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Determination of lipid profile in meningococcal polysaccharide using reversed-phase liquid chromatography

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Abstract

A fast and sensitive HPLC method using fluorescence detection is developed to quantitate 1-pyrenyldiazomethane (PDAM) derivatized fatty acids derived from the lipid components of both the capsular meningococcal polysaccharide and other impurities such as endotoxin in various meningococcal vaccine samples. The HPLC method is capable of well resolving 13 relevant fatty acids within 40 min by using a multi-stage acetonitrile/water gradient. Endotoxin values measured by HPLC well correlated with results from the standard Limulus amebocyte lysate (LAL) assay. Furthermore, the fatty acid profiles of various process intermediate samples as well as final purified polysaccharide products were determined to better understand and characterize the purification process.

Keywords: Lipid profile; Polysaccharides

1. Introduction

The capsular meningococcal polysaccharide (MnPs) of *Neisseria meningitidis* is the antigenic component of the current meningitis vaccines for adults [1] and of the polysaccharide-protein conjugate vaccine for infants [2]. The capsular meningococcal polysaccharide (MnPs) consists of covalently attached phospholipid moiety [3], which serves to anchor the polysaccharide to the outer membrane of the bacterium.

In order for pediatric polysaccharide-protein vaccines to be highly efficacious and stable, the MnPs should be covalently conjugated to the protein carrier [2]. The purification of MnPs for use in conjugate vaccines requires the effective removal of lipids which would otherwise create mere physical binding. The purification must also remove endotoxin, a pyrogenic lipopolysaccharide impurity that is released from the membrane of the gram negative host cell [4]. Finally, additional lipid impurities have to be cleared in order to meet the product purity specifications.

Purified MnPs of acceptable quality and safety can be achieved by developing a purification process including a phospholipase enzymatic delipidation reaction to delipidate and purify the MnPs, and also remove endotoxin [5]. Therefore, an efficient assay method to identify and quantitate lipids across the purification process is critical. The constituent fatty acids of lipids are distinctive and therefore fatty acid analysis is the basis of most lipid assays. In its native form, MnPs is comprised of only even-chain fatty acids [3,6]. Endotoxin differs in that the C12 and C14 fatty acids are hydroxylated in the gamma position [7]. Consequently, a method that differentiates fatty acids provides an assay for both of these lipids.

Current lipid or fatty acid assays are not yet optimal for the analysis of process intermediate samples on demand. A colorimetric-based assay for lipid determination, based on reaction of unsaturated hydrocarbon bonds groups with phosphovanillin [8], cannot differentiate the various fatty acids. GC–MS has been widely employed for lipid analysis [9], but has not yet been extensively implemented in monitoring process intermediates in real time. Alternatively, reversed-phase HPLC combined with high sensitive fluorescence detection has also been used to quantitate the fatty acid profile of lipids derived from various sample matrices [10]. Detection by fluorescence provides sensitive quantitation and numerous derivatization options [10–12]. Documented applications include analysis of free fatty acids, phospholipids, and endotoxin from blood, microorganisms, human and animal serum, food and other sources [13–16]. In this study, 1-pyrenyldiazomethane (PDAM), the most

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recent generation of aryldiazoalkane labeling reagents was chosen for its structural stability and excellent fluorescence yield [17].

In this paper, the HPLC separation and the fluorescence detection method was separately optimized using a pure standard mixture of even-chain fatty acids (C12:0–C24:0). Next, the sample treatment by lipid hydrolysis/extraction/labeling was developed using a pure phospholipid analogue (based on [3], 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine, $C_{40}H_{80}NO_8P$) in addition to pure fatty acids. The developed protocol was then challenged with a partially purified MnPs (intermediate powder). The results were verified by spike recovery tests. Finally, the HPLC assay was used against process intermediate samples to assist the purification development.

2. Experimental

2.1. Chemicals

1-Pyrenyldiazomethane was purchased from Molecular Probe Inc. (Eugene, OR, USA). Fatty acids along with their methyl esters, and phospholipid standard ($C_{40}H_{80}NO_8P$) were purchased from Sigma (St. Louis, MO, USA). 3-Hydoxyl of C12:0 and C14:0 were purchased from Matreya Inc. (Pleasant Gap, PA, USA). Anhydrous methanol and acetyl chloride were obtained from Alltech (State College, PA, USA). All other reagents were of GC grade from Sigma (St. Louis, MO, USA).

