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Profiling of lipids in *Leishmania donovani* using hydrophilic interaction chromatography in combination with Fourier transform mass spectrometry

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There is evidence from our current research on resistance to stibigluconate and from some previous observations that lipid composition may be altered in resistant *Leishmania donovani* and in order to explore this we required a comprehensive lipidomics method. Phospholipids can be analysed by direct infusion into a mass spectrometer and such methods can work very well. However, chromatographic methods can also be very effective and are extensively used. They potentially avoid ion suppression effects, associate lipid classes with a retention time range and deliver good quantitative accuracy. In the current study three chromatography columns were compared for their ability to separate different classes of lipid. Butylsilane (C-4), Zic-HILIC and a silica gel column were compared. The best results were obtained with a silica gel column used in hydrophilic interaction chromatography (HILIC) mode with a mobile phase gradient consisting of (A) 20% isopropyl alcohol (IPA) in acetonitrile (v/v) and (B) 20% IPA in 0.02 M ammonium formate. Using these conditions separate peaks were obtained for triglycerides (TG), phosphoinositols (PI), inositol phosphoceramides (IPC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylcholines (PC), sphingosines (SG), lysophosphatidyethanolamines (LPE) and lysophosphatidylcholines (LPC). The methodology was applied to the analysis of lipid extracts from *Leishmania donovani* and by coupling the chromatography with an LTQ Orbitrap mass spectrometer. It was possible to detect 188 lipid species in the extracts with the following breakdown: PC 59, PE 38, TG 35, PI 20, CPI 13, LPC 11, LPE 2 and SG 10. The fatty acid composition of the more abundant lipids was characterised by MS^2 and MS^3 experiments carried out by using an LCQ Deca low-resolution ion trap instrument coupled with the silica gel column. The separation of lipids into well-defined groups gives extra confidence in their identification and minimises the risk of ion suppression effects. High-resolution mass spectrometry was necessary in order to be able to differentiate between acyl- and acyl-alkyl-lipids. Copyright © 2010 John Wiley & Sons, Ltd.

The genus *Leishmania* is composed of a group of protozoan parasites which are responsible for a number of human diseases. The parasites alternate between promastigote stages in the sand fly vector and an intramacrophagic amastigote stage in the mammalian host. The ability of *Leishmania* to survive both in the sand fly and mammalian host is crucially dependent on complex glycoconjugates based on phosphoinositol (PI) lipids to which are added hexose units and the glyconjugate may then be anchored to proteins.[1,2] These glycoconjugates have attracted interest both as potential antigens for vaccine development and as potential drug targets where a drug might intervene in their maturation. In addition to the inositol lipids *Leishmania* contain phosphatidylcholine (PC) and phosphatidyethanolamine (PE) lipids and these are of interest since alterations in membrane fluidity may be a component in drug resistance. Alteration in membrane lipid composition has not been an extensive topic of research but there is evidence from our current research on resistance to stibigluconate[3] and from some previous observations that lipid composition may be altered in resistant cells. When *L. donovani* was subjected to selection pressure with amphotericin in order to promote resistance it was found that the percentage of saturated fatty acid in the resistant strains rose from 2–55%, mainly at the expense of oleic acid levels. Steroid profiles were also altered and the changes were accompanied by decreased membrane permeability.[4] Treatment of *L. donovani* and *L.amazonesis* with pentamidine caused an alteration in membrane permeability with a decrease in the phospholipid content of the membrane under observation.[5] Alterations in membrane permeability were observed in relation to promotion of resistance in *L. infantum* promastigotes by selection pressure using

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atovaquone.[6] In this case there was a decrease in membrane permeability in the resistant strain associated increased levels of cholesterol and there were also alterations in the overall fatty acid profile of the membrane although no marked change in the level of saturation of the lipids. Miltefosine, an ether lipid, has been developed quite recently as an orally bioavailable therapeutic agent for treating *Leishmania*. A study compared wild-type and miltefosine-resistant *L. donovani* and found a decrease in membrane fluidity compared with wild type and this was associated with an increase in the level of saturation in the fatty acids composing the membrane lipids.[7] Thus in order to support our work on metabolomic profiling of stibogluconate resistant *Leishmania* we required a comprehensive method for lipid profiling providing wide coverage of the different lipid classes in the organism.

Phospholipids can be analysed by direct infusion into a mass spectrometer and such methods can work very well.[8,9] However, chromatographic methods can also be very effective and are extensively used. They potentially avoid ion suppression effects, associate lipid classes with a retention time range and deliver good quantitative accuracy. Both reversed-phase and normal-phase chromatography have been used in the separation of phospholipids prior to MS analysis. Recent examples of chromatographic methods are described below.

A C4 alkyl column was used for the analysis of phospholipids in yeast. A complex binary gradient was used at a flow rate of 115 μL/min. This method was successful in separating PI, PC, PE and phosphatidylserine (PS) lipids and lipids within each class with different chain lengths. However, there was no defined group separation between the PE and PC lipid classes which overlapped. Run times were long requiring up to 90 min to ensure complete elution of all lipids.[10] In a study of lipids in rat liver a fused-silica capillary column packed in-house with a C18 phase was used for analysis of phospholipids. The method was able to identify 19 PE and 37 PC lipids in rat liver using collision-induced dissociation (CID) with an ion trap instrument.[11] The same method was applied to the analysis of PE and PC lipids in human urine where 21 PC and 12 PE lipids could be identified.[12] A fused-core C8 column was used to analyse the phospholipids and lysolipids in human bronchial lavage fluid.[13] The peak shapes obtained on the C8 column were better than those obtained on a C18 column. PE and PC lipids with the same acyl groups attached were separated but eluted quite closely even when elution time was around 40 min. A fused-core C8 column was used to separate 160 lipids in eight different classes in samples of human and rat plasma.[14] The method used a complex binary run over 30 min; PI and PS lipids were not considered in this method. A C18 X-bridge column was used to analyse the strongly acidic phospholipids phosphatidic acid and PS.[15] A complex mobile phase composition including 5 mM phosphoric acid was required in order to obtain good peak shapes for these analytes. A C18 column with 1.7 μm particle size was used to analyse phospholipids in plasma.[16] All the lipids eluted at around 6 min and it was mainly the PC lipids that were detected.

