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GC–MS detection and quantification of lipopolysaccharides in polysaccharides through 3-O-acetyl fatty acid methyl esters

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ABSTRACT

The most common method used to quantify lipopolysaccharide (LPS) in polysaccharide samples is the Limulus amebocyte lysate (LAL) test. It is a very sensitive and simple, although not accurate with samples containing carbohydrates, such as widely distributed (1 → 3)-linked β-glucans. Another method, the Polymyxin B assay, suffers interference with samples containing negatively charged polysaccharides. We have now developed a method to detect and quantify LPS in carbohydrate-containing samples, using GC–MS of derived acetylated 3-OH fatty acid methyl esters. The method proved to be robust, highly specific and sensitive, allowing detection of LPS at 1 ng, 100 times less than the amount of LPS frequently used as positive control in immunological experiments. In order to demonstrate the applicability of the method, 14 polysaccharide samples were analyzed. On two of them, the presence of LPS was detected at concentrations of 16.1 and 12.7 ng/300 μg polysaccharide.

1. Introduction

Compounds obtained from natural sources can be contaminated with lipopolysaccharide (LPS), since bacteria are ubiquitous organisms and interfere with biological evaluation experiments (Beutler, 2004). To check contamination of polysaccharides with LPS few tests are available (Kataoka, Muta, Yamazaki, & Takeshige, 2002; Novitsky, 1998), and these are based on chemical reactions taking into account the structure of the LPS and the polysaccharides analyzed. One test is the Limulus amebocyte lysate (LAL), an aqueous extract from amebocytes of the North American horseshoe crab, *Limulus polyphemus*, which reacts in a quite sensitive way with LPS. The nonspecificity of the LAL assay depends on an enzyme present in the lysate reagent, called factor G. This is activated by (1 → 3)-linked β-glucans, which in turn activate the proclotting enzyme (Novitsky, 1998). An alternative is the polymyxin assay, which depends on the capability of Polymyxin B to bind to charged regions of LPS, neutralizing its activity in the nitric oxide (NO), TNF-α and IL-1 related pathways in macrophages (Kataoka et al., 2002). The problem resides in the fact that this binding is not exclusive to LPS, since other negatively charged molecules can also be present, such as poly-*N*-acetylglucosamine and sulfated polysaccharides (Amini, Goodarzi, & Tavazoie, 2009).

LPS is present in the outer membrane of most Gram-negative bacteria and is composed of an outer O-antigen and a middle core region covalently bound to a glycolipid moiety (lipid A). This contains a di- or tri-glucosamine group, which is covalently bound to 2- and 3-hydroxy fatty acids. The latter (3-OH-FAs) have been used as chemical markers of LPS (Pomorska, Larsson, Skórska, Sitkowska, & Dutkiewicz, 2007; Saraf, Park, Milton, & Larsson, 1999). 3-OH-$C_{14:0}$ is the dominant 3-OH-FA in the LPS of *Enterobacteriaceae* (Szponar, Norin, Midtvedt, & Larsson, 2002; Wilkinson, 1988). The minimal core structure of LPS consists of KDO (3-deoxy-keto-octulosonic acid) linked to lipid A (Helander et al., 1988). Its presence in LPS is critical for the survival of most bacteria, since few surviving bacterial mutant exists without KDO and lipid A in their LPS (Rybka & Gamian, 2006; Tan & Darby, 2005; Zamze, Ferguson, Moxon, Dwek, & Rademacher, 1987).

Attempts to detect the presence of LPS by the use of GC–MS has been developed mainly directed at detection of KDO as a specific marker (Rybka & Gamian, 2006), but the methodology often involves several steps, such as enzymatic dephosphorylation (Zamze et al., 1987), implying interference by the glycosidic components of LPS. The use of gas chromatography–mass

Abbreviations: LPS, lipopolysaccharide; LAL, limulus amebocyte lysate; NO, nitric oxide; TNF-α, tumor necrosis factor-alpha; IL-1, interleukin-1; 3-OH-FAs, 3-hydroxy fatty acids; KDO, keto-3deoxy-octulosonic acid; FAME, fatty acid methyl ester; SIE, selected ion extraction; EI, electron ionization; LOD, limit of detection; LOQ, limit of quantification.