2.2. Equipment

The HPLC system consists of an Agilent 1100 HPLC with micro vacuum degasser, autosampler, quaternary pump, and a Jasco FP920 fluorescence detector. A 4.6 mm × 250 mm Agilent Eclipse XDB-C8 column was used for HPLC analysis. Water and acetonitrile were chosen as mobile phase buffers.

2.3. Meningococcal polysaccharide purification

A purification flow diagram including all sample information is presented in Fig. 1. *N. meningitidis* fermentation cultures were clarified by centrifugation and sampled, and then concentrated before selective alcohol fractionation to recover intermediate powder. The clarified broth was treated with a hydrophobic adsorbent to study its effect on lipid removal. Delipidation and further purification was accomplished by the method described in [5]. The delipidated MnPs was recovered as dried final powder.

2.4. Characterization of MnPs intermediate and final powder

The MnPs concentration of process intermediate samples (such as clarified fermentation broth) was determined

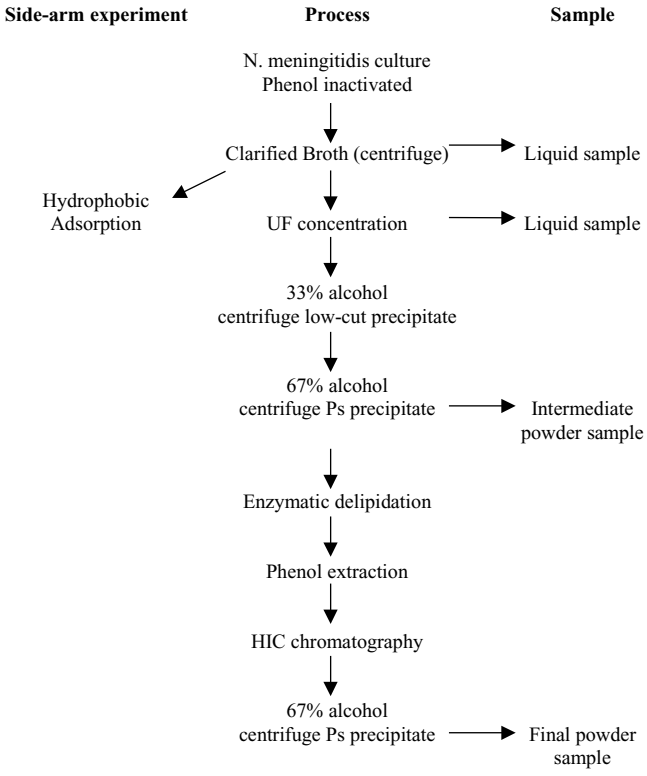

Fig. 1. MnPs purification flow diagram.

by Rate Nephelometry using BECKMAN Array 360 System (Brea, CA, USA). *N. meningitidis* atisera were obtained from DIFCO Laboratories (Detroit, MI, USA). Purified final MnPs powders were used as standard. All other reagents were purchased from BECKMAN. The vendor's recommended procedure was used.

The standard Limulus amebocyte lysate (LAL) assay was also performed on MnPs powders to determine endotoxin level. Assay reagents (ENDOSAFE®) were purchased from Charles River (SC, USA).

2.5. Sample preparation for HPLC analysis

Samples were either powder solution (powder dissolved in water) or liquid in-process samples containing 1–5 mg. MnPs powder. All samples were hydrolyzed either by base (2–6 N NaOH) or acid (6 N HCl) for 2–4 h at 90–100 °C. The hydrolyzed solution was rapidly cooled in ice water, and acidified (in the case of base hydrolysis) with concentrated HCl solution (12 N). The sample was then thoroughly mixed with 2 ml chloroform for at least one minute, and the mixture was centrifuged at 1000 rpm for 10 min. The water (top) phase was carefully withdrawn, avoiding the interface and discarded. The chloroform phase was washed with 5 ml DI water and the extraction step was repeated. The volume of chloroform phase was recorded after phase separation, and a fraction (based on estimated MnPs concentration) was transferred to a 2 ml amber vial for derivatization. PDAM

(in chloroform) was added at a molar ratio of at least 20:1 (PDAM: estimated fatty acids). The sample was incubated in the dark at room temperature for at least 90 min. Standards were prepared by derivatizing free fatty acids (in chloroform) with PDAM under the same conditions and the linearity of standard curves was confirmed ($R^2 \geq 0.99$). Ten microliters of each sample was injected onto the HPLC. The excitation and emission wavelengths were set at 340 and 375 nm, respectively. The Gain setting of the fluorescence detector between 1 and 1000 was chosen based on estimation of fatty acid concentrations. More details about the HPLC and sample preparation procedure will be discussed in the following sections.