Normal-phase methods have often been applied to the analysis of phospholipids by liquid chromatography/mass spectrometry (LC/MS) and some recent examples are described below. A diol column was used to measure the phospholipids in diabetes nephropathy.[17] The method was able to produce wide separation of seven classes of phospholipid and over 100 phospholipids could be identified in human blood. The disadvantage of this type of method is that hexane was required in the eluent and it is not desirable as an eluent in mass spectrometry because of its toxicity and unpleasant smell. A similar system based on a diol column was used to analyse phospholipids in blood, again the eluent contained hexane.[18] The run time was 45 min and five different classes of phospholipids were separated, although PS and PI lipids were closely eluting.

Hydrophilic interaction chromatography (HILIC) is a relatively recent development, also termed reverse normal phase chromatography, which uses a polar stationary phase and its associated layer of water to promote chromatographic retention with partitioning against a mobile phase which has a high content of organic solvent.[19,20] In this mode polar compounds are retained strongly and their elution is promoted by gradually increasing the aqueous content of the mobile phase. We have found HILIC to be very useful in metabolomic profiling.[21–24] HILIC has been used in lipid profiling although not widely. A silica gel column was used to analyse phospholipids in bronchoalveolar lavage fluid.[25] The method was not specifically described as being based on HILIC; however, it used mobile phase A consisting of ACN/methanol/1 M ammonium formate (78:20:2, v/v/v) and mobile phase B consisting of ACN/methanol/1 M ammonium formate (49:49:2, v/v/v) which are HILIC-like conditions and was able to separate six phosphoplipid classes. Recently, a silica gel column was used in HILIC mode to separate sphingomylin lipids extracted from meats.[26]

The application of mass spectrometry in lipid profiling is well known and there are many well-established mass spectrometry routines for elucidating lipid structures.[27,28] When it comes to determining the side chains of lipids the most informative fragmentations are obtained by operating in negative electrospray ionisation (ESI) mode since this produces negative ion fragments which are due to the fatty acid substituents of the lipid. In positive ion mode the information obtained for PC lipids is limited since the predominant mode of fragmentation produces an ion at *m/z* 184 which is due to the phosphatidylcholine head group.[27,28] In negative ion mode the PC lipids form negatively charged adducts by combining with acidic modifiers, usually acetate or formate, used in the chromatographic mobile phase or infusion solution. In tandem mass spectrometry (MS/MS) mode these adducts can be fragmented producing an ion due to loss of formate or acetate plus a methyl group from the choline head group as well as the negatively charged fatty acid acyl groups. This fragmentation can be either achieved in the ion source or in the second quadrupole via CID[27] in MS/MS The negatively charged acyl groups are observed when using an ion trap instrument by using MS^2 to remove the acidic modifier plus a methyl group and then MS^3 on the ion formed to promote formation of the acyl ions. Apart from PC lipids the side chains of PE, PI and PS lipids can be observed directly following MS/MS or MS^2 fragmentation.

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Most of the previous work on MS-based lipid profiling in *Leishmania* has focused on the characterisation of lipophosphoglycans which are important as potential drug targets and antigenic vaccine components.[29–31] The more generalised profiling of lipids in *Leishmania* has focused on fatty acid and sterol profiling in membranes using GC/MS methods.[3–7] Due to our interest in the potential role of changes in membrane lipid composition in resistant *Leishmania* a method for profiling lipids was required and the goal of the work described below was to explore the development of a HILIC-type method for the chromatographic separation of different classes of phospholipids for use in combination with detection by Fourier transform mass spectrometry (FT-MS).

EXPERIMENTAL

Materials

The phospholipid standards dipalmityl phosphatidyl choline (16:0/16:0 PC) >99%, oleyl palmityl phosphatidyl choline (16:0/18:1 PC) >99%, dioleyl phosphatidyl choline (18:1/18:1 PC) >99%, dioleylphosphatidyl ethanolamine (18:1/18:1 PE) >98%, linoleyl palmityl phosphatidyl inositol (18:2/16:0 PI) >98% and dioleyl phosphatidyl serine (18:1/18:1 PS) >95% were purchased from Sigma-Aldrich (Dorset, UK). Analar grade ammonium formate was also purchased from Sigma-Aldrich. Formic acid (HPLC grade) was purchased from VWR (Dorset, UK). Methanol, acetonitrile (ACN) and isopropyl alcohol (IPA) (all HPLC grade) were obtained from ThermoFisher (Leicestershire, UK). Water was obtained from an in-house Milli-Q water purification station. The lipid standards were dissolved in methanol at a concentration of 1 mg/mL and diluted with ACN to a concentration of 0.01 mg/mL prior to injection into the LC/MS system.

Chromatography

ACE C4, silica gel columns (3 mm × 150 mm × 3 μm), a Zic-HILIC column (4.6 mm × 150 mm × 5 μm) and Zic-HILIC guard columns (20 mm × 2.1 mm i.d., 5 μm) were obtained from HiChrom (Reading UK).

Mobile phase composition

Elution of the C4 column was carried out with (A) 0.02 M ammonia formate in water (pH 6.2) and (B) methanol. The flow rate was at 0.4 mL/min and the gradient was as follows: 0 min 80% B, 40 min 90% B, 50 min 90% B with a 10 min re-equilibration time at the end of the run.

Elution of the Zic-HILIC column was carried out with (A) 0.02 M ammonium formate in ACN (pH 6.2) and (B) 0.02 M ammonium formate in water. The flow rate was 0.4 mL/min and the gradient was as follows: 0 min 5% B, 30 min 10% B, 40 min 5% B.

