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Application News

High Performance Liquid Chromatograph Mass Spectrometer

OAD-MS/MS for the Structural Identification of Double-Bond Positions in Lipids Associated with Alcohol Toxicity

Emily Armitage[1], Alan Barnes[1], Olga Deda[2], Christina Virgiliou[2], Neil Loftus[1], Helen Gika[2], Ian Wilson[3]
1 Shimadzu Corporation, Manchester, UK, 2 Aristotle University of Thessaloniki, Greece
3 Imperial College, London, UK

User Benefits

- Oxygen Attachment Dissociation (OAD) is a novel MS/MS fragmentation technology that induces (C=C) position-specific fragmentation.
- Simultaneous acquisition of OAD-MS/MS and CID-MS/MS in positive or negative ESI mode, enables lipid identification to the structural level. Automated analysis of OAD-MS/MS data is supported in MS-DIAL.
- OAD-MS/MS increases reporting confidence in lipid identification.

■ Introduction

In metabolomics and lipidomics studies, the structural characterisation of lipids requires the identification of carbon number as well as the number and position of double bonds. Identifying carbon-carbon double bond (C=C) position(s) presents a major challenge in unsaturated lipid characterisation, however it is vital to understand a biological mechanism, since minor structural differences between positional isomers can alter the biochemical function of a lipid.

Oxygen Attachment Dissociation (OAD) MS/MS is a novel method for specific fragmentation of C=C bonds, providing additional fragmentation to CID in positive and negative ESI mode on the LCMS-9050. It involves the generation of O/OH• radicals by microwave discharge of water vapour which are introduced into the collision cell via an inductively coupled plasma through a quartz tube. Neutral radicals interact with precursor ions to specifically oxidise/dissociate the double bonds between carbons. A key advantage is that spectra can be acquired with simultaneous OAD-MS/MS and CID-MS/MS (in either positive or negative ion mode) to generate sufficient information to identify lipids to the structural level (Fig. 1).

In this application, OAD-MS/MS was applied to an untargeted metabolomics study following ethanol-induced exposure in mice. Tissue extracts from gut, liver and pancreas were analysed using UHPLC-OAD-MS/MS to increase reporting confidence in identification for organ-specific changes in the lipid profiles of gut, liver and pancreas of mice in response to ethanol toxicity.

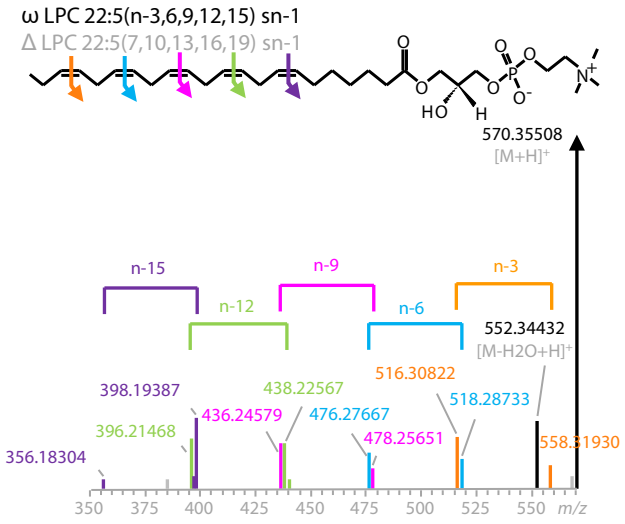

Fig. 1 Oxygen Attachment Dissociation (OAD) MS/MS is a novel fragmentation method, providing additional fragmentation to CID on the LCMS-9050. In this example, C=C bond position-specific fragmentation by OAD enables lipid identification to the structural level identifying this lipid as LPC 22:5(n-3,6,9,12,15) sn-1 [or LPC 22:5(7,10,13,16,19) sn-1 using the delta nomenclature].

■ Materials and Methods

Tissue extracts (gut, liver and pancreas) were obtained from C57BL/6 mice that were either subjected to ethanol exposure or un-dosed controls. The study was conducted in accordance with EU and National ethical guidelines and approved by the Aristotle University of Thessaloniki. Phenotypic tissue samples were analysed by OAD-MS/MS to structurally characterise lipids found to be significantly differentiated in ethanol treated mice compared to healthy controls from a previous study. The analysis was performed using the analytical conditions described in Table 1.