3. Results and discussion

3.1. HPLC method development: derivatization of fatty acid standards

A mixture of even-chain fatty acids (C12:0–C24:0; hydroxyl C12:0 and C14:0) was dissolved in chloroform and derivatized directly with PDAM for this study. The fatty acids were prepared at a concentration (0.5 μg/ml) around the estimated level in the MnPs powder. The C8 column with a simple acetonitrile gradient resolved all fatty acids, however, a stepwise gradient and column temperature of 40 °C. allowed a shorter run time without compromizing resolution.

The resulting chromatogram in Fig. 2 shows that the 13 fatty acids were well resolved in 40 min and separated from excess PDAM which eluted much earlier. The baseline is also steady over the entire HPLC run. Standard curves for all fatty acids based on this method show good linearity ($R^2 \geq 0.99$). The derivatized fatty acids are eluted in the order of carbon chain number, suggesting that the hydrophobic binding is dominated by fatty acid carbon backbones. In addition, the unsaturated bond(s) or side group (three OH) reduce the retention time as they introduce polarity to the molecules.

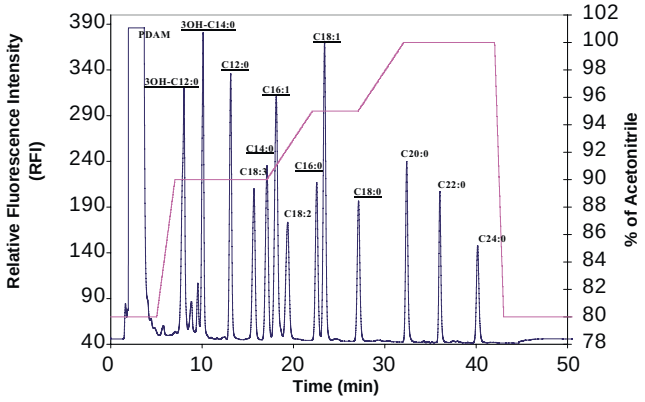

Fig. 2. Chromatography of standard even-chain fatty acids with Agilent C8 column. Underscored markers represent those fatty acids previously identified in MnPs samples [3]; sample concentration: 0.5 μg/ml for each fatty acid; injection volume: 10 μl; detector gain = 100; mobile phase: water with acetonitrile multi-stage gradient (80–100%); column temperature: 40 °C.

3.2. Development of the sample preparation procedure: hydrolysis, extraction, labeling

Fatty acids need to be released from their lipid source by hydrolysis and transferred into an organic solvent before PDAM derivatization and HPLC quantitation. Both acidic and basic acid conditions have been used for hydrolysis of biological lipids [9,18]. Acid hydrolysis of MnPs sample generated dark colored particles, presumably decomposed polysaccharide, which interfered with subsequent steps (such as extraction). Other studies also showed that acid hydrolysis leads to varying degrees of decomposition [19]. To investigate the base hydrolysis procedure, free fatty acids or phospholipid were incubated in 2 N NaOH at 90 °C for 2 h, acidified with excess HCl, extracted into chloroform, derivatized with PDAM, and recovery was evaluated by HPLC, respectively.

Free fatty acids or fatty acids derived from the phospholipid separated by the HPLC method as described above yielded chromatograms such as the example shown in Fig. 3A. Although individual peaks can be identified, peak