Elution of the silica gel column was carried with (A) 20% isopropyl alcohol (IPA) in aACN (v/v) and (B) 20% IPA in 0.02 M ammonium formate (v/v). The flow rate was 0.3 mL/min and gradient was as follows: 0–1 min 8% B, 5 min 9% B, 10 min 20% B, 16 min 25% B, 23 min 35% B, 26–40 min 8% B.

Mass spectrometry

High-resolution measurements were carried out by using a Finnigan LTQ Orbitrap instrument fitted with a Surveyor HPLC pump (ThermoElectron, Hemel Hempstead, UK). Sample analysis was carried out in positive and negative ESI modes with a needle voltage of 4.5 kV. The mass scanning range was *m/z* 50–1200, while the capillary temperature was 250°C and the sheath and auxiliary gas flow rates were 30 and 10, respectively (units not specified by the manufacturer). The LC/MS system was controlled by Xcalibur version 2.0 (Thermo Fisher Corporation). MS^2 and MS^3 fragmentation experiments were carried out by using a LCQ DECA mass spectrometer (ThermoElectron UK), equipped with an ESI source fitted with an Agilent 1100 HPLC pump. The ion spray voltage was set to 4.5 kV in the positive mode and negative mode. The capillary voltage and temperature was set to 35 V and 260°C, respectively, and gas flow was set at 20 and 60 mL/min for the sheath and auxiliary gas, respectively. Tube lens was 25 V for +ve mode and −20 V for –ve mode, respectively. The instrument was optimised for sensitivity with freshly prepared 16:0/16:0 PC solution. Data acquisition was controlled with Xcalibur version 2.0. In order to improve fragmentation performance, the run time was divided into eight time segments during which different ion ranges were selected for fragmentation. Each lipid class (PC, PE, PI, IPC) occupied one segment. PI and IPC segments used a CID of 59 V, PE a CID of 24 V and PC a CID of 40 V. These four segments used a MS^3 data-dependent scan with an activation Q of 0.18.

Extraction of *Leishmania* samples

Stationary phase *Leishmania donovani* promastigotes were harvested, the number of cells in the flask were counted and, in order to promote rapid quenching of metabolism, the flasks were cooled in a dry-ice/ethanol bath to 0°C while monitoring the temperature with a digital thermometer (should take no longer than 1 min). The flask was transferred immediately into ice and kept in ice during extraction. An aliquot of culture containing 4×10^7 cells was taken and transferred into pre-chilled Eppendorf tubes (kept in ice). The samples were spun down at 13 000 rpm, 0°C for 10 min, supernatant was removed and the pellet was then washed with 1 mL of phosphate-buffered saline (PBS). The sample was spun down again (13 000 rpm, 0°C, 10 min) and the supernatant removed and the pellet was again washed with 1 mL of PBS. The washing step with PBS was repeated and the supernatant was then thoroughly removed. Then 200 μL of cold (0°C) chloroform/methanol (50:50 v/v) was added to the cell pellet and the sample was extracted for 1 h in a thermomixer at 4°C (1400 rpm). The sample was spun down (13 000 rpm, 0°C, 10 min) and the supernatant was immediately transferred into vials with (glass) inserts. The supernatant was stored at −70°C until analysis which was carried out within 24 h of extraction. During analysis the sample was stored at 4°C in the autosampler tray and 10 μL of sample was injected in the LC/MS system.

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RESULTS AND DISCUSSION

In comparison to commonly used octdecyl (C18) reversed-phase columns, C4 columns are modified with shorter acyl chains and thus have a thinner surface coating of stationary phase with 60% less carbon load. Lipid molecules can diffuse through a short porous layer more rapidly thus providing faster mass transfer and hence a better peak shape than is achievable on C18 columns. Five phospholipids, 16:0/16:0 PC, 16:0/18:1 PC, 18:1/18:1 PC, 18:1/18:1 PE and 16:0/18:2 PI, were selected were tested as standards. The lipids 16:0/16:0 PC, 16:0/18:1 PC, 18:1/18:1 PC and 18:1/18:1 PE had very similar retention times and all eluted at around 39 min; the PI lipid eluted much earlier. Thus there was no clear separation between the PE and PC lipids (Fig. 1).

The Zic-HILIC column separates compounds by hydrophilic partitioning and electrostatic interactions and provides strong retention of polar metabolites.[21–24] The Zic-HILIC phase contains both sulfonic acid groups and quaternary ammonium groups and in theory is charge neutral; however, in practice, it does exhibit ion-exchange interactions. In this study, five phospholipids, 16:0/16:0 PC, 16:0/18:1 PC, 18:1/18:1 PC, 18:1/18:1 PE, 16:0/18:2 PI, were examined for their chromatographic behaviour. As seen in Fig. 2, PE and PI have short retention times on the Zic-HILIC phase and good peak shapes. However, the PC lipids were more strongly retained on the column since they contain a choline group which has a quaternary amine functionality which interacts strongly with the anionic groups of the stationary phase. In LC/MS mode ammonium is the only counter cation available for improving elution characteristics in ion-exchange mode; a more strongly basic counter ion might be more effective in producing good peak shape.