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spectrometry (GC–MS) to detect and quantify 3-OH-FA in LPS has been extensively used (Lin, Huang, Hsieh, Chen, & Chen, 2007; Rybka & Gamian, 2006; Saraf et al., 1999; Szponar et al., 2002), but has never been applied directly to determine the LPS content in samples containing carbohydrates, such as polysaccharides from natural sources. Adapting the methods showing monosaccharide composition, one can simultaneously observe the presence of 3-OH-FA (Sassaki et al., 2008). 3-OH-FAs are mainly analyzed by GC–MS as acetate or trimethylsilyl derivatives of their methyl esters (FAME), since the presence of the hydroxyl group sometimes interferes with the elution on GC–MS (Lin et al., 2007; Szponar et al., 2002). Acetylation provides stable derivatives (Sassaki et al., 2008), and the fragment ions formed on GC–MS can show the position of the OH group, whether α or β.

In order to overcome these problems, we have now developed a GC–MS assay that specifically detects and quantifies LPS in the presence of carbohydrate-containing material. This is based on 3-OH-FAs as specific chemical markers for LPS. Selected ion extraction (SIE) (Dodds, McCoy, Rea, & Kennish, 2005), using 3-OH-FA specific ions, increases the sensitivity and reduces interference arising from other components. It was then possible to detect LPS in concentrations lower than 1 ng, several times less than those frequently used as positive control in biological assays performed with carbohydrate-containing samples (Kataoka et al., 2002).

2. Materials and methods

2.1. Samples and derivative preparation

The LPS standard from *Escherichia coli* serotype O111:B4 was obtained from Sigma (St. Louis, MO, USA) and diluted in deionized water and the solution was then sonicated (two cycles of 15 min). An aliquot of LPS (0.3 mg) was collected and placed in reinforced Pyrex tubes with 4 mL Teflon lined screw-cap vessels (Supelco, Bellefonte, PA, USA). The solution was dried under a N_2 stream, and the residue was dissolved in 400 μL MeOH (Merck, Darmstadt, Germany) and subjected to methanolysis by the addition of 100 μL of 3 M MeOH–HCl (Supelco, Bellefonte, PA, USA). The vial was vortexed (1 min) and kept at 80 °C for 20 h. The solution was then partitioned between hexane (1 mL) and deionized water (0.5 mL). The hexane phase was evaporated under a gentle N_2 stream, and acetylated with a mixture of pyridine (100 μL) and acetic anhydride (100 μL) at 100 °C for 1 h. Serial dilutions of the acetylated product were then prepared, using acetone as solvent, corresponding to concentrations of 75, 50, 37.5, 25, and 10 ng of LPS, which were analyzed by GC–MS. In order to demonstrate the applicability of the method, 14 polysaccharide samples that had been previously tested in biological assays and which represent different carbohydrate structures, were submitted to analysis. Each one was diluted in deionized water and vigorously shaken for 12 h. An aliquot containing 0.3 mg of each sample was then collected, the procedure being that described above up to the acetylation step. Samples were gently evaporated under a N_2 stream, and the residues dissolved in acetone (5 μL), concentrated down to 1 μL, and analyzed by GC–MS. In addition to these experiments, in order to check the procedure, a GC–MS analysis was performed in the presence of the polysaccharide samples spiked with LPS (50 ng).

Table 1
Retention times and EI–MS reporter fragments obtained from fatty acid methyl esters (FAMEs).

