Analysis of Ubiquinones, Dolichols, and Dolichol Diphosphate-Oligosaccharides by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

Teresa A. Garrett, Ziqiang Guan and Christian R. H. Raetz. Methods in Enzymology. Volume 432, 2007, Pages 117-14.

https://doi.org/10.1016/S0076-6879(07)32005-3

Materials

High performance liquid chromatography (HPLC)–grade solvents were from VWR (International Leicestershire, England). Ammonium acetate was from Mallinckrodt (Hazelwood, MO). Zorbax SB-C8 (2.1 × 50 mm, 5 μm) reverse-phase column was from Agilent (Palo Alto, CA). Coenzyme Q6 and Q10 standards were from Sigma-Aldrich (St. Louis, MO). Synthetic nor-dolichol was from Avanti Polar Lipids (Alabaster, AL). Dolichol-diphosphate-oligosaccharide purified from pig pancreas was a gift from Dr. R. Gilmore (University of Massachusetts Medical School, Worcester, MA). All extractions were done in glass tubes equipped with Teflon-lined screw caps using disposable glass pipettes.

Liquid Chromatography-Mass Spectrometry

LC-MS analysis was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps, and a SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight (TOF), tandem mass spectrometer (Applied Biosystems/MDS Sciex). A Zorbax SB-C8 reversed-phase column (5 μm, 2.1 × 50 mm) was used for all LC-MS analyses. LC was operated at a flow rate of 200 μl/min with a linear gradient as follows: 100% mobile phase A (methanol:acetonitrile:aqueous 1 m*M* ammonium acetate; 60:20:20) was held for 2 min, then linearly increased to 100% mobile phase B (100% ethanol containing 1 m*M* ammonium acetate) over 14 min and held at 100% mobile phase B for 4 min. The column was re-equilibrated to 100% mobile-phase A for 2 min prior to the next injection. The post-column split diverted 10% of the LC flow to the ESI source. All MS data were analysed using Analyst QS software (Applied Biosystems/MDS Sciex). The mass spectrometer was calibrated in both the negative and positive mode using PPG3000 (Applied Biosystems).

Preparation of Lipid Extracts

RAW cells, cultured were extracted using the method of Bligh and Dyer (1959), as shown in **Figure 1**. A 150-mm tissue culture plate at about 90% confluence was washed with 10 ml phosphate buffered saline (PBS) and then scraped into 5 ml PBS (137 m*M* NaCl, 0.027 m*M* KCl, 0.01 m*M* Na_2HPO_4, 0.0018 m*M* KH_2PO_4). The cell suspension was centrifuged at 400×*g* to harvest the cells. This culture plate size typically yields about 100 × 10^6 cells or ∼0.1 g of cells (wet weight). The cell pellet is resuspended in 1 ml of PBS and transferred to a 16 × 150-mm-glass centrifuge tube with a Teflon-lined cap. Next, 1.25 ml of chloroform and 2.5 ml of methanol are added to generate a single-phase Bligh-Dyer mixture (final ratio: 1:2:0.8, C:M:PBS). This mixture is vigorously mixed with a vortex and, if necessary, subjected to sonic irradiation in a bath apparatus for 2 min to break up any chunks of cells. The single-phase extraction mixture is incubated at room temperature for 15 min, then centrifuged at ∼500×*g* for 10 min in a clinical centrifuge to pellet the cell debris (**Fig. 1**). The supernatant is transferred to a fresh tube; 1.25 ml chloroform and 1.25 ml PBS are added to generate a two-phase system (final ratio; 2:2:1.8, C:M:PBS). After vigorous mixing, the tubes are centrifuged as previously stated to resolve the phases. The upper phase and any interface that may be present is removed and discarded. The interface is generally minimal. Next, the lower phase is washed with 4.75 ml pre-equilibrated neutral upper phase, vortexed, and centrifuged as previously stated to resolve the phases. After removal of the upper phase, the lower phase is dried under a stream of nitrogen, and the dried lipids are stored at −20° until analysis. Generally, from 0.1 g of cells or tissue, about 10 mg of dried lipid is obtained.

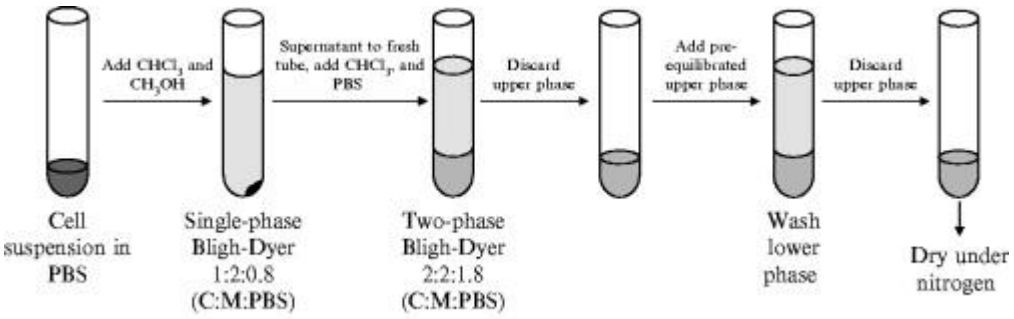
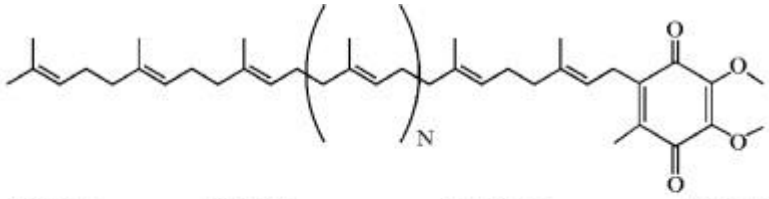


Figure 1. Lipid extraction procedure.