LabSolutions Insight Explore™ and MS-DIAL were used to process data. DDA spectra acquired in positive and negative ESI mode for each lipid were analysed to assign C=C double bond positions and matched to the OAD database in MS-DIAL. Lipids were identified according to the omega and delta nomenclature, counting carbons from the methyl end or the carboxylic end of the fatty acyl, respectively. Fig. 2 highlights an example of the identification in MS-DIAL.

Table 1 Analytical conditions

UHPLC:	Nexera X2
Column	C18 (2.1x100 mm 1.7 µm); column maintained at 50 ˚C
Flow Rate	0.4 mL/min
Mobile Phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Rinse (R0/R1/R2)	IPA/MeOH/MeCN/H_2O (1:1:1:1) + 1% formic acid
Gradient	2% B (0-1 min); 40% B (2 min); 90% B (25 min, curvi-linear gradient); 100% B (27.5-31 min); 2% B (31.01-35 min)
Injection	1 µL (ESI+) / 2 µL (ESI-); autosampler maintained at 4 ˚C
Mass Spectrometer:	LCMS-9050 with OAD Radical Source I
Interface	300 ˚C, 4.0 kV (ESI+) / –3.0 kV (ESI–)
Heat block, DL	400 ˚C, 250 ˚C
Gas flow rates	Nebulizing gas 3 L/min; Heating gas 10 L/min Drying gas 15 L/min
OAD Radical Source	Atomic oxygen (O) and hydroxyl radicals (OH•) are generated in a radical source. Collision gas pressure 17 kPa
MS	TOF-MS (*m/z* 60-1250)
MS/MS	3 DDA-MS/MS (*m/z* 40-1250; 1 second cycle time; collision energy spread 6-30 V; Precursor isolation 0.8 Da (ESI+) / 3 Da (ESI–)