Silica gel has frequently been used in normal-phase mode to separate lipids but, as discussed in the introduction, normal-phase methods have the disadvantage that the solvents used, such as hexane and chloroform, produce toxic vapors when used with LC/MS. Five phospholipid standards, 16:0/16:0 PC, 18:1/18:1 PE, 18:1/18:1 PS and 16:0/18:2 PI, were analysed and, as can be seen in Fig. 3, peak shapes for all five lipids were good. The retention mechanism operating on the silica gel is open to some debate. In the case

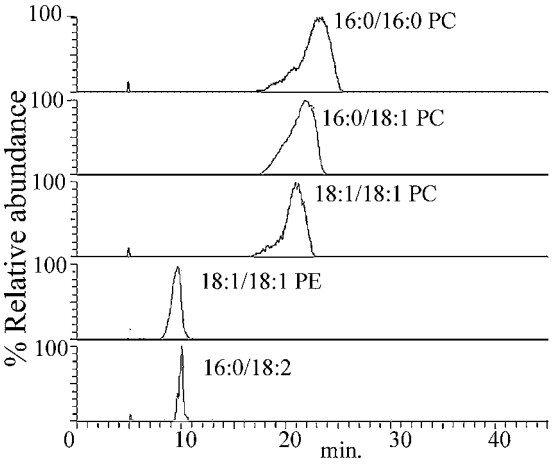

Figure 2. Analysis of phospholipid standards on a Zic-HILIC column eluted with a gradient between (A) 0.02 M ammonium formate in ACN and (B) 0.02 M ammonium formate in water (pH 6.2) at 0.4 mL/min.

of the PC and PE lipids it is most likely the result of silanophilic interaction with the silanol groups in the phase which will be to some extent ionised at the pH of the mobile phase (ca. 6.2 for ammonium formate solution). This would fit with the PC lipids eluting later than PE lipids. However, there may also be a purely HILIC-type mechanism at work since the PI lipid peak, which is negatively charged, elutes at 5 min. With a flow rate of 0.3 mL/min, the void time for the column is ca. 2.5 min. At pH 6.2 the PS lipid is also predominantly negatively charged, since both the phosphate group and carboxylate group within its structure carry negative charges, yet it is retained by the column more strongly than the PE lipid.

The lipid-rich extract from *L. donovani* was run on the silica gel column coupled with an LTQ-Orbitrap mass spectrometer. The samples were run in both positive and negative ion mode and the deviations in the mass accuracies were generally <2 ppm and <1 ppm for the more intense lipid peaks. For high molecular weight compounds such as lipids even good mass accuracy results in a wide range of possible elemental compositions. However, many compositions are

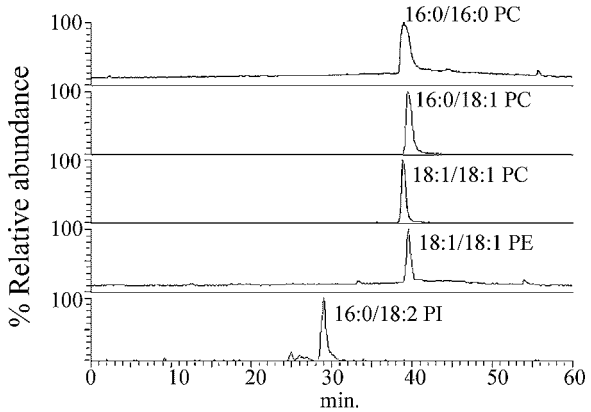

Figure 1. Standard phospholipids on an ACE C4 column eluted with a gradient between (A) 0.02 M ammonia formate in water (pH 6.2) and (B) methanol at 0.4 mL/min.

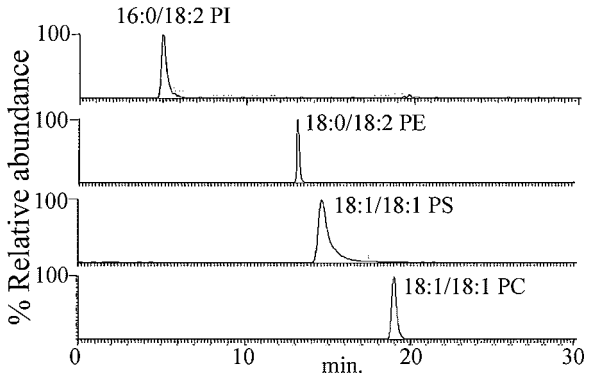

Figure 3. Separation of lipid standards on an ACE silica column eluted with a gradient between (A) 20% IPA in ACN (v/v) and (B) 20% IPA in 0.02 M ammonium formate (v/v) at 0.3 mL/min.

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implausible and in addition the characteristic retention times for the groups of lipids in the samples gave confidence in identification. Figure 4 shows extracted ion traces obtained for representative lipids extracted from *L.donovani*. Three lipid classes relating to the standards could be observed but there was no evidence for the presence of PS lipids. PS lipids play an interesting role in the biology of *Leishamania* since there is evidence that in the metacyclic stage of the growth cycle exposure of PS lipids on the cell surface of the organism mimics apoptosis and encourages the macrophages in the host organism to phagocytose the parasite.[32,33] However, in the promastigote stage extracted in the current study the presence of PS lipids may not be required. In addition to PI, PE and PC lipids the sample contained triglycerides, which eluted at around the void volume of the column, inositol phosphoceramide (IPC), sphingo (SG), lysophosphatidyl ethanolamine (LPE) and lysophosphatidyl choline (LPC) lipids which were identified on the basis of their elemental compositions. The LPC lipids eluted ca. 2 min later than the PC lipids and appeared as two peaks with identical ions suggesting that the *sn*-1- and *sn*-2-substituted lysolipids were chromatographically resolved. There was no strong evidence of separation within lipid groups apart from in the case of the PC lipids which almost separated into two groups with the higher molecular weight PC lipids eluting in the earlier peak (Fig. 4). Using the current methodology 188 lipids could be identified in the sample with the following breakdown: PC 59, PE 38, TG 35, PI 20, IPC 13, SG 10 LPC 11, LPE 2.