Note on scaling up: This method of extraction can be used with larger quantities of cells or tissues. Volumes should be scaled accordingly; however, a three- to five-fold concentration of the initial PBS suspension of cells or tissue should not affect extraction efficiency. With larger volumes, 250 ml Teflon-lined centrifuge bottles (VWR) are useful for centrifugation and separation of the phases.

LC-MS Detection and Quantification of Coenzyme Q



N	CoQ species	Formula [M]	$[M+H]^+$ Observed	$[M+H]^+$ Exact	$[M+NH_4]^+$ Observed	$[M+NH_4]^+$ Exact	$[M+Na]^+$ Observed	$[M+Na]^+$ Exact
1	CoQ6	$C_{39}H_{58}O_4$	591.434	591.441	608.469	608.467	613.423	613.423
4	CoQ9	$C_{54}H_{82}O_4$	795.647	795.629	812.678	812.655	817.640	817.611
5	CoQ10	$C_{59}H_{90}O_4$	863.713	863.691	880.734	880.718	885.689	885.673

Figure 2. Structures and masses of coenzyme Q6, Q9, and Q10.

In order to develop LC-MS methods for the detection of CoQ in total lipid extracts, we needed to:

(1) determine the linear range for MS response with respect to the amount of standard analysed;
(2) establish that this linear range applied to the MS response when analysing the standard in total lipid extracts;
(3) show that the number of isoprene units on the CoQ did not alter the MS response; and
(4) determine the amount of standard to add to our sample to get approximately the same peak area of the extracted ion current for the standard and the analyte. We found that we could use a CoQ with a different number of isoprene units as a standard instead of using deuterated CoQ.

We obtained two CoQ standards, one with 6 isoprenoid units, CoQ6, and the other with 10 isoprenoid units, CoQ10 (Sigma) (**Fig. 2**). For LC-MS analysis, the standard was re-dissolved in $CHCl_3$ and diluted into DMSO:MeOH (9:1 v/v) at a concentration of 0.1, 0.2, 0.5, 1, 2, or 5 ng/µl. Next, 10 µl of each dilution were injected, in turn, onto a Zorbax SB-C8 (2.1 × 50 mm, 5 mm) column at 200 µl/min and eluted as described above. CoQs were detected in the positive ion mode as $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ ions using the following mass spectrometer settings: ESI voltage, +5500 V; declustering potential, 60 V; and focusing potential, 265 V; and nebulizer gas, 20 psi.

Figure 3A shows the extracted ion current (EIC) for the $[M+H]^+$ ion of CoQ6 and CoQ10. Each standard was analysed in separate LC-MS runs, and the spectra were overlaid. The CoQ6 elutes from the reverse phase column at about 11.1 min, and the CoQ10 elutes at about 13.7 min, consistent with the difference in the polyprenol chain length. **Figure 3B** and **C** show the positive ion MS and collision-induced dissociation MS (CID-MS) for the $[M+H]^+$ CoQ6 standard. The collision energy was set at 52 V (laboratory frame of reference), and nitrogen was used as the collision gas. The major fragment ion is *m/z*197.079, which corresponds by exact mass to a proton

adduct of the quinone ring and is formed by the elimination of the isoprenoid chain. The CID-MS of the CoQ10 standard produces a similar fragment ion pattern (data not shown). As shown in **Fig. 3D**, the peak area of the EICs of the CoQ6 and CoQ10 standards are linear for 1 ng to 50 ng injected onto the column. This also shows that, for CoQs, the number of isoprenoid repeats does not alter the ionization efficiency, as is sometimes seen with other lipids analysed by MS. In addition, the peak area of the EIC of the CoQ6 standard increased linearly when increasing amounts of the standard were co-extracted with the RAW cells (data not shown). Therefore, the linear range established using the standard also applies to the analysis of CoQs from total lipid extracts.

