmons were invaluable in shaping the direction of this work and in providing useful suggestions for experiments and interpretation. Pfizer generously supported this research through an Eng. D. sponsorship and the Engineering and Physical Sciences Research Council provided critical backing.

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