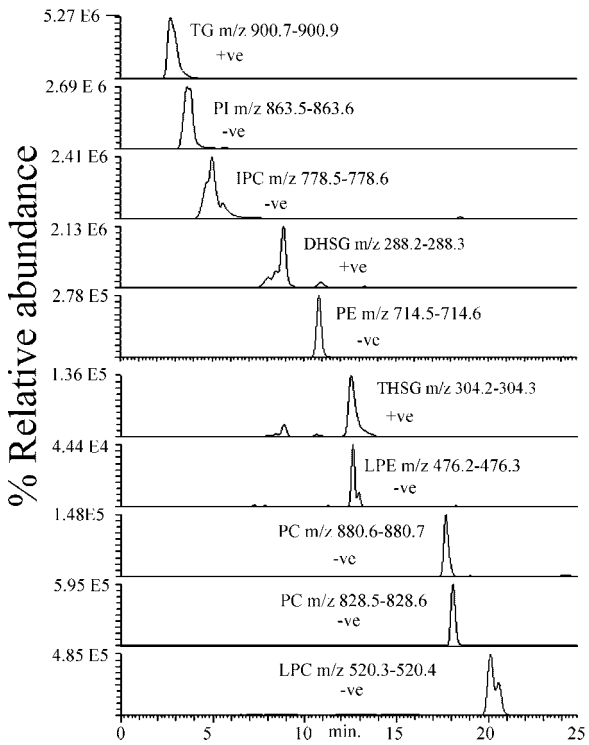

Figure 4. Extracted ion traces for the different lipid classes extracted from *L. donovani* run in negative ion and positive ion mode on an LTQ-Orbitrap using an ACE silica column eluted with a gradient between (A) 20% IPA in ACN (v/v) and (B) 20% IPA in 0.02 M ammonium formate (v/v) at 0.3 mL/min. DHSG = dihydroxysphingosine, THSG = trihydroxysphingosphingosine.

A LCQ Deca mass spectrometer was used in negative ion mode with data-dependent MS^2/MS^3 in order to determine the acyl groups in the more abundant phospholipids; targeted fragmentation would be required to characterise the less abundant ions. The PI, PS and PE lipids gave diagnostic fragments in MS^2 in both positive and negative ion mode. MS^3 in negative ion mode was used to characterise PC lipids; an example is shown in Fig. 5. Data-dependent MS^2 fragmentation results in the loss of 60 amu from the formic acid adduct of the molecular ion (*m/z* 830) of 18:0, 18:2 PC due to loss of formic acid and a methyl group from the choline head group of the PC lipid. This produces an ion at *m/z* 770 which is picked up by the data-dependent scan as the most intense ion in the MS^2 spectrum and fragmented further resulting in the formation of ions at *m/z* 508 and 279 which result from the loss of $C_{17}H_{30}CO$ (ketene type fragment) from *m/z* 770 and from the acyl ion $C_{17}H_{33}COO^-$, respectively. In contrast, in positive ion mode the main fragment ion produced is at *m/z* 184 due to the phosphocholine head group. The PC and LPC lipids observed in the sample are shown in Table 1. It is possible that some of the less abundant PCs listed which appear to contain odd-numbered fatty acids are the result of demethylation of the phosphocholine group in the mass spectrometer.

Figure 6 shows the MS^2 spectrum of 18:1/16:1 PE in negative ion mode. In this case the MS^2 spectrum is indicative of the acyl groups within the structure with prominent ions at *m/z* 436 due to loss of $C_{17}H_{32}CO$ from the molecular ion at *m/z* 698 and at *m/z* 279 due to $C_{17}H_{31}COO^-$. The PE lipid fraction in *Leishmania* contains a mixture of acyl-lipids and acyl-alkyl-lipids which are not distinguishable by molecular weight alone. Fragmentation methodologies have been developed in order to distinguish between them[34] but it is also possible to readily tell them apart using their accurate masses since the fractional masses of the acyl-lipids are considerably smaller than those of ether lipids with the

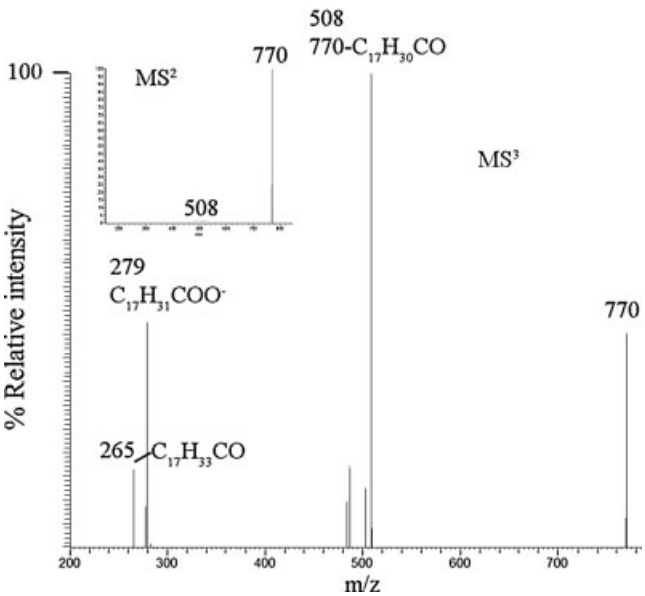

Figure 5. MS^2 and MS^3 spectra of 18:0/18:2 PC lipid at 40 V.

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Table 1. PC and LPC lipids extracted from *L. donovani* eluting in the peaks at 17–18.5 min and 20 min in Fig. 4