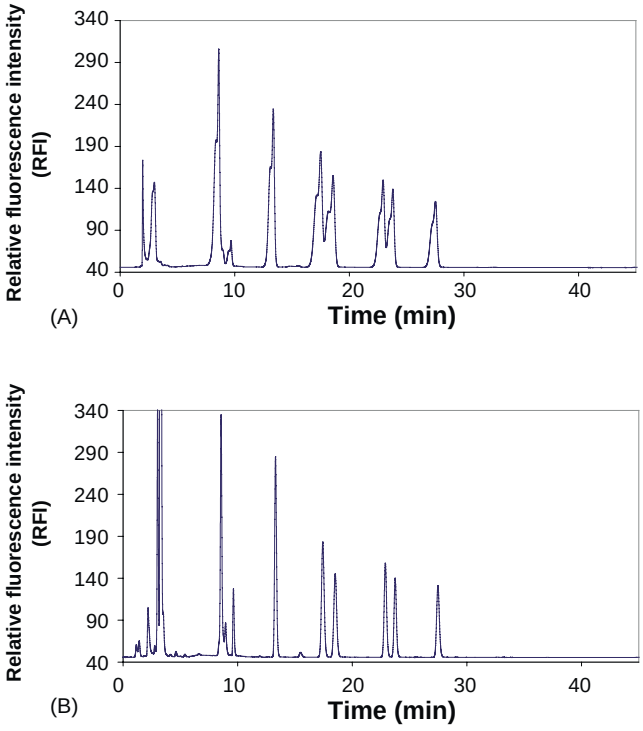

Fig. 3. Comparison between chromatogram containing split peaks/shoulders (A) and normal peaks (B). The injected sample for each figure was prepared from the same blend of fatty acids, incubated under base hydrolysis conditions, acidified, extracted, and derivatized with PDAM in chloroform at a final concentration 0.5 μg/ml (each fatty acid). However, in the case of (B) 20% (v/v) methanol was introduced to the sample prior to injection. Injection volume: 10 μl; detector gain = 100.

splitting compromises the resolution and prevents accurate quantitation. Peak areas for same sample injections varied by 20–200% from run to run. To locate the cause of split peaks, an investigation was performed by spiking free fatty acid at each step in the procedure: hydrolysis, acidification, extraction, and derivatization, and then evaluating the resulting chromatograms. In all cases, split peaks were obtained. It is likely that the problems are in the late stage of the sample treatment protocol. In a separate experiment, the derivatized fatty acid was dried and reconstituted in methanol prior to HPLC injection. Sharp peaks identical to those of direct derivatization (similar to Fig. 2) were obtained. Presuming that the methanolic reconstitution was responsible for this improvement, the chloroform extract of sample which had shown split peaks (Fig. 3A) was constituted to 20% methanol and injected to HPLC again. As shown in Fig. 3B, sharp peaks and baseline resolution were obtained.

These experiments indicated that the hydrolysis did not cause chemical modification to fatty acids. Peak splitting was resulted from heterogeneous binding/elution due to the heterogenicity in either the sample or the column. Fatty acid solutes may exist in an aggregated state, such as reverse micelles, and residual water entrained during the chloroform extraction may participate in stabilizing the micelle core. It is also possible that localized high concentration of chloroform or undissolved droplet modified part of the column, and displaced or interrupted the binding of fatty acid derivatives. Addition of 20% methanol disrupts the micelle structure and enhanced mobile phase solvation of chloroform, resulting in homogeneity and single peak. By simply using methanol as an HPLC sample cosolvent, the procedure avoided solvent exchange via lyophilization/evaporation which could lead to recovery loss (data not shown).

3.3. MnPs sample: polysaccharide powder

The developed procedure was evaluated by using MnPs intermediate powder. In contrast to the case of pure phospholipid (Section 3.2), fatty acid recovery was inconsistent with replicate assays originating from the same powder. The base hydrolysis and the chloroform extraction steps were identified as loss points.

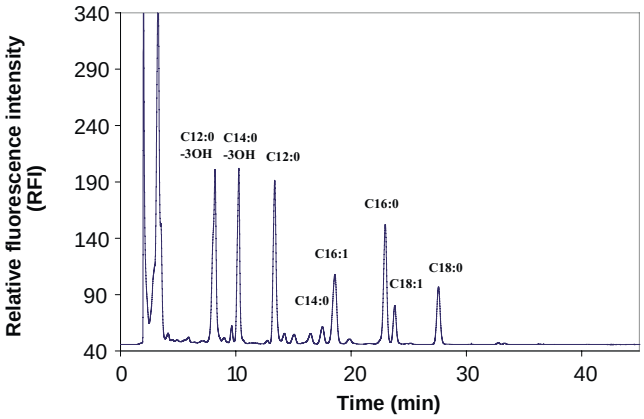

Fig. 4. HPLC of meningococcal polysaccharide serogroup C sample (powder solution, 7.2 mg/ml in water). Sample amount: 0.2 ml; injection volume: 10 μl; detector gain = 100.