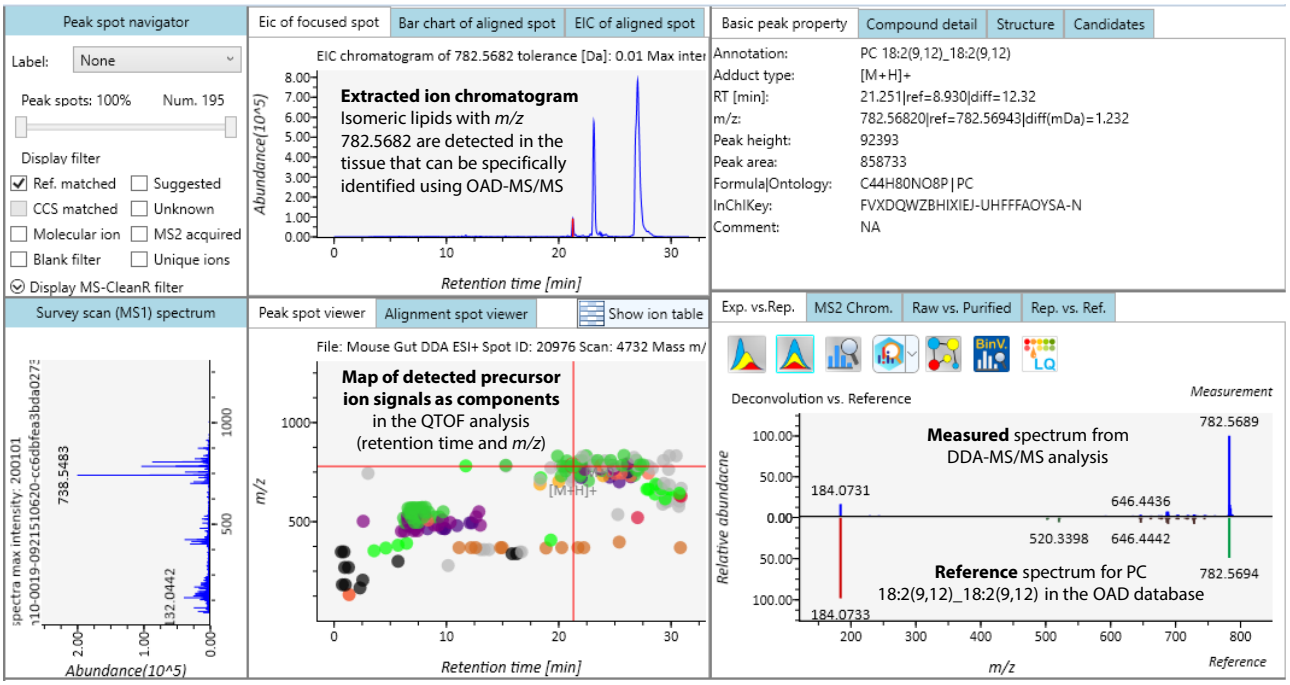

Fig. 2 Data processing and lipid identification using the OAD database in MS-DIAL. This example highlights the identification of PC 18:2(9,12)_18:2(9,12), also known as PC(18:2(n-6,9)/18:2(n-6,9)), in the gut.

■ Results

For each of the lipids with C=C double bonds found to be dysregulated by ethanol toxicity in gut, liver or pancreas in a previous untargeted metabolomics study, the UHPLC-OAD-MS/MS spectral data were used to positively assign the position(s) of each double bond. This involved identification of OAD-MS/MS characteristic neutral loss fragments for each of the candidates as described for lipids[1]. Structurally characterised lipids that were increased by ethanol exposure in one or more of the tissues are given in Table 2, while those that were decreased are presented in Table 3.

OAD-MS/MS enabled the structural characterisation of specific lipid isomers, indicating the exact structures affected by ethanol toxicity. As one example, four retention time separated components were annotated as LPC 22:5 in the untargeted metabolomics analysis, with only the last two components being significant.

From the analysis of CID-MS/MS in positive ion fragment spectra, sn-1 and sn-2 isoforms were identified given the relative fragment ion intensity at *m/z* 104 (a characteristic fragment ion of the sn-1 isoform known to exceed a 30-fold difference in intensity relative to the same ion in the sn-2 isoform[2]). However, the CID-MS/MS spectra could not distinguish between each of the sn-1 components (2 and 4) or sn-2 components (1 and 3). Negative ion CID-MS/MS provided diagnostic fragments to identify the acyl chain, but the spectra were similar for retention time separated components (Fig. 3 highlights the same distribution and intensity of fragments ions in sn-1 components 2 and 4).

OAD-MS/MS fragment spectra on the other hand resulted in C=C-positional information and enabled the identification of four components; LPC 22:5(n-3,6,9,12,15) sn-2 and sn-1, LPC 22:5(n-6,9,12,15,18) sn-2 and sn-1. Figure 4 highlights the different sn-1 isoforms, distinguishable by OAD-MS/MS.