*PC −ve	PC +ve	Comp.	Fragments	PC −ve	PC +ve	Comp.	Negative ion fragments
746.497	702.507	30:2		874.560	830.568	22:6/18:2,	552 = 814- $C_{17}H_{30}CO$
748.514	704.522	30:1		876.575	832.582	22:6/18:1,	552 = 816- $C_{17}H_{32}CO$
750.529	706.537	30:0		878.592	834.599	22:6/18:0,	552 = 818- $C_{17}H_{34}CO$
772.513	728.542	32:3		880.609	836.614	22:5/18:0	510 = 820- $C_{21}H_{30}CO$
774.528	730.5378	32:2		882.625	838.629	40:4	
776.545	732.553	32:1		884.637	840.646	40:3,	
786.565	742.574	EL 34:3		886.656	842.661	40:2,	
788.582	744.589	EL34:2	*m/z* 480 = 718–$C_{15}H_{30}CO$	-	852.550	42:11	
790.599	-	EL 34:1		-	854.567	42:10	
-	752.520	34:5		-	856.583	42:7	
798.528	754.536	34:4		-	858.598	42:8	
800.545	756.553	34:3		-	860.614	42:4	
802.560	758.569	16:0/18:2	*m/z* 504 = 742–$C_{15}H_{30}CO$	-	862.629	42:3	
804.577	760.584	16:0/18:1	*m/z* 506 = 744–$C_{15}H_{30}CO$	-	864.646	42:2	
806.592	762.600	16:0/18:0	*m/z* 508 = 746–$C_{15}H_{30}CO$	-	866.663	42:1	
810.533	766.536	35:5		-	868.677	42:0	
812.544	768.554	35:4		-	856.583	42:6	
814.595	770.571	EL 36:3		-	858.598	42:5	
816.576	772.586	35:2		-	876.5537	44:13	
816.612	772.621	EL 36:2		-	878.5699	44:12	
818.593	774.600	35:1		-	880.5842	44:11	
820.513	776.522	36:7		-	894.695	44:10	
822.530	778.538	36:6		-	896.708	44:1	
824.545	780.551	18:2/18:3	504 = 764–$C_{17}H_{28}CO$	**LyPC**	**LyPCC**		
826.559	782.568	18:2/18:2	504 = 766–$C_{17}H_{30}CO$	562.315	518.823	18:3	
828.575	784.583	18:3/18:0 18:1,18:2	508 = 768–$C_{17}H_{28}CO$ 506 = 768- $C_{17}H_{30}CO$	564.331	520.339	18:2	279
830.591	786.600	18:2/18:0	508 = 770–$C_{17}H_{30}CO$	566.347	524.355	18:1	281
832.607	788.614	18:1/18:0,	508 = 772–$C_{17}H_{32}CO$	568.361	526.370	18:2	283
850.561	806.568	22:6/16:0	480 = 790–$C_{21}H_{30}CO$	-	544.339	20:4	
852.576	808.584	22:5/16:0 and 20:3,18:2	480 = 792–$C_{21}H_{32}CO$ 504 = 792- $C_{19}H_{32}CO$	590.346	546.355	20:3	367
854.591	810.598	38:4		592.362	548.371	20:2	
856.606	812.614	20:2/18:1	532 = 796–$C_{17}H_{32}CO$	-	550.386	20:1	
858.620	814.540	20:1/18:0,	508 = 798–$C_{19}H_{34}CO$	612.331	568.339	22:6	
860.638	816.645	20:0/18:0	508 = 800–$C_{19}H_{36}CO$	614.347	570.355	22.5	
872.546	828.554	22:6/18:3	552 = 812–$C_{17}H_{28}CO$	-	572.371	22:4	

EL = ether-lipid.
* The PC lipids are observed as their formate acid adducts in negative ion mode.

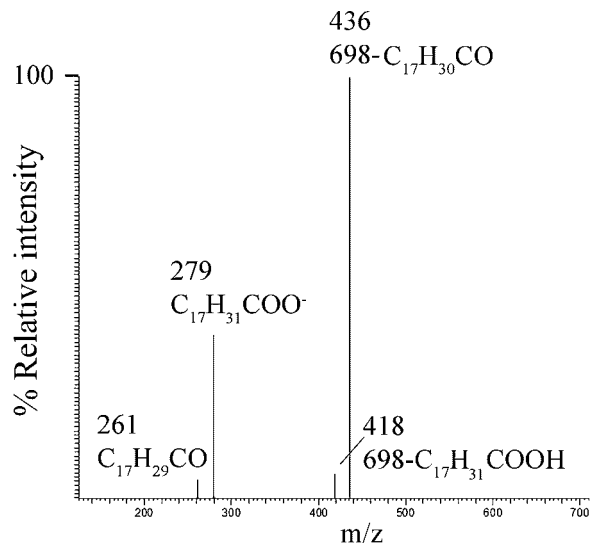

Figure 6. MS^2 spectrum of 18:2/16:1 PE lipid in negative ion mode at 40 V.

same nominal mass. The PE lipids observed in the sample are shown in Table 2.

PI lipids, which contain a phosphatidylinositol head group without any basic centre, yielded deprotonated anions in the negative ion mode. Figure 7 shows the MS^2 spectrum of 18:0/18:1 PI. The ions at *m/z* 581 and 599 arise from the losses of the oleic acid (18:1) and an oleic acid ketene type fragment. Loss of inositol from the ion at *m/z* 581 gives rise to an ion at *m/z* 419. A small ion at *m/z* 241 is due to inositol-1,2-cyclic phosphate. Such information-rich spectra meant that many of the PI lipids in the *Leishmania* sample could be completely assigned. Many of the minor PI lipids were acyl-alkyl-lipids. The PI lipids detected in the sample are shown in Table 3.

The fourth major group of lipids in the *Leishmania* extract was comprised of IPCs. These compounds along with PI lipids are important anchors for the surface glycoproteins of trypanosomatids,[35,36] although they have not been reported to have this role in *Leishmania* despite it falling within this class of organisms. It is difficult to get an exact structural I.D. for these compounds since both the fatty acid bonded to the amino group of the sphingosine base and the chain length of the sphingosine portion of the molecule can vary. Figure 8

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Table 2. PE lipids eluting in the peak at ca. 10.5 min in Fig. 4