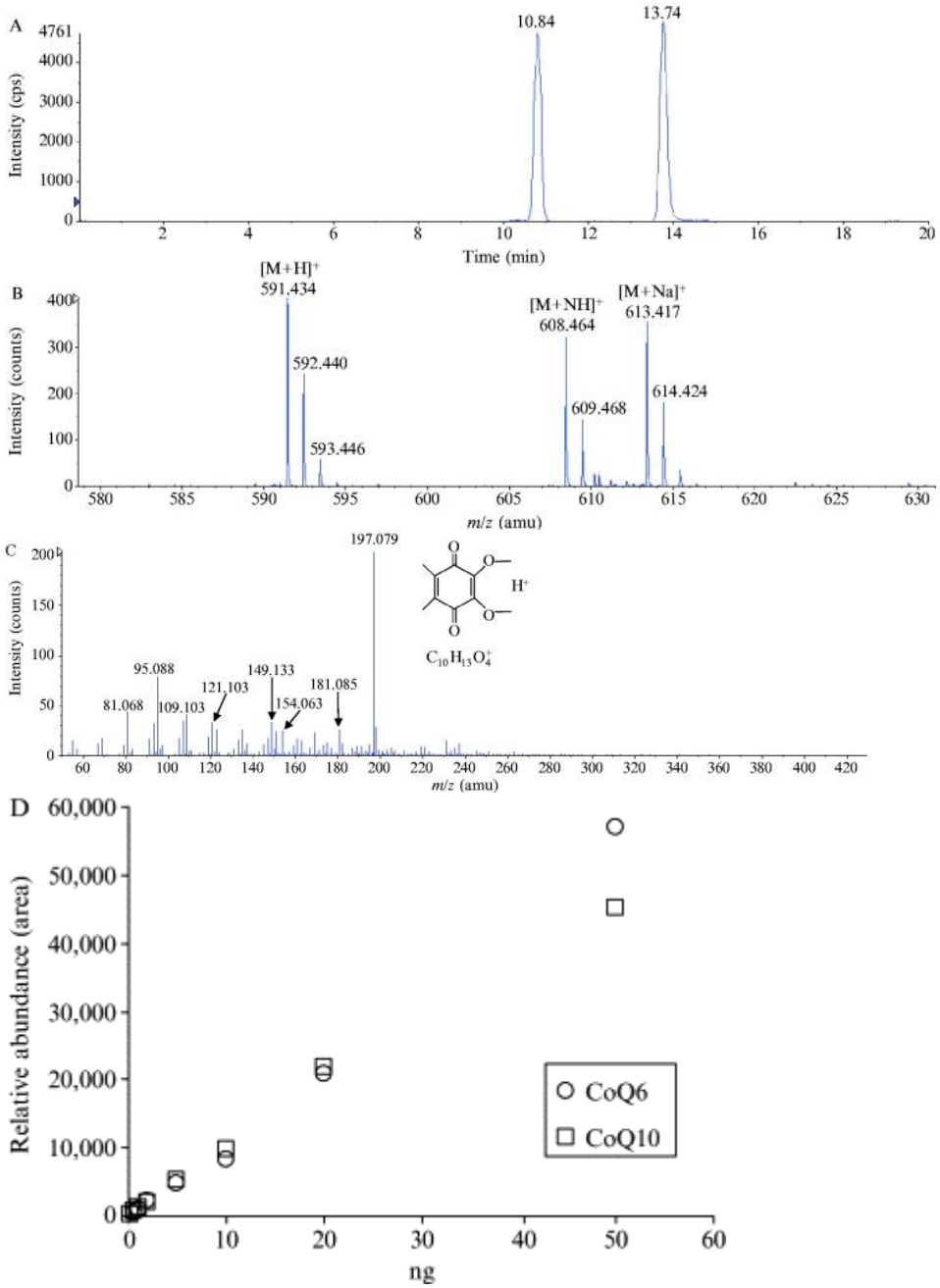


Figure 3. Positive-ion liquid chromatography-mass spectrometry (LC-MS) analysis of CoQ6 and CoQ10 standards. (A) The extracted ion current (EIC) for the $[M+NH_4]^+$ ions of CoQ6 (608.464 *m/z*) and CoQ10 (880.734 *m/z*) standards. Under these liquid chromatography conditions, the CoQ6 elutes at about 10 minutes while the CoQ10 elutes at about 14 minutes. (B) The mass spectrum of the material eluting from minutes 10.6 to 11.1, representing the CoQ6 standard. The CoQ6 form $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$adduct ions. (C) The collision-induced mass spectrometry (CID-MS) of the $[M+H]^+$ ion of the CoQ6 standard. The major fragment ion (*m/z* 197.079) corresponds to the quinonering and is formed by the elimination of the isoprenoid chain. (D) The plot of the peak area of the extracted ion current of the $[M+H]^+$ ion of CoQ6 and CoQ10 versus the nanogram (ng) of standard injected onto the Zorbax C-8 reverse phase column. The peak areas of the CoQ6 and the CoQ10 were similar when equivalent amounts of standard were analysed.

Because the major CoQs found in mouse tissue are CoQ9 and CoQ10, we chose to use CoQ6 as the internal standard for our quantitative analysis CoQ6 is not found in RAW cells and is not isobaric with other ions eluting at a similar retention time.
In addition to establishing the linear range of the MS response, we needed to determine the amount of CoQ6 standard to add to the extraction mixture in order for the peak area of the EIC for the standard to be about equal to the peak area of the EIC for the CoQs being quantified in the sample. By varying the amount of CoQ6 added during the co-extraction, we determined that, for a 150-mm culture dish grown to ~90% confluence, 5 μg of CoQ6 standard gave an EIC peak area about equal to the EIC peak of the major coenzyme species of the cell, CoQ9.

Overall, our quantification procedure for CoQs in RAW cells is as follows: 5 μg of CoQ6 (dissolved in $CHCl_3$ at 1 mg/ml) standard is added to the cell suspension (1 ml) prior to the addition of chloroform (1.25 ml) and methanol (2.5 ml) to form the single-phase extraction mixture; the lipids are extracted as previously described, dried under a stream of nitrogen, and stored at −20° until analysis; the dried lipids are re-dissolved in 0.2 ml of chloroform:methanol (1:1, v:v) and diluted twofold into chromatography solvent A; 10 μl is injected onto the reverse-phase column and analysed using the LC-MS procedure previously shown. The extracted ion current and mass spectra for the CoQ6 standard and endogenous CoQ9 are shown in **Figs. 4A–D**. For CoQ9, the major ion species are observed as $[M+H]^+$ at *m/z* 795.647, $[M+NH_4]^+$ at *m/z* 812.678, and $[M+Na]^+$at *m/z* 817.640 (**Fig. 3D**). For CoQ10, the major ion species are observed as $[M+H]^+$ at *m/z* 863.713, $[M+NH_4]^+$ at *m/z* 880.734, and $[M+Na]^+$ at *m/z* 885.673 (data not shown). The peak area of the EIC for the $[M+NH_4]^+$ is used to quantify the total content of CoQ9 and CoQ10 in the RAW cells. For example, the quantification of the CoQ9 is accomplished using the following equation:
[(CoQ9 peak area/CoQ6 standard peak area) × 5 μg]/MW of CoQ9 = μmol CoQ9

in the sample. Using this method, we have been able to detect CoQs in all cells and tissues we have analysed. LC-MS Detection and Quantification of Dolichol