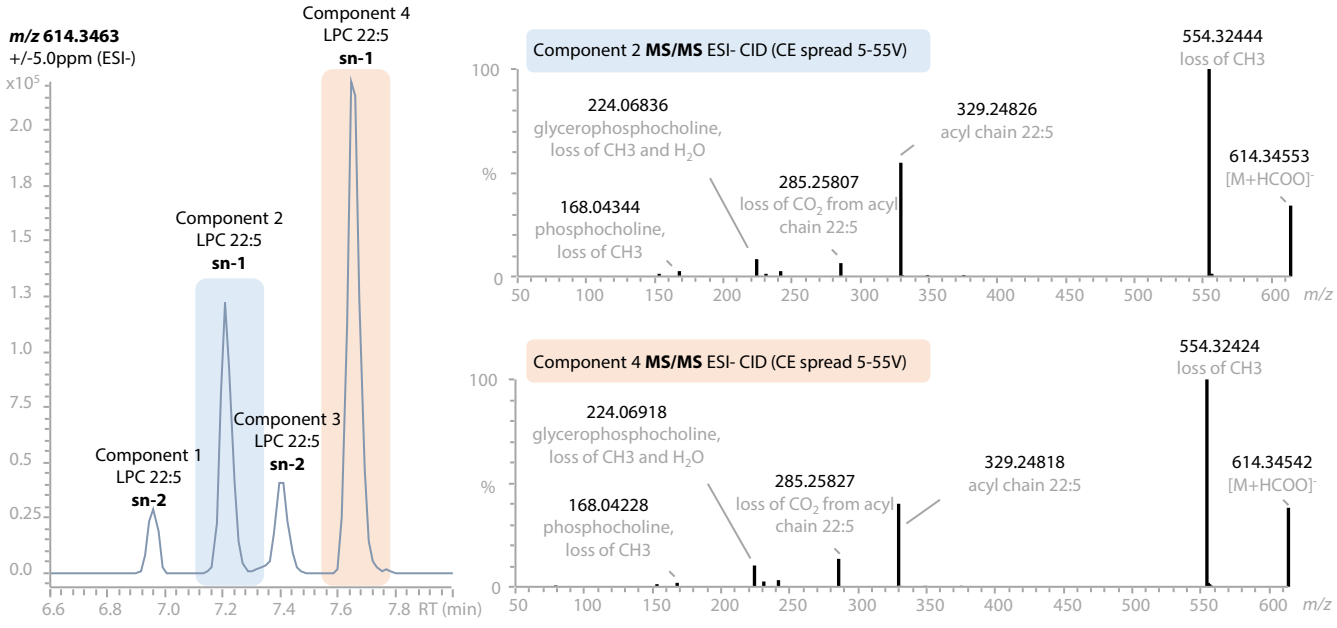

Fig. 3 Detection of LPC 22:5 isomers with CID-MS/MS in negative ion mode. TOF-MS extracted ion chromatogram for *m/z* 614.3463 [M+HCOO-H]- in pancreas tissue extract. Negative ion spectra enable the identification of the acyl chain, but retention time separated isomers (components 2 and 4) cannot be distinguished by CID-MS/MS. CID-MS/MS acquired with a gas pressure of 230 kPa and a collision energy spread of 5-55 V.