PE –ve	PE +ve	Comp.	Fragments	PE –ve	PE +ve	Comp.
696.498	698.512	AAL16:2/18:2		754.575	756.589	AAL 38:3
698.513	700.530	AAL16:1/18:2	436 = 698–$C_{17}H_{30}CO$	-	758.605	EL 38:2
700.529	702.543	AAL16:0/18:2	438 = 700–$C_{17}H_{30}CO$	-	762.505	38:7
702.545	704.528	AAL16:0/18:1	438 = 702–$C_{17}H_{32}CO$	762.510	764.521	38:6
710.513	712.527	AAL 35:4		764.524	766.537	38:5
712.529	714.543	AAL17:1/18:2	450 = 712–$C_{17}H_{30}CO$	766.542	768.553	38:4
714.545	716.560	AAL17:0/18:2	452 = 714–$C_{17}H_{30}CO$	768.557	770.569	38:3
722.513	724.525	AAL 36:5		770.571	772.585	38:2
724.525	726.541	AAL 36:4		-	774.601	38:1
726.544	728.558	AAL18:1/18:2	464 = 726–$C_{17}H_{30}CO$	784.492	786.505	40:9
728.560	730.574	AAL18:1/18:1	464 = 728–$C_{17}H_{32}CO$	786.508	788.522	40:8
730.566	732.589	AAL 36:1		788.523	790.537	40:7
736.492	738.505	36:5		790.538	792.5539	40:6
738.508	736.489	36:6		792.558	794.569	40:5
740.529	742.538	36:3		-	796.583	40:4
742.539	744.553	36:2		-	798.599	40:3
744.556	746.569	36:1		-	798.544	AAL 42:8
-	750.541	AAL 38:6		-	802.631	40:1
750.545	752.558	AAL 38:5		Lyso PE		
752	754.544	AAL 38:4		476.278	478.294	18:3
				478.294	480.310	18:2

AAL = acyl-alkyl-lipid.

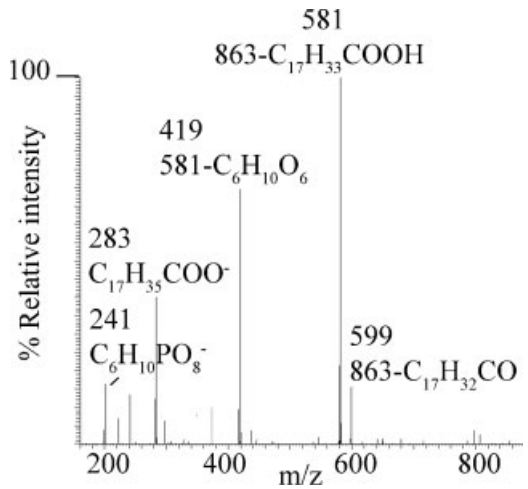

Figure 7. The MS^2 spectrum of 18:0/18:1 PI lipid in negative mode at 40 V.

shows the MS^2 spectrum of the most abundant IPC compound in the extract which had a molecular ion at *m/z* 778 in negative ion mode. The MS^2 spectrum of the IPC lipids all show an ion at *m/z* 241 which is due to the inositol 1,2-cyclic phosphate. In Fig. 8 the ion at *m/z* 616 is due to neutral loss of inositol-H_2O from the molecular ion and the ion at *m/z* 598 results from loss of the complete inositol moiety. The ion at *m/z* 241 can be readily used to characterise the various ions in the peak as being due to IPC lipids. The modifying fatty acid is attached to the sphingosine nucleus via a relatively strong amide bond thus there are no reliable fragments indicative of the fatty acid substitution. In some cases losses of the fatty acid portion of the molecule could be observed but this was not a robust method of characterisation. In Table 4 the IPC lipids are listed as being based on di- or trihydroxylated sphingosines which are readily distinguishable on the basis of their accurate masses. Trihydroxylated IPC lipids have not been reported before in *Leishmania* and their identification here is tentatively based on an elemental composition within 1.5 ppm of that expected for this class of lipid. Long-chain amino alcohols are also present in the

Table 3. PI lipids eluting in the peak at 3.8 min in Fig. 4

PI –ve	Comp.	Fragments	PI –ve	Comp.	Negative ion fragments
817.521	AAL16:0/18:3	539 = 817 – $C_{17}H_{29}COOH$	861.550	18:0/18:2	581 = 861 - $C_{17}H_{31}COOH$
819.540	AAL16:0/18:2	539 = 819 – $C_{17}H_{31}COOH$	863.566	18:0/18:1	581 = 863 - $C_{17}H_{33}COOH$
821.556	AAL16:0/18:1	539 = 821 – $C_{17}H_{33}COOH$	865.573	18:0/18:0	581 = 865 - $C_{17}H_{35}COOH$
823.570	AAL16:0/18:0	539 = 823 – $C_{17}H_{35}COOH$	875.569	37:2	
833.5214	16:0/18:2	553 = 833 – $C_{17}H_{31}COOH$	877.581	37:1	
835.533	16:0/18:1	553 = 835 – $C_{17}H_{33}COOH$	889.580	38:2	
845.553	AAL 36:3		891.597	38:1	
847.569	AAL18:0/18:2	567 = 847 – $C_{17}H_{31}COOH$	933.682	AAL42:1	
849.552	AAL18:0/18:1	567 = 849 – $C_{17}H_{33}COOH$	935.693	AAL42:0	
859.534	36:3		961.713	AAL44:1	

AAL = acyl-alkyl-lipid.

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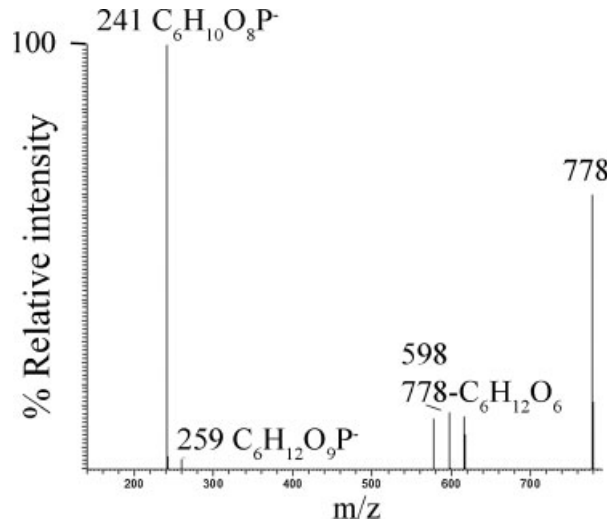

Figure 8. MS^2 spectrum of the most abundant IPC lipid in *Leishmania* extracts at 59 V.

extract (Table 5) and several of these are trihydroxylated which gives them a phytosphingosine-type structure; this ties in with the observation of small amounts of trihydroxylated IPC lipids.