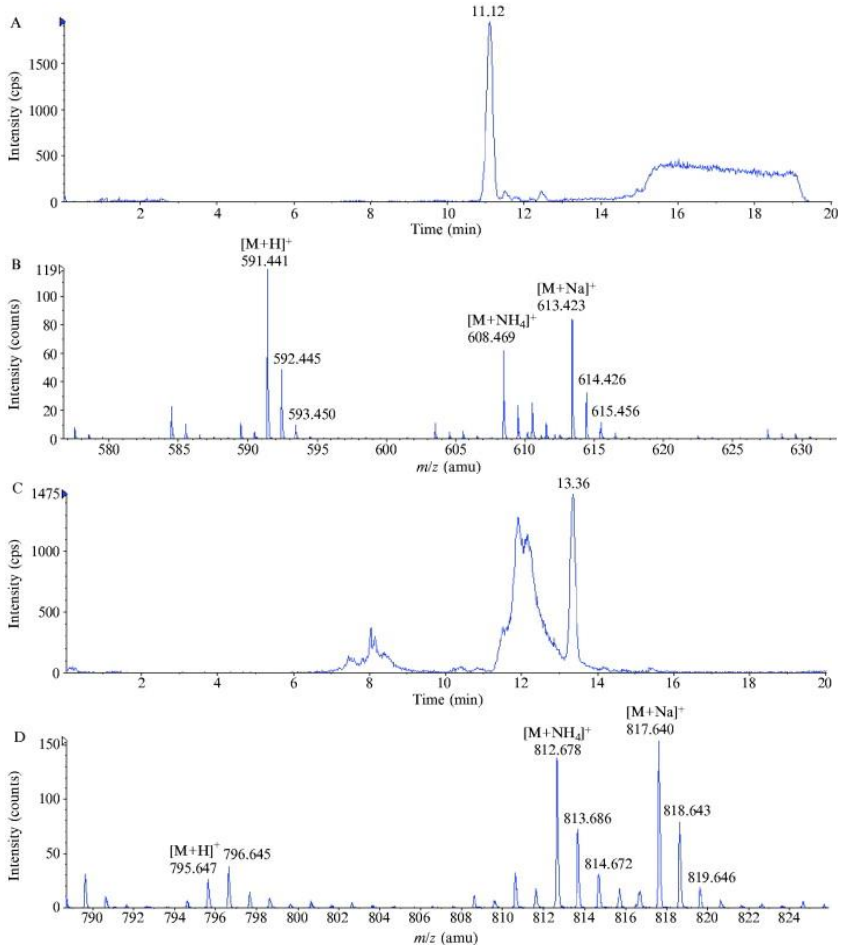
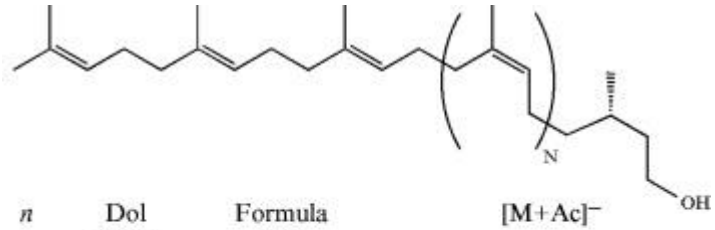


Figure 4. Positive ion liquid chromatography-mass spectrometry (LC-MS) analysis of CoQs in RAW lipid extracts. (A) The extracted ion current (EIC) for the $[M+NH_4]^+$ ion of CoQ6 standard after 1 μg of standard is co-

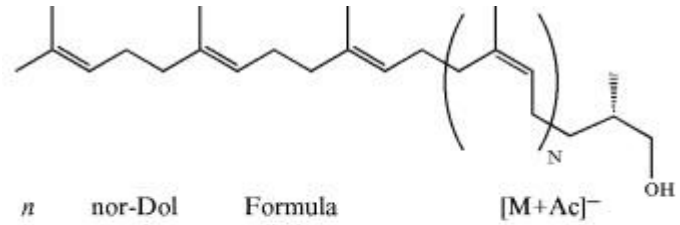
extracted in the presence of RAW cells from one 150-mm plate. The retention time (11.12 min) is consistent with the retention time seen when the standard is injected alone (**Fig. 3A**). (B) The mass spectrum of the material eluting between 10.9 and 11.3 min. The $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ ions of the CoQ6 standard are easily resolved. (C) The extracted ion current of the $[M+NH_4]^+$ ion of the major CoQ of RAW cells, CoQ9. The CoQ9 elutes at approximately 13.3 min under these liquid chromatography conditions. The peaks at approximately 8 and 12 min are due to isobaric lipids found in the lipid extract and are not due to CoQ9. (D) The mass spectrum of the material eluting between 13.3 and 14.3 min. The $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ ions of the CoQ9 standard are detected.



n	Dol species	Formula [M]	[M+Ac]⁻ Observed	[M+Ac]⁻ Exact
13	Dol-17	$C_{85}H_{140}O$	1236.076	1236.104
14	Dol-18	$C_{90}H_{148}O$	1304.133	1304.166
15	Dol-19	$C_{95}H_{156}O$	1372.178	1372.229
16	Dol-20	$C_{100}H_{164}O$	1440.239	1440.292

Figure 5. Structures and masses of the acetate adducts of dolichols-17, -18, -19, and -20.

Quantitative, chemically defined standards of dolichol are not commercially available. In order to quantify the dolichol content of the RAW cells, a nor-dolichol standard with 17 to 20 isoprene units, which differs from dolichol (Figure 5.1, Figure 5.7, Figure 5.8) by the absence of one CH_2 unit at the hydroxyl end of the molecule, was prepared by modification of natural dolichol at Avanti Polar Lipids.



n	nor-Dol species	Formula [M]	[M+Ac]⁻ Observed	[M+Ac]⁻ Exact
13	nor-Dol-17	$C_{84}H_{138}O$	1222.137	1222.086
14	nor-Dol-18	$C_{89}H_{146}O$	1290.196	1290.151
15	nor-Dol-19	$C_{94}H_{154}O$	1358.255	1358.214
16	nor-Dol-20	$C_{99}H_{162}O$	1426.316	1426.276

Figure 6. Structures and masses of the acetate adducts of nor-dolichols-17, -18, -19, and -20.