	R_t	Reporter ions, *m/z*
FAME[a]		
$C_{12:0}$	17.63	214-185-171-157-143-129-87-74-55
$C_{14:0}$	21.75	242-213-199-185-171-157-143-129-87-74-55
$C_{16:0}$	25.70	270-241-227-213-199-185-171-157-143-129-87-74-55
Ac-FAME[a]		
2-OAc-$C_{10:0}$	19.27	201-165-146-124-111-97-89-83-55-43
$C_{14:0}$	21.75	242-213-199-185-171-157-143-129-87-74-55
3-OAc-$C_{12:0}$	22.73	243-166-143-127-123-113-110-103-96-81-74-69-55-43
$C_{16:0}$	25.70	270-241-227-213-199-185-171-157-143-129-87-74-55
3-OAc-$C_{14:0}$	26.49	257-166-143-123-113-110-103-96-81-74-69-55-43

[a] FAMEs were obtained by methanolysis of LPS from *E. coli* serotype O111:B4. The hexane phase is that obtained by partition with water of the methanolyzate of LPS standards. It was evaporated to dryness and the residue acetylated.

2.2. GC–MS analysis

GC–MS analyses were carried out using a Varian 3800 gas chromatograph with a 4000 MS detector (ion trap) equipped with 30 m × 0.25 mm i.d. low-bleed/MS capillary column (VF-1MS), both from Agilent/Varian, Santa Clara, CA, USA. The temperature ramp

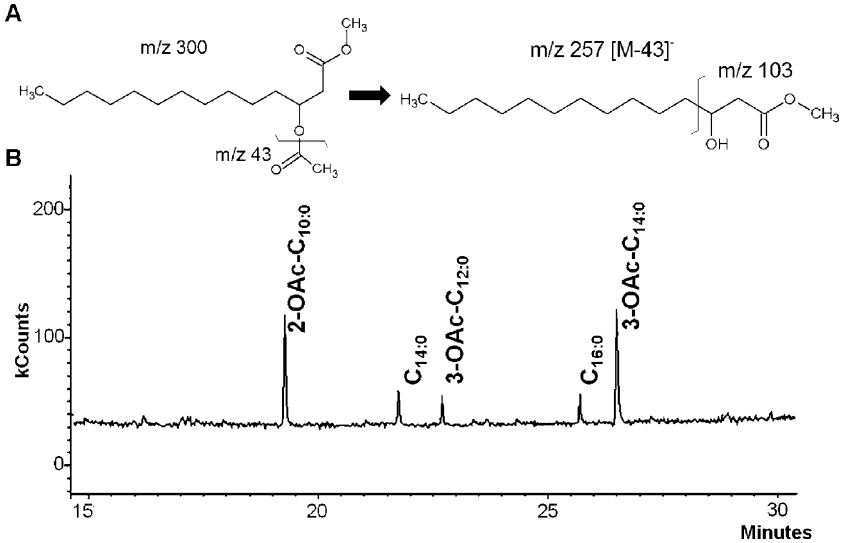

Fig. 1. GC–MS of acetylated hexane phase containing methanolyzed LPS. (A) Fragmentation mechanism proposed for methyl 3-OAc-$C_{14:0}$ and its $[M-43]^-$ ion, resulting from loss of its acetyl group. (B) GC–MS of LPS samples subjected to methanolysis and acetylation. Identification was based on the spectrum and retention time of each peak.

was: injector 250 °C, oven initially at 50 °C, held for 2 min, heated to 90 °C (20 °C min^{-1}, then held for 1 min) and then to 280 °C (5 °C min^{-1}, then held for 2 min). Electron ionization (EI) spectra were obtained at 70 eV at 200 °C. The injection volume was 1 μL, with a split ratio of 1:10. Post-run analysis was performed with a SaturnWorkstation 5.1 (Sassaki et al., 2008).

3. Results and discussion

After methanolysis and solvent partition of the LPS-containing samples between hexane and water, GC–MS of hydroxyl fatty acid methyl esters in the hexane phase was unsuccessful, since they were not eluted. Identified were only non-hydroxylated fatty acid esters, the usual components of the lipid A moiety of LPS molecules (Raetz, 1990). These were, as main components, $C_{12:0}$, $C_{14:0}$, and $C_{16:0}$ fatty acids (Table 1) in a 9:4:1 ratio. This agrees with previously obtained results, where saturated chains of lauric and myristic acid were those esterified with the hydroxyl group of β-OH fatty acids present in the lipid A from *E. coli* LPS (Morrison & Leive, 1975).