The triglycerides (TGs) in the sample eluted at the void volume of the column and could only be observed in positive ion mode as their ammonium adducts. Table 6 shows the TGs detected in the sample. Data-dependent fragmentation only managed to characterise a few of the most abundant TGs which had C18 or C16 acids at each position which fragmented to give ions due to each fatty acid substituent. There were a number of abundant TGs which gave elemental compositions indicating that they must carry fatty acids with odd numbers of carbon atoms which is perhaps consistent with the presence of fatty acids with odd numbers of carbons in some of the phospholipids.

Table 4. IPC lipids eluting in the peak at ca. 5 min in Fig. 4

IPC –ve	Comp.	IPC –ve	Comp.
778.523	d34:1	822.584	d37:0
780.535	d34:0	824.565	t36:0
792.540	d35:1	832.570	d38:2
796.535	t34:0	834.586	d38:1
806.555	d36:1	836.602	d38:0
808.565	d36:0	852.593	t38:0
820.571	d37:1 or t 36:2		

d = dihydroxy, t = trihydroxy.

Table 5. Sphingosine bases eluting in the peaks at ca. 8.5 min and 12.5 min in Fig. 4

Molecular ion	Mass deviation (ppm)	MW
$C_{16}H_{34}NO_2$	−0.168	272.2584
$C_{17}H_{38}NO_2$	−0.749	288.2895
$C_{18}H_{38}NO_2$	−0.419	300.2900
$C_{19}H_{42}NO_2$	−0.177	316.3210
$C_{20}H_{42}NO_2$	−0.079	328.3210
$C_{16}H_{36}NO_3$	−0.484	290.2688
$C_{17}H_{38}NO_3$	−0.035	304.2846
$C_{18}H_{40}NO_3$	−0.222	318.3002
$C_{20}H_{44}NO_2$	−0.349	346.3315
$C_{22}H_{48}NO_3$	−0.169	374.3628

MW: molecular weight.

Table 6. TG lipids eluting in the peak at ca. 2.6 min in Fig. 4

Molecular ion of adduct	Composition	MW
$C_{51}H_{100}NO_6$	48:1	822.753
$C_{51}H_{98}NO_6$	48:2	820.738
$C_{51}H_{96}NO_6$	48:3	818.722
$C_{51}H_{94}NO_6$	48:4	816.706
$C_{53}H_{104}NO_6$	50:1	850.784
$C_{53}H_{102}NO_6$	50:2	848.769
$C_{53}H_{100}NO_6$	50:3	846.753
$C_{53}H_{98}NO_6$	50:4	844.738
$C_{53}H_{96}NO_6$	50:5	842.722
$C_{53}H_{94}NO_6$	16:0/18:3/18:3	840.709
$C_{55}H_{108}NO_6$	52:1,	878.814
$C_{55}H_{106}NO_6$	52:2,	876.800
$C_{55}H_{104}NO_6$	16:0/18:1/18:2	874.785
$C_{55}H_{102}NO_6$	16:0/18:2/18:2	872.769
$C_{55}H_{100}NO_6$	52:5	870.754
$C_{55}H_{98}NO_6$	52:6	868.738
$C_{57}H_{108}NO_6$	18:2/18:1/18:0	902.813
$C_{57}H_{106}NO_6$	18:2/18:1/18:1	900.799
$C_{57}H_{104}NO_6$	18:2/18:1/18:1	898.784
$C_{57}H_{102}NO_6$	54:6	896.769
$C_{57}H_{100}NO_6$	18:3/18:2/18:2	894.754
$C_{57}H_{98}NO_6$	54:8	892.738
$C_{59}H_{110}NO_6$	56:4	928.830
$C_{59}H_{108}NO_6$	56:5	926.815
$C_{59}H_{106}NO_6$	56:6	924.799
$C_{59}H_{104}NO_6$	56:7	922.784
$C_{59}H_{102}NO_6$	56:8	920.770
$C_{59}H_{100}NO_6$	56:9	918.753
$C_{61}H_{112}NO_6$	58:5	954.835
$C_{61}H_{110}NO_6$	58:6	952.831
$C_{61}H_{108}NO_6$	58:7	950.815
$C_{61}H_{106}NO_6$	58:8	948.800
$C_{61}H_{104}NO_6$	58:9	946.784
$C_{61}H_{102}NO_6$	58:10	944.768
$C_{61}H_{100}NO_6$	58:11	942.755

The triglycerides were detected as their ammonium adducts which explains the presence of nitrogen in the molecular ion.

Although it is possible to obtain structural information on lipids using direct infusion methods there are distinct advantages in carrying out a chromatographic separation of different lipid classes. These include avoidance of ion suppression effects, removing overlap between isobaric compounds and quantitative accuracy. The current method gives a clear separation of eight lipid classes using a commonly available HPLC column without the need to use environmentally and MS-unfriendly solvents. The main purpose of the work was to develop a separation method to simplify the characterisation of lipid mixtures and, without any particular attempt to optimise structural elucidation of the different phospholipids, it was possible observe 188 lipids in *L. donovani* and characterise many of these. A more focused approach to structural elucidation would yield further information. The paper thus presents a convenient and straightforward method for profiling lipids in parasites where alterations in membrane permeability, which may be linked to changes in phospholipid profiles, might have a role in parasite drug resistance.

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