For the LC-MS analysis, we established, as with the coenzyme Q, the linear range of the MS response with respect to the amount of nor-dolichol injected onto the column. Using the same LC-MS system as previously shown and detecting in the negative ion mode, 5, 20, 50, or 100 ng in 10 µl of chromatography solvent A were injected onto the Zorbax SB-C8 (2.1 × 50 mm, 5 µm) reverse-phase column and eluted using the same scheme as previously described. The mass spectrometer settings are as follows: ESI voltage, −4400 V; declustering potential, −55 V; focusing potential, −265 V; neubuliser gas, 18 psi. The semi synthetic nor-dolichol is a mixture of species with the number of isoprene units ranging from mainly 16 to 21. The smaller nor-dolichols, such as nor-dolichol with 17 isoprene units, eluted from the column first, followed by the larger nor-dolichol species. The nor-dolichols are detected as the acetate adduct $[M+Ac]^-$ ions in the negative ion mode using the LC-MS method as previously described. **Figure 7A** shows the EIC for the predominant nor-dolichol-19. The MS of 16.3 to 17.3 min when the nor-dolichol-19 acetate adduct $[M + Ac]^-$ elutes from the column is shown **(Fig. 7B)**. Small amounts of contaminants at *m/z* 1334.213 and 1344.241 may correspond to oxidative loss of additional carbon atoms from the dolichol during the preparation of the nor-dolichol. Similar to the CoQ standard, the peak area of the EIC for the dolichol standard was linear for 5 ng to 50 ng injected on the column (**Fig. 7C**).

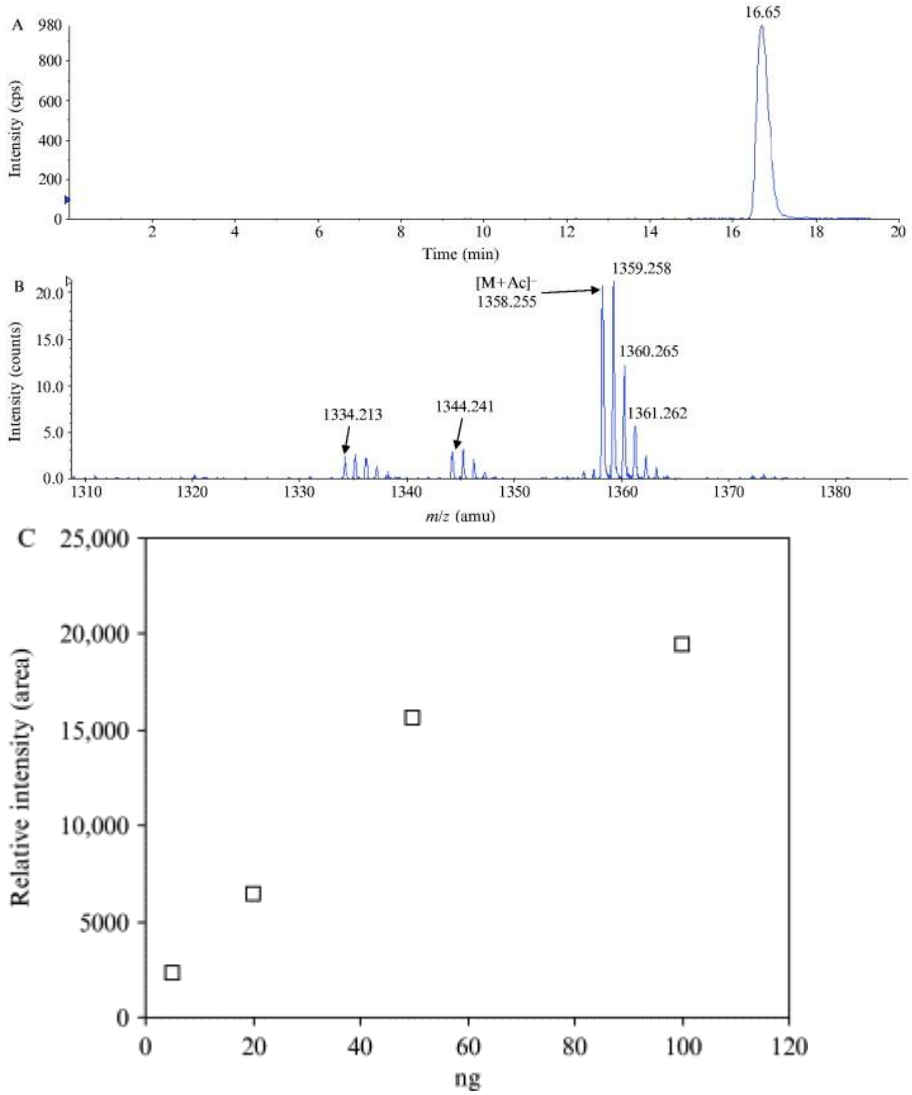


Figure 7. Negative-ion liquid chromatography-mass spectrometry (LC-MS) analysis of nor-dolichol standard. (A) The extracted ion current (EIC) for the $[M+Ac]^-$ ions of the nor-dolichol-19 (1358.255 *m/z*) standard. Under these liquid chromatography conditions, the nor-dolichol-19 elutes at approximately 16.7 min. The shorter nor-dolichols, nor-dolichol-17 and 18, elute slightly earlier, while the nor-dolichol-20 elutes slightly later. (B) The mass spectrum of material eluting between 16.3 to 17.3 min, representing mainly nor-dolichol-19. The small peaks at *m/z* 1334.213 and 1344.241 are likely due to loss of additional carbons from the dolichol during the preparation of the nor-dolichol standard. (C) The plot of the peak area of the extracted ion current of the $[M+Ac]^-$ ion of nor-dolichol-19 versus the nanogram (ng) of standard injected onto the Zorbax C-8 reverse-phase column.