The residue in the hexane phase was therefore acetylated to give -OAc derivatives, which were examined by GC–MS. Identified were methyl esters of mainly 3-OAc-$C_{14:0}$ (Fig. 1), with 3-OAc-$C_{12:0}$ and 2-OAc-$C_{10:0}$. Also identified were $C_{14:0}$ and $C_{16:0}$ fatty acid methyl esters. As in previous studies (Szponar et al., 2002), 3-OAc-$C_{14:0}$ methyl ester was chosen as the chemical marker for LPS because this showed the best correlation with the standard procedure for detecting LPS in samples (LAL assay). It was more reliable than 3-OH $C_{10:0}$, $C_{12:0}$, $C_{16:0}$ and C_{18} fatty acids (Saraf et al., 1999).

GC–MS analyses were carried out using various dilutions of the acetylated residue, thus providing a standard curve of LPS concentration vs. integrated area of the extracted-ion chromatograms (Ferguson, 1992). This gave a high correlation factor of 0.97 (Fig. 2), so with the use of SIE we could determine the presence of the 3-OAc-$C_{14:0}$ methyl ester and confirm the position of the hydroxyl group from its characteristic ions with *m*/*z* 257 and 103. SIE improved the S/N ratio, similar effects were found employing *m*/*z* 103 with 257 ions, and that with *m*/*z* 257 ([M-43]) alone (Table S1) (Dodds et al., 2005). Detection and quantification were possible to limits as low as 100 fmol (Table S1). The limit of detection (LOD) was calculated as the mass of LPS which gave a signal 3σ above the mean blank signal, where σ is the standard deviation of the blank signal. The limit of quantification (LOQ) is the mass of LPS that gives a signal 10σ above the mean blank signal (Priego-Capote, Ruiz-Jiménez, & Luque de Castro, 2007). At all tested concentrations, the values of LOD and LOQ were at least 10 times lower (data not shown).

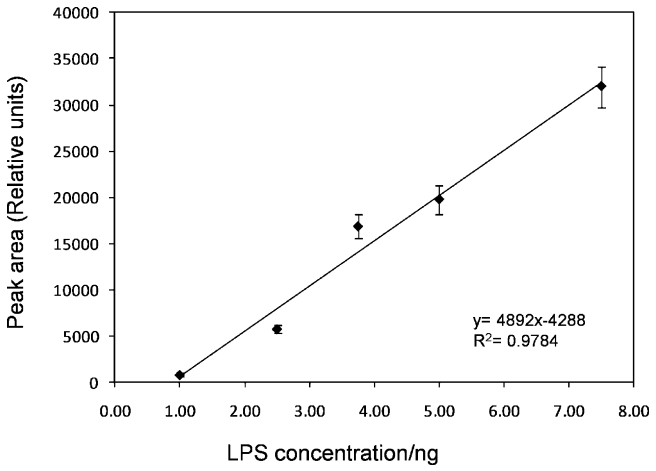

Fig. 2. Standard curve for LPS in the 1.0–7.5 ng range, with the use of SIE chromatogram for the *m*/*z* 257 ion. Serial dilutions of the reference sample of LPS from *E. coli* 0111:B4 were subjected to analysis. The quantity of LPS was calculated assuming that its molecular mass was ~10 kDa and contained 3,4-OH-$C_{14:0}$ residues (976 Da), similar to ~10.0% (w/w) of 3-OH-$C_{14:0}$ in LPS.

