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4 **Liquid extraction surface analysis field asymmetric waveform ion**
5 **mobility spectrometry mass spectrometry for the analysis of dried**
6 **blood spots**
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31 **Abstract**

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33 Liquid extraction surface analysis (LESA) is a surface sampling technique that allows electrospray
34 mass spectrometry analysis of a wide range of analytes directly from biological substrates. Here, we
35 present LESA mass spectrometry coupled with high field asymmetric waveform ion mobility
36 spectrometry (FAIMS) for the analysis of dried blood spots on filter paper. Incorporation of FAIMS in
37 the workflow enables gas-phase separation of lipid and protein molecular classes, enabling analysis
38 of both haemoglobin and a range of lipid (phosphatidylcholine or phosphatidylethanolamine, and
39 sphingomyelin species) from a single extraction sample. The work has implications for multiplexed
40 clinical assays of multiple analytes.
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48 Keywords: liquid microjunction sampling, dried blood spot, FAIMS
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Introduction

Liquid extraction surface analysis (LESA) mass spectrometry is a direct surface sampling technique originally developed by Van Berkel and co-workers^{1,2}. Analytes are extracted from the surface via a liquid micro-junction between a pipette tip and the sample surface. This approach enables direct sampling of a variety of biological analytes, such as drugs³, lipids^{4,5} and proteins⁶⁻⁸, from a range of solid surfaces prior to electrospray ionisation (ESI). Substrates analysed by LESA include thin tissue sections^{3,6,9}, bacterial colonies grown on agar⁷, dried blood spots on card^{8,10,11} and polymeric surfaces^{4,12}. Other direct analysis approaches that have been applied to the analysis of dried blood spots include desorption electrospray ionisation (DESI)¹³, direct analysis in real time (DART)^{14,15} and paperspray¹⁶⁻¹⁸.

Previous analyses of dried blood spots (DBS) on card have shown that peaks corresponding to the intact α -globin and β -globin sub-units can be detected via LESA MS^{8,11}. More recently, we have shown that intact tetrameric haemoglobin can be detected by LESA MS of dried blood spots¹². LESA MS therefore has potential applications in the analysis of haemoglobinopathies, the most commonly inherited disorders. Edwards *et al.* showed that LESA MS of dried blood spots was suitable for the screening of variants HbS, HbC, HbD, HbE, and β -thalassemia^{8,19}.

Haemoglobin is highly abundant in blood samples (between 120 and 130 mg/mL²⁰), however a range of other analytes, such as lipid species, are also present and have been implicated as biomarkers for disease profiling. Analysis of DBS samples, rather than whole blood, can be preferential in a clinical setting owing to the reduced sample volumes required²¹, the relative ease of storage compared to whole blood or plasma²² and due to the reduced risk of infection from certain blood borne diseases²³.

Generally lipids are prepared from DBS samples by taking a "punch" and extracting in methanolic solution before performing mass spectrometry analysis²⁴⁻²⁷. Sandler *et al.* used an LC MS/MS approach to monitor 26:0 lyso-phosphatidylcholine as a biomarker of X-linked adrenoleukodystrophy²⁴ in samples eluted from DBS. Koulman *et al.* showed that the lipid profile in samples from DBS was a reliable indicator of lipid metabolism in infants²⁵. These researchers also reported that monitoring of lipid species from DBS samples yielded more precise results in comparison to plasma samples, owing to the prevention of degradation processes upon drying²⁵. Faller *et al.* have shown that the monitoring of phosphatidylethanolamine species extracted from DBS samples by LC MS/MS can be determined as reliably as from whole blood²⁶, which could be a viable method of monitoring alcohol misuse. Recently, Denes *et al.* reported the detection of a wide

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3 range of metabolites, including lipids, from similarly prepared samples²⁷. They also showed that
4 their methods were sufficiently robust for disease profiling.
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8 A method which combines the benefits of direct sampling of dried blood spots (elimination of
9 sample preparation) with the ability to separate molecule classes, e.g., lipids and proteins, would be
10 of great benefit: Such a method would overcome the challenge of interference from haemoglobin
11 enabling direct analysis of lipids. Furthermore, separation of the different sub-units of haemoglobin
12 could be advantageous in the diagnosis of haemoglobinopathies.
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17 Here, we combine field asymmetric waveform ion mobility spectrometry (FAIMS) with LESA of DBS.
18 We have recently demonstrated the benefits of coupling of FAIMS to LESA MS analysis of thin tissue
19 sections and bacterial colonies growing on agar²⁸. FAIMS separates ions at atmospheric pressure on
20