We determined the amount of nor-dolichol standard to add to the extraction mixture in order to have the peak area of the EIC for the standard be about equal to the peak area of the EIC for the dolichols being quantified in the sample. By varying the amount of nor-dolichol added during the co-extraction, we determined that, for a 150-mm culture dish grown to ~90% confluence, 1 μg of the nor-dolichol standard gave an EIC peak area of the nor-dolichol-18 about equal to the EIC peak of the major dolichol species of the cell, dolichol-18.

The ion intensities of dolichol species present in RAW cell lipid extracts are relatively low compared to those of CoQs. Dolichol cannot be detected by direct injection of total lipid extracts without prior chromatography. In addition, while CoQ ions can be readily detected when approximately 5% of the total lipid extract is analysed using the above LC-MS system, endogenous dolichol ions are only effectively detected when 15% of the total lipid extract is injected onto the reverse-phase column.

Overall, our method for quantifying dolichol is as follows: 1 μg of nor-dolichol (dissolved in $CHCl_3$ at 1 mg/ml) is added to the cell suspension (1 ml) just prior to the addition of chloroform (1.25 ml) and methanol (2.5 ml) to form the single-phase extraction mixture; the lipids are extracted as previously described, dried under a stream of

nitrogen, and stored at −20° until analysis; the dried lipids are redissolved in 0.2 ml of chloroform/methanol (2:1, v/v), and 30 µl is injected onto the reverse-phase column and analyzed using the LC-MS procedure previously described**. Figure 8A to D** shows the EIC and MS for the endogenous dolichol with 17, 18, 19, and 20 isoprene units. The peak area of the EIC for the $[M + Ac]^-$ ion of the nor-dolichol-17 was used to quantify dolichol-17; nor-dolichol-18 was used to quantify dolichol 18 and so forth. Because the nor-dolichol standard is a mixture, we needed to determine which fraction of the standard was nor-dolichol-17, nor-dolichol-18, etc. To do this, we summed the peak area of the EIC of nor-dolichol-16 to nor-dolichol-21 to obtain the total peak area when 1 µg of standard was injected onto the column. The total amount of nor-dolichol-18, for example, was then calculated by dividing the peak area of the EIC for nor-dolichol-18 by the summed peak areas. Using this method, we determined the total amount of dolichol in the sample using the following equation:

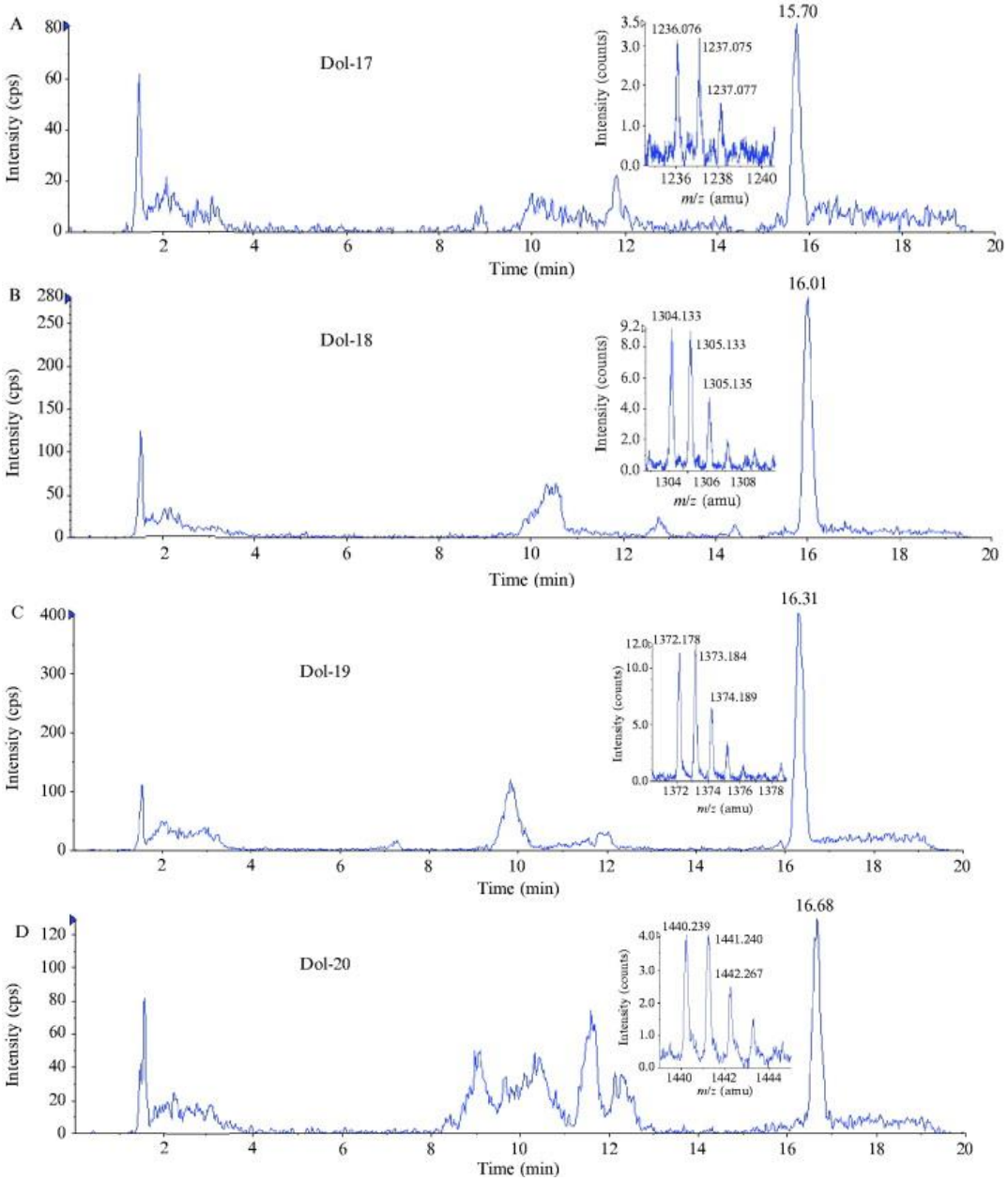


Figure 8. Negative ion liquid chromatography-mass spectrometry (LC-MS) analysis of dolichols in RAW lipid extracts. (A–D) The extracted ion current (EIC) of the acetate adducts of dolichol-17, -18, -19, and -20, respectively. For each, the peak labelled with a retention time corresponds to the relevant extracted ion current peak for a given dolichol. The inset on each panel shows the mass spectra of each of the dolichols detected in the RAW lipid extract.

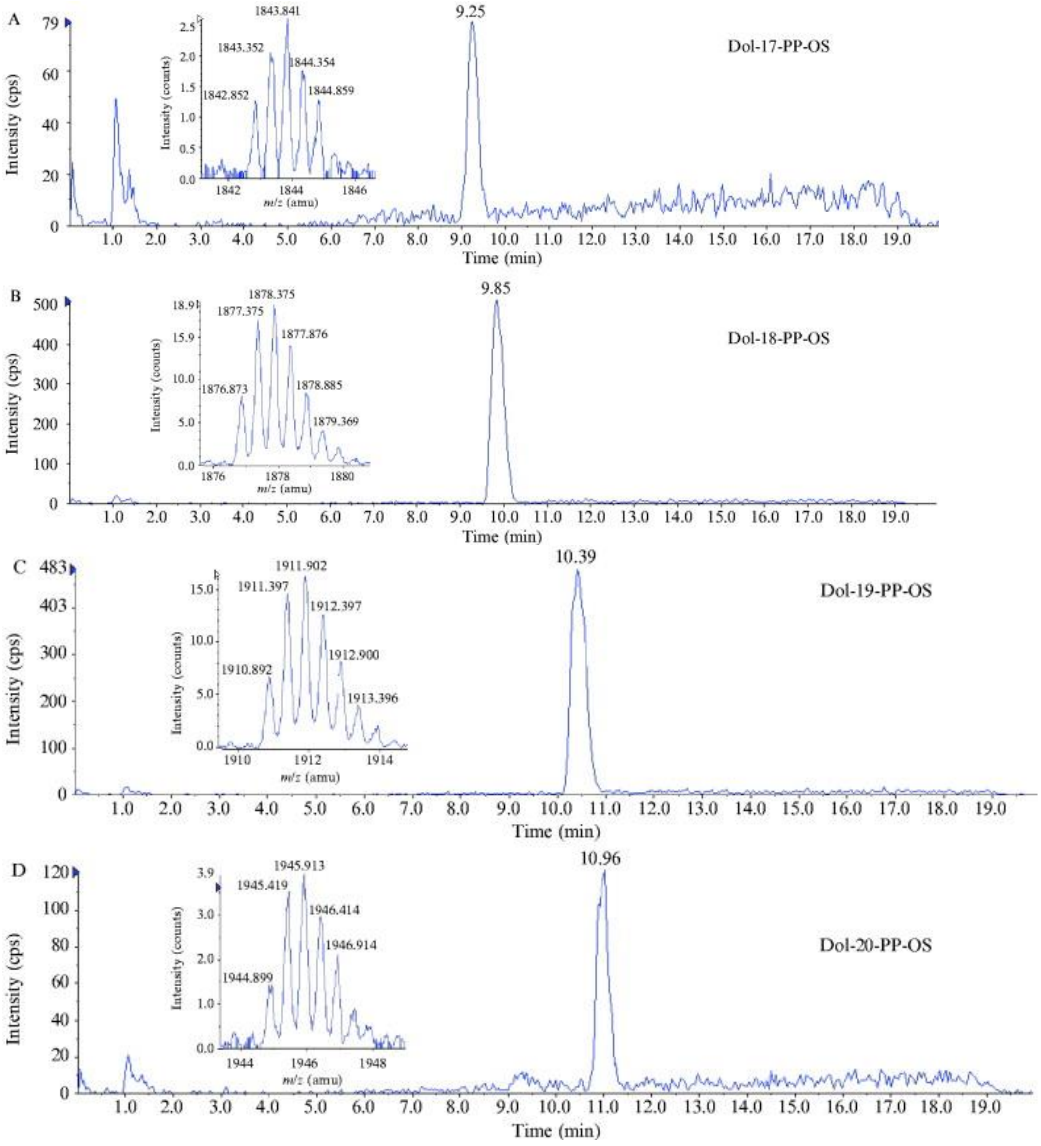
[(Dol-18 peak area/nor-Dol-18 standard peak area) × 1 µg]/MW of Dol-18 = µmol Dol-18

in the sample. Because large amounts of total lipid extract are being injected onto the column, a blank is run between each sample being analysed to prevent carry-over between samples.

Note: Separate cell suspensions are not necessary for the quantification of the CoQs and dolichols. Routinely, a single cell suspension is co-extracted with both the nor-dolichol and CoQ6 standard. After extraction, the sample is divided in two and analysed using positive-ion LC-MS for quantification of the CoQs and negative-ion LC-MS for quantification of the dolichols.