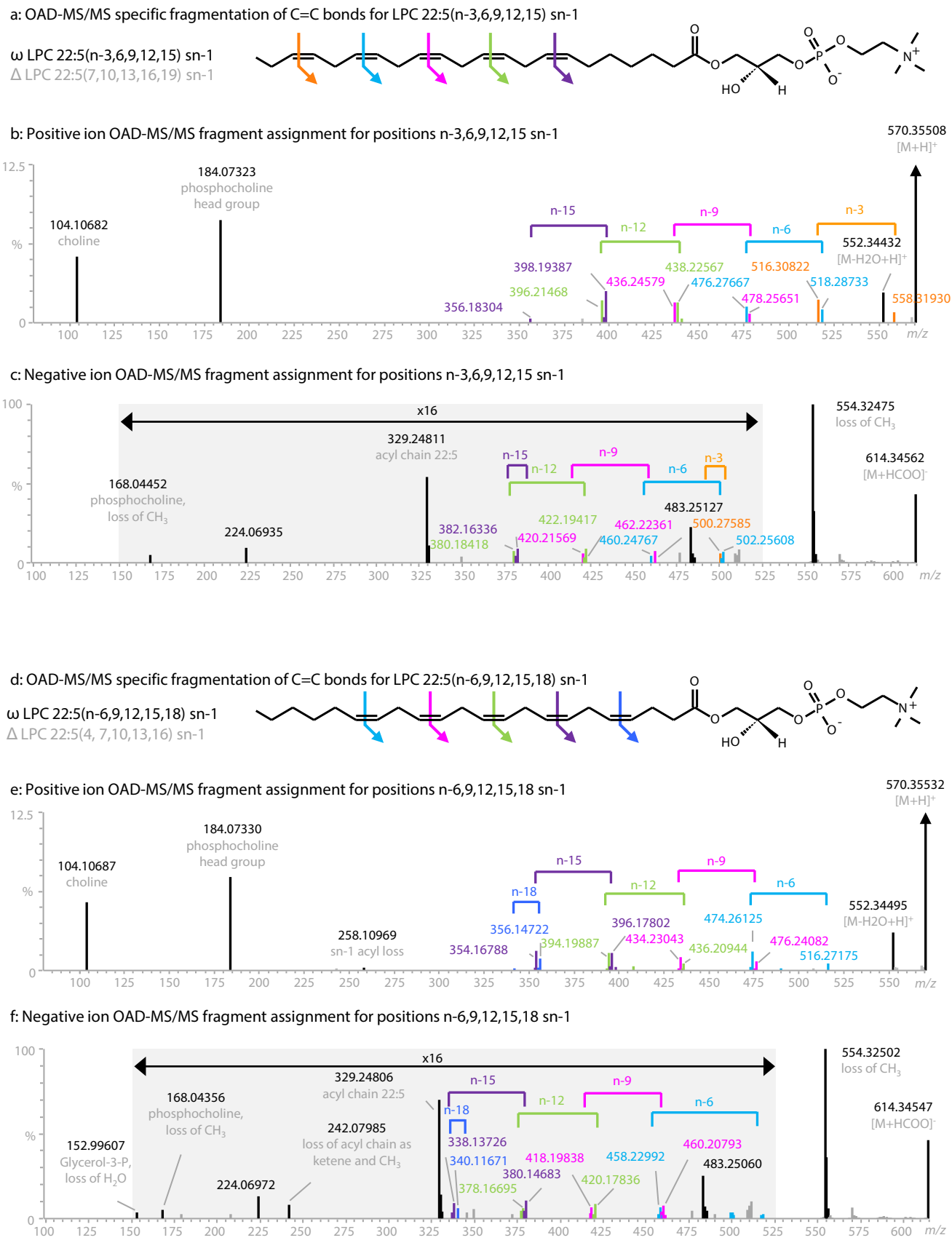

Fig. 4 LPC 22:5(n-3,6,9,12,15) sn-1 (a: molecular structure with omega and delta nomenclature, b: OAD-MS/MS in positive ion, c: OAD-MS/MS in negative ion). LPC 22:5(n-6,9,12,15,18) sn-1 (d: molecular structure with omega and delta nomenclature, e: OAD-MS/MS in positive ion, f: OAD-MS/MS in negative ion).

Simultaneous CID and OAD-MS/MS spectra resulted in a specific identification for the feature which is differentially expressed following ethanol treatment in mice as identified LPC 22:5(n-6,9,12,15,18) sn-1 in a pancreatic tissue sample. CID-MS/MS specific fragments highlighted in black, OAD-MS/MS highlighted in colours as indicated.

Annotated significant results from UHPLC-MS/MS untargeted metabolic profiling of mouse gut, liver, and pancreas following chronic ethanol exposure were considered for OAD-MS/MS analysis to increase the level of confidence in identification. Lipid annotations in the original study considered library matching between the experimental CID-MS/MS (DIA-MS/MS and DDA-MS/MS) with external databases such as LIPID MAPS, MassBank and HMDB. The level of confidence in identification as determined by the Metabolomics Standards Initiative[3] is level 2; comparison of experimental data to literature or external laboratory data. To enhance the identification of unsaturated lipids in this study, C=C bond positions were derived from specific OAD-MS/MS spectra for all unsaturated lipids that were significant.

Lipids which were significantly increased or decreased following ethanol exposure are listed in Tables 2 and 3 respectively.

The response to ethanol toxicity was largely tissue specific, OAD-MS/MS highlighted common constituents such as notable increases in omega-6 containing phosphatidylcholines (18:2 n-6,9) in response to ethanol toxicity as well decreases in polyunsaturated phospholipids containing fatty acids with four or more double bonds.

Table 2. Identification of C=C double bond position(s) in unsaturated lipids found to be **significantly increased** in gut, liver and/or pancreas tissue extracts following ethanol exposure. The tissue(s) in which the lipid was found to be significant in the original study is indicated (☐) along with the detected retention time. OAD-MS/MS data were analysed in positive and negative ion mode to confidently identify the double bond positions in each of the lipids.