The recovery of fatty acids from hydrolysis was improved by using a combination of higher NaOH concentration in the hydrolysis along with a 25% (v/v) concentration of methanol. The structure of fatty acids was well preserved under such conditions, as indicated by controls using pure fatty acids. Evidently, for the case of *lipid containing MnPs*, both solvation and base strength are important for consistent and complete hydrolysis of lipid-polysaccharide structure. In the absence of methanol, the lipid may be hidden if normal micelles are formed due to preferential solubility of the polysaccharide component. During chloroform extraction, the impurities in MnPs samples (i.e. proteins) caused an interfacial emulsion. An additional water wash of the chloroform extract corrected this problem. Consistent and reproducible recoveries were obtained after these two adjustments.

A chromatogram of meningococcal polysaccharide serogroup C sample which was treated with the optimized procedure is shown in Fig. 4. The peaks are identified as C12:0, C14:0, C16:0, C16:1, C18:0, C18:1. This is consistent with published data for MnPs [3]. The 3 hydroxyl

Table 1
Spike recovery of fatty acids and phospholipid in MnPs serogroup C powder sample

Fatty acids or phospholipid	Low-level spike concentration (w/w (%) of MnPs powder)	Recovery (%)	High-level spike concentration (w/w (%) of MnPs powder)	Recovery (%)
C12:0–3OH	0.05	103	–	–
C14:0–3OH	0.05	88	–	–
C12:0	0.05	83	0.70	102
C14:0	0.05	100	0.70	106
C16:1	0.05	85	–	–
C16:0	0.05	76	0.70	107
C18:1	0.05	81	–	–
C18:0	0.05	81	0.70	108
$C_{40}H_{80}NO_8P$[a] (C16:0)	0.07	108	0.84	91

[a] C16:0 is the fatty acid component of phospholipid standard $C_{40}H_{80}NO_8P$.

Table 2
HPLC results[a] of fatty acid contents in in-process samples during MnPs serogroup W135 purification

Experimental set #1		
Fatty acid	Clarified broth	Sample after hydrophobic adsorption treatment
C12:0–3OH	1.25 ± 0.02	0.62 ± 0.03
C14:0–3OH	1.00 ± 0.02	0.41 ± 0.01
C12:0	0.97 ± 0.03	0.32 ± 0.01
C14:0	0.73 ± 0.02	0.13 ± 0.01
C16:1	3.00 ± 0.05	0.51 ± 0.03
C16:0	2.94 ± 0.04	0.61 ± 0.03
C18:1	0.81 ± 0.03	0.14 ± 0.01
C18:0	0.06 ± 0.01	<0.0015

Experiment set #2			
Fatty acid	Concentrated broth	Intermediate powder	Final powder
C12:0–3OH	>0.1	>0.1	0.019 ± 0.001
C14:0–3OH	>0.1	>0.1	0.016 ± 0.001
C12:0	>0.1	>0.1	0.003 ± 0.001
C14:0	0.09 ± 0.01	0.009 ± 0.001	<0.0015
C16:1	0.37 ± 0.02	0.033 ± 0.005	<0.0015
C16:0	0.37 ± 0.02	0.038 ± 0.006	<0.0015
C18:1	0.098 ± 0.006	0.012 ± 0.002	<0.0015
C18:0	0.0044 ± 0.0001	<0.0015	<0.0015

The information about samples and process is described in Section 2.3 and Fig. 1 (fatty acid concentrations are expressed as w/w (%) of MnPs[#] in each sample). [#]MnPs concentrations (1–10 mg/ml) were determined by Rate Nephelometry.

[a] Sample number '*n*' equals to 3 (performed in three different days). All data expressed as (mean) ± (standard deviation); 0.5–2 ml in-process samples (containing 1–10 mg MnPs) were used for analysis.

C12:0–3OH and C14:0–3OH are indicators of lipid A of endotoxin. All components eluted within 30 min with baseline resolution. The presence of impurities (such as 3% (w/w) proteins) does not interfere with the chromatography.

To provide adequate information for purification where step impurity clearance need to be quantitated, the assay recovery besides the resolution is critical. Thus fatty acid spiking studies at concentrations approximates or much higher than (i.e. 10× greater) the typical fatty acid concentrations observed in MnPs, were investigated. Average recovery of above 85% were obtained (Table 1), indicating that the assay was not sensitive to the polysaccharide or other matrix components.

3.4. Process intermediate samples from MnPs purification process

The HPLC method thus has to be challenged by crude process intermediate samples at each stage which could represent different samples matrices. Fatty acid assay values for intermediate samples derived from the purification process (Section 2.3 and Fig. 1) are shown in experiments 1 and 2 of Table 2. Also included are results from alternative hydrophobic adsorption step. The individual fatty acid concentrations are determined as percentage of MnPs for each sample, as shown in Table 2. The lipid clearance across purification step is assessed by the fatty acid amount relative to MnPs content. Apparently, both hydrophobic adsorption and alcohol precipitation are capable of removing free lipids and endotoxin from clarified fermentation broth, but to different degrees. Other lipids present in the early stages of purification are composed of all types of fatty acid from C12:0 to C18:1 and are likely derived from cell membrane. No other type of fatty acid was found at significant levels. The overall process including adsorption, alcohol precipitation, organic extraction, plus enzymatic delipidation achieves more than an order of magnitude greater removal of free lipids, endotoxin, and bound lipid in MnPs, as required for final product specifications. Chromatograms of all samples showed no interference of impurities from those in-process crude samples.

Endotoxin level is a major quality control attribute of MnPs product. The three OH fatty acid concentrations obtained from HPLC method were compared with standard LAL testing results for various purified MnPs powders as shown in Table 3. Note that the LAL testing is measuring specific biological activity of endotoxin toward Limulus amebocyte lysate while HPLC is measuring the physical presence of three OH groups in the lipid A portion of endotoxin. The two assay results are in agreement and the C12:0–3OH and C14:0–3OH are in relatively constant, expected ratio of 1:1 suggesting that endotoxin is possible the only source of these fatty acids.

Table 3
The comparison between HPLC results of 3-hydroxyl fatty acid concentrations and LAL results for various MnPs powders (intermediate or final powders)

Sample ID	Serogroup	C12:0–3OH (w/w (%) powder)	C14:0–3OH (w/w (%) powder)	LAL (unit per μg powder)
1	Y	0.012 ± 0.0003	0.013 ± 0.0005	24
2	Y	>0.1	>0.1	3710
3	A	0.011 ± 0.001	0.012 ± 0.001	2.7
4	B	<0.0015	<0.0015	0.3
5	W135	0.017 ± 0.002	0.017 ± 0.001	6.8
6	W135	>0.1	>0.1	2115
7	W135	0.023 ± 0.001	0.020 ± 0.001	29.1
8	C	0.043 ± 0.004	0.044 ± 0.007	117.8
9	C	<0.0006	<0.0006	0.016

Samples number is 3 (performed in three different days). All data expressed as (mean) ± (standard deviation); 0.1–7 mg dry powders were used for HPLC analysis and 20–50 mg dry powders were used for LAL testing.

4. Conclusion

A simple HPLC method using an Agilent, C8 column and a multi-stage acetonitrile gradient was developed to resolve 13 even-chain fatty acids within 40 min. The method, involving fluorescent labeling for detection, is capable of measuring the composition and quantity of all of the fatty acids derived from the lipid component of meningococcal polysaccharide as well as other lipid part in the host cell (e.g. endotoxin). It offers greater sensitivity (around 0.1 pg of fatty acid) than other published HPLC method [20]. Further improvements should include automation for high throughput, use of mass spectrometry to better characterize the fatty acids [21], and implementation of internal standards (e.g. both hydroxyl and non-hydroxyl fatty acids) for validation purposes.

The assay demonstrated its ability to facilitate the purification development by good recovery and suitability to handle crude samples such as cell lysate. The reported assay development strategy is quite effective. Pure fatty acids, pure phospholipid and then complex MnPs samples, were used sequentially to develop the HPLC gradient method and the sample hydrolysis/extraction/labeling protocol. Therefore limited and valuable pure vaccine materials were less consumed. The use of methanol, both in hydrolysis and chromatography is likely to break up micellar aggregates of the amphipathic MnPs or the deriviatized fatty acids. In the hydrolysis, aggregates can shield lipids while in the chromatography; they may bind heterogeneously and cause peak splitting.

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