LC-MS and LC-MS/MS Characterization of Dolichol Diphosphate-Linked Oligosaccharides

We wanted to develop a MS-based technique to detect and confirm the structure of the intact Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$. A sample of Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$, purified from porcine pancreas, was kindly provided by Dr. R. Gilmore. Due to the amphiphatic nature of this molecule, we modified the LC-MS method previously described. Specifically, a new solvent mixture was used as chromatography solvent B (cholorform:methanol:1 m*M* ammonium acetate [2:3:1 v/v/v], supplemented with 0.1% piperidine). With this modification, we were able to detect the Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$ eluting between 9 and 12 min, depending on the length of the dolichol to which the 14-sugar oligosaccharide was attached. **Fig. 9A-D** shows the EIC and MS for the $[M–2H]^{2–}$ ion for the oligosaccharide attached to Dol-17, 18, 19, and 20. The exact mass predicted from the proposed structure matches the mass of the major $[M–2H]^{2–}$ and $[M–3H]^{3–}$ ions (not shown) within experimental error. Using this technique, we also performed CID-MS on the most abundant isotope of the triply charged ion of the Dol-19-PP-$(GlcNAc)_2(Man)_9(Glc)_3$. This spectrum, shown in Fig. 5.11E, shows the sequential loss of 162 u, which corresponds to the loss of one, two, three, or four hexoses. A fragment ion corresponding to Dol-19-P is also detected as a singly charged ion at *m/z*1392.169. In the lower mass region, fragment ions corresponding to a single hexose (*m/z* 179.051) and two hexoses (*m/z* 323.096) are also detected.



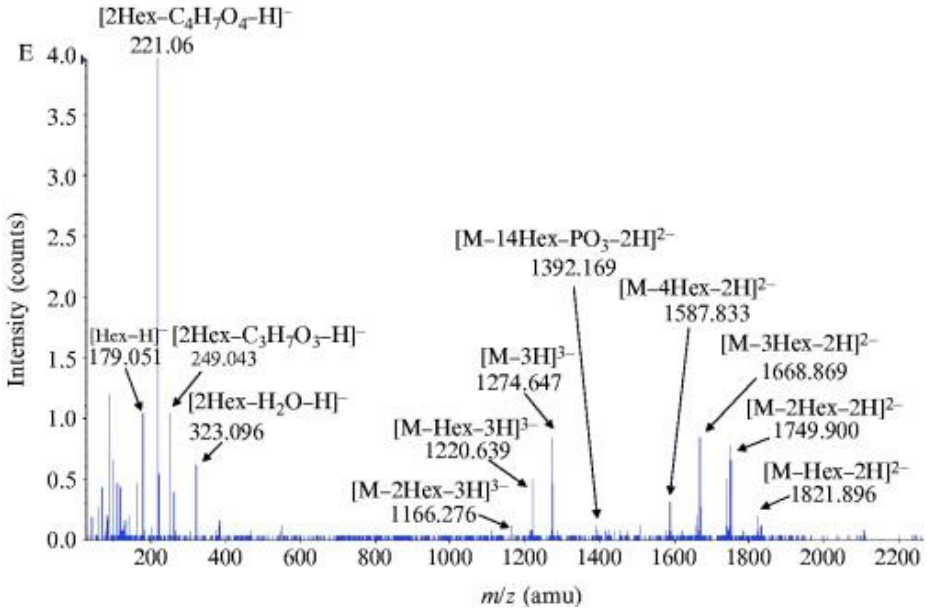


Figure 9. Negative-ion liquid chromatography-mass spectrometry (LC-MS) analysis of purified Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$. (A–D) The extracted ion current (EIC) of Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$ with 17, 18, 19, and 20 isoprenoid units on the dolichol, respectively. The inset shows the mass spectrum of the $[M\text{-}2H]^{2-}$ ion for the Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$ detected in the purified sample. (E) The collision-induced mass spectrometry (CID-MS) of the most abundant isotope of the $[M–3H]^{3-}$ ion of the Dol-19-PP-$(GlcNAc)_2(Man)_9(Glc)_3$ (*m/z* 1274.647). Sequential loss of 162 u, which corresponds to the loss of one, two, three, or four hexoses, is observed. A fragment ion corresponding to Dol-19-P is also detected as a singly charged ion at *m/z* 1392.169. Fragment ions corresponding to a single hexose (*m/z* 179.051) and two hexoses (*m/z* 323.096) are detected in the low mass region.

Table 1. Masses of the $[M\text{-}2H]^{2-}$ Ions of Dol-PP-$(GlcNAc)_2(Man)_9(Glc)_3$

			$[M–2H]^{2-}$	
n	**Dol-PP-OS species**	**Formula [M]**	**Observed (*m/z*)**	**Exact (*m/z*)**
11	Dol-17-PP-OS	$C_{173}H_{288}N_2O_{77}P_2$	1842.852	1484.908
12	Dol-18-PP-OS	$C_{178}H_{296}N_2O_{77}P_2$	1876.873	1876.939
13	Dol-19-PP-OS	$C_{183}H_{304}N_2O_{77}P_2$	1910.892	1910.971
14	Dol-20-PP-OS	$C_{188}H_{312}N_2O_{77}P_2$	1944.899	1445.002

While we have been able to detect purified dolichol-phosphate and dolichol phosphate mannose (provided by Charles J. Waechter at University of Kentucky College of Medicine) using the LC-MS conditions described above (data not shown), our inability to detect dolichol phosphate, dolichol diphosphate, dolichol phosphate-hexose, or dolichol diphosphate-N-acetylglucosamine in the total lipids of RAW cells suggests that the endogenous levels of these compounds are extremely low.