Annotation	Formula	RT	ESI+ *m/z*	ESI- *m/z*	Gut	Liver	Pancreas
						☐	
Docosahexaenoic acid ethyl ester (n-3,6,9,12,15,18)	C24H36O2	14.49	357.27881	355.26425			
Linoleic acid ethyl ester (n-6,9)	C20H36O2	15.77	309.27881	307.26425		☐	
LPC 18:1(n-9) sn-1	C26H52NO7P	7.81	522.35542	566.34634	☐		
LPC 18:1(n-9) sn-2	C26H52NO7P	7.49	522.35542	566.34634	☐		
LPC 18:2(n-6,9) sn-1	C26H50NO7P	6.78	520.33977	564.33069	☐		
LPC 18:2(n-6,9) sn-2	C26H50NO7P	6.50	520.33977	564.33069	☐		
LPC 20:3(n-6,9,12) sn-1	C28H52NO7P	7.36	546.35542	590.34634		☐	
LPC 20:3(n-6,9,12) sn-2	C28H52NO7P	7.09	546.35542	590.34634	☐		
LPC 20:4(n-6,9,12,15) sn-2	C28H50NO7P	6.53	544.33977	588.33069	☐		
LPC 20:5(n-3,6,9,12,15) sn-1	C28H48NO7P	6.01	542.32412	586.31504		☐	
LPE 18:2(n-6,9) sn-1	C23H44NO7P	6.72	478.29282	476.27826	☐	☐	
LPE 18:2(n-6,9) sn-2	C23H44NO7P	6.44	478.29282	476.27826	☐		
LPE 20:3(n-6,9,12) sn-1	C25H46NO7P	7.31	504.30847	502.29391		☐	
LPE O-18:1(n-9)	C23H48NO6P	8.19	466.32920	464.31465			☐
LPE P-18:2(n-6,9)	C23H44NO6P	7.12	462.29790	460.28335			☐
LPI 18:2(n-6,9) sn-1	C27H49O12P	6.85	597.30344	595.28889		☐	
Oleic acid ethyl ester	C20H38O2	18.04	311.29446	309.2799	☐		
PC 14:0_18:2(n-6,9)	C40H76NO8P	19.74	730.53813	728.52358	☐		
PC 15:0_18:2(n-6,9)	C41H78NO8P	21.40	744.55378	788.54471	☐		
PC 16:0_18:2(n-6,9)	C42H80NO8P	23.14	758.56943	802.56036	☐		
PC 16:0_18:3(n-6,9,12)	C42H78NO8P	21.28	756.55378	800.54471	☐		
PC 16:0_20:5(n-3,6,9,12,15)	C44H78NO8P	20.31	780.55378	824.54471	☐	☐	
PC 16:1(n-7)_18:2(n-6,9)	C42H78NO8P	20.13	756.55378	800.54471	☐		
PC 18:0_18:2(n-6,9)	C44H84NO8P	26.97	786.60073	830.59166	☐	☐	
PC 18:1(n-9)_18:2(n-6,9)	C44H82NO8P	23.51	784.58508	828.57601	☐		
PC 18:1(n-9)_18:2(n-6,9)	C44H82NO8P	24.62	784.58508	828.57601	☐		
PC 18:2(n-6,9)/18:2(n-6,9)	C44H80NO8P	20.75	782.56943	826.56036	☐		
PC 18:2(n-6,9)_20:4(n-6,9,12,15)	C46H80NO8P	20.26	806.56943	850.56036	☐		
PC 36:2(n-6,9)	C44H84NO8P	26.67	786.60073	830.59166	☐		
PC P-16:0/18:2(n-6,9)	C42H80NO7P	24.33	742.57452	786.56544	☐		
PE 18:0_18:2(n-6,9)	C41H78NO8P	27.23	744.55378	742.53923	☐		
PE 18:1(n-9)_18:2(n-6,9)	C41H76NO8P	23.76	742.53813	740.52358	☐	☐	
PE P-16:0_20:5(n-3,6,9,12,15)	C41H72NO7P	21.45	722.51192	720.49736	☐		

Table 3. Identification of C=C double bond position(s) in unsaturated lipids found to be **significantly decreased** in gut, liver and/or pancreas tissue extracts following ethanol exposure. The tissue(s) in which the lipid was found to be significant in the original study is indicated (☐) along with the detected retention time. OAD-MS/MS data were analysed in positive and negative ion mode to confidently identify the double bond positions in each of the lipids.