In order to test the applicability of the above procedure for detecting LPS in polysaccharide samples, various polysaccharides, obtained from different natural sources and different extraction procedures, were examined. Employed was a procedure similar to that for the LPS samples, GC–MS data being analyzed using both full-scan and the SIE chromatograms, employing *m*/*z* 257 and 103 ions. With two of the polysaccharides analyzed, LPS was detected and quantified (Table 2). In analysis of the spiked samples, the full-scan chromatogram contained many ion peaks from the carbohydrate methanolyzate (Fig. 3A), occasionally present in the hexane phase following acetylation. However, using the extracted-ion chromatogram (*m*/*z* 257 and 103 + 257 ions), detected specifically was the 3-OAc-$C_{14:0}$ methyl ester, with little interference from other components (Fig. 3B). Occasionally, one could find

Table 2
Polysaccharides subjected to LPS determination analysis.

Polysaccharide	Main chemical structure	LPS/polysaccharide (ng/300 μg)	LPS spiked samples (LOD in ng)	LPS spiked samples (LOQ in ng)	Reference
XGC	Branched α (1 → 6)-D-xylan	[a]	2.0	5.3	Rosário et al. (2010)
XGJ	Branched α (1 → 6)-D-xylan	[a]	2.3	6.1	Rosário et al. (2010)
ABN	Branched β (1 → 4)-D-xylan	[a]	1.9	4.4	[c]
ARAGAL	Branched β (1 → 3)-D-galactan	[a]	1.8	4.3	Moretão, Zampronio, Gorin, Iacomini, and Oliveira (2004)
GMPOLY	Branched α (1 → 6)-D-mannan	[a]	1.5	1.9	Noleto et al. (2002)
MPOLY	Branched α (1 → 6)-D-mannan	[a]	1.4	3.4	Noleto, Mercê, Iacomini, Gorin, and Oliveira (2004)
A1	Branched β (1 → 3)-D-glucan	16.15	[b]	[b]	Smiderle et al. (2008a)
A2	Branched β (1 → 3)-D-glucan	[a]	1.9	4.4	[c]
A3	Branched β (1 → 3)-D-glucan	[a]	1.8	4.3	[c]
A4	β (1 → 6)-D-glucan	12.73	[b]	[b]	[c]
A5	Branched α (1 → 6)-D-galactan	[a]	2.0	5.3	Smiderle et al. (2008b)
A6	β (1 → 6)-D-glucan	[a]	2.3	6.1	Smiderle et al. (2011)
A7	[d]	[a]	1.5	1.9	[c]
A8	[d]	[a]	1.4	3.4	[c]

The LOD and LOQ are relative to the volume injected (1 μL), and were calculated on the basis of the volume that actually were analyzed (0.1 μL), as determined by the split ratio (1:10).
[a] Not detected.
[b] Not analyzed.
[c] Structure not yet elucidated.
[d] Not published yet.