Annotation	Formula	RT	ESI+ *m/z*	ESI- *m/z*	Gut	Liver	Pancreas
DG 18:1(n-9)_18:2(n-6,9)	C39H70O5	29.27	641.51155	N/A	☐		☐
Linoleic acid(n-6,9)	C18H32O2	11.04	281.24751	279.23295	☐		
Linolenic acid methyl ester(n-6,9)	C19H32O2	12.87	293.24751	291.23295			☐
LPC 18:3(n-6,9,12) sn-1	C26H48NO7P	6.11	518.32412	562.31504			☐
LPC 20:4(n-6,9,12,15) sn-1	C28H50NO7P	6.77	544.33977	588.33069			☐
LPC 20:4(n-6,9,12,15) sn-2	C28H50NO7P	6.53	544.33977	588.33069		☐	☐
LPC 22:4(6,9,12,15) sn-1	C30H54NO7P	8.01	572.37107	616.36199			☐
LPC 22:4(6,9,12,15) sn-2	C30H54NO7P	7.74	572.37107	616.36199		☐	☐
LPC 22:5(n-6,9,12,15,18) sn-1	C30H52NO7P	7.56	570.35542	614.34634		☐	☐
LPC 22:5(n-6,9,12,15,18) sn-2	C30H52NO7P	7.31	568.33977	614.34634		☐	☐
LPC O-18:1(n-9)	C26H54NO6P	8.25	508.37615	552.36708	☐		
LPC O-18:2(n-6,9)	C26H52NO6P	7.14	506.36050	550.35143	☐		
LPC P-18:1(n-9)	C26H52NO6P	8.26	506.36050	550.35143	☐		
LPE 22:5(n-6,9,12,15,18) sn-1	C27H46NO7P	7.51	528.30847	526.29391	☐		☐
LPE 22:5(n-6,9,12,15,18) sn-2	C27H46NO7P	7.26	528.30847	526.29391		☐	☐
LPE O-18:1(n-9)	C23H48NO6P	8.19	466.32920	464.31465	☐		
LPE P-18:1(n-9)	C23H46NO6P	8.21	464.31355	462.29900	☐		
LPE P-18:2(n-6,9)	C23H44NO6P	7.12	462.29790	460.28335	☐		
LPE P-20:1(n-9)	C25H50NO6P	10.01	492.34485	490.33030	☐		
LPE P-22:1(n-9)	C27H54NO6P	12.13	520.37615	518.36160	☐		
LPI 20:4(n-6,9,12,15) sn-2	C29H49O12P	6.56	621.30344	619.28889			☐
Oleic acid (n-9)	C18H34O2	12.73	283.26316	281.24860	☐		☐
Oleic acid methyl ester (n-9)	C19H36O2	16.66	297.27881	295.26425			☐
Palmitoleic acid methyl ester (n-7)	C17H32O2	13.88	269.24751	267.23295			☐
PC 16:0_22:4(n-6,9,12,15)	C46H84NO8P	25.25	810.60073	854.59166	☐		
PC 16:0_22:5(n-3,6,9,12,15)	C46H82NO8P	23.97	808.58508	852.57601	☐		
PC 16:1(n-7)_20:4(n-6,9,12,15)	C44H78NO8P	19.66	780.55378	824.54471	☐		☐
PC 16:1(n-7)_22:6(n-3,6,9,12,15,18)	C46H78NO8P	18.89	804.55378	848.54471		☐	
PC 18:1(n-9)_20:4(n-6,9,12,15)	C46H82NO8P	22.88	808.58508	852.57601		☐	
PC 20:4(n-6,9,12,15)_22:6(n-3,6,9,12,15,18)	C50H80NO8P	18.89	854.56943	898.56036		☐	
PE 16:0_22:5(n-6,9,12,15,18)	C43H76NO8P	24.21	766.53813	764.52358	☐	☐	
PE 18:0_22:6(n-3,6,9,12,15,18)	C45H78NO8P	24.56	792.55378	790.53923		☐	

■ Conclusion

OAD-MS/MS is a novel fragmentation method providing complementary structural information to CID in positive and negative ion mode. It is less hazardous than other methods and provides higher efficiency fragmentation, especially for singly charged positive and negative ion precursors. OAD-MS/MS enables C=C positional assignments in lipids and has been applied to structurally characterise lipids found to be potential markers of ethanol toxicity in a previous study using high resolution metabolomics analysis by UHPLC-CID-MS/MS. The enhanced level of structural identification provided by OAD-MS/MS allows the potential to improve our understanding of the biological roles of lipids.

■ References

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