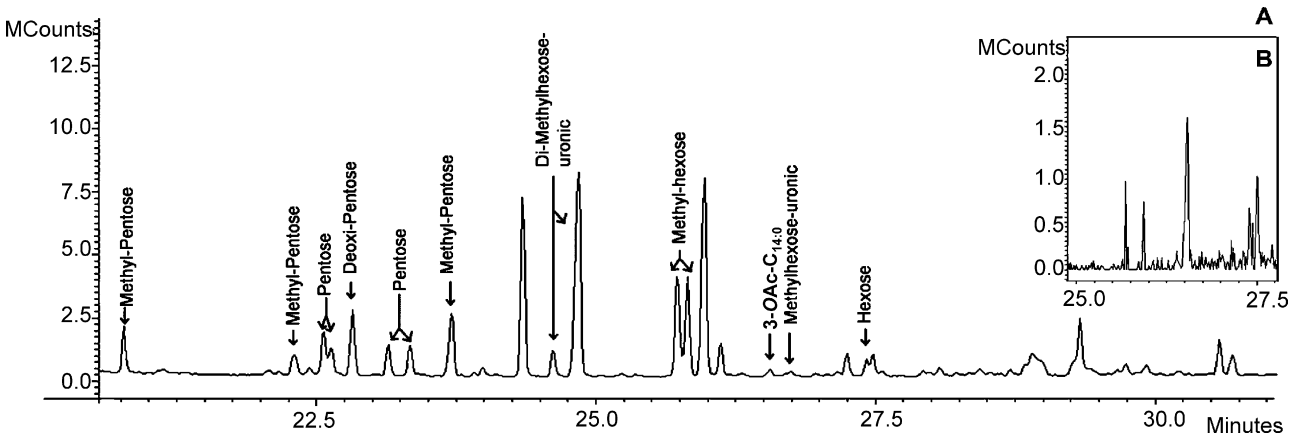

Fig. 3. Partial ion chromatogram of a polysaccharide sample spiked with 50 ng of LPS. (A) The polysaccharide sample chromatogram, without any filters and in (B), an insert of the same chromatogram filtered with *m*/*z* 257. (A) has a high incidence of contaminants derived from the carbohydrates released by methanolysis and which underwent acetylation. With the use of SIE with an *m*/*z* 257 ion, (B) an enhanced peak with a retention time of 3-OAc-$C_{14:0}$ methyl ester is shown. The identity of the compounds was determined by examination of the EI-MS spectra.

carbohydrate-derived molecules, giving vestiges of the *m*/*z* 257 or 103 ions, like those present in the sample analyzed in Fig. 3A, but neither of them occurred in the filtered chromatogram co-eluted with the 3-OAc-$C_{14:0}$ methyl ester (Fig. 3B). Comparing the intensity of the *m*/*z* 257 ion, with the others, the components clearly arose from the carbohydrate methanolyzate (Fig. S1A–C). To confirm this, the EI-MS spectrum of a standard LPS sample was compared with the EI-MS spectrum derived from the peak corresponding to the 3-OAc-$C_{14:0}$ methyl ester in the spiked sample (Fig. S1A and C, respectively). The mass spectrum showed that the SIE with the *m*/*z* 257 ion indicates the exact elution time of the 3-OAc-$C_{14:0}$ methyl ester, which had its structure confirmed by the presence of ion fragments with *m*/*z* 103 and 143. Since that with *m*/*z* 143 could be problematical, due to its ubiquitous presence on lipid-containing samples, the use of glycolipids in these kind of assays is very uncommon. The present results agree with previous ones, and confirm the broad applicability of our method when applied to different carbohydrate samples. These should validate results obtained from biological studies, specifically those performed with negatively charged polysaccharides and (1 → 3)-linked β-glucans, where the presence of LPS could not be detected by previously available tests (Kataoka et al., 2002; Novitsky, 1998).

The LPS from *E. coli* serotype O111:B4 has a molecular mass (*M*), estimated to be ~10 kDa (Aurell & Wistrom, 1998), and contains, attached to lipid A in each individual LPS molecule, 4 molecules of 3-OH-$C_{14:0}$ fatty acid, which corresponds to 976 Da, representing ~10% of the total LPS mass. Thus, the amount detected of 3-OAc-$C_{14:0}$ methyl ester would have to be increased tenfold to show the real LPS concentration detected (Rybka & Gamian, 2006). This means that even when detecting only a minor component of the LPS molecule, detection and quantification reached the femtomol range, proving that GC–MS assays are extremely sensitive, if allied to the unequivocal identification parameters, GC retention time and mass spectra. This estimate can be used to determine the LPS content of unknown samples, since the M_W of *E. coli* serotype O111:B4 is similar to others, and even with other *Enterobacteriaceae*, described in the literature (Romeo & Rosano, 1970).

Concerning the origin of LPS detected in two of our polysaccharide samples, one can infer that it could be present in the biological material prior to extraction and purification. However, there are several other factors involved that could originate this contamination, such as incorrect extraction procedures and storage of the purified polysaccharides. Thus, even with all the care taken during the entire extraction and purification process, some of these steps could provide more opportunity of contamination. Consequently, it is incorrect to affirm that when a contamination of the polysaccharide with LPS is detected, the main cause of the problem is the biological source of the material.

4. Conclusion

The results presented herein provide methodology to detect the presence of LPS in polysaccharide samples, as well as monosaccharide composition determinations. The robustness, sensitivity, and wide applicability allow its application to other biologically important carbohydrate preparations, used in immunological experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.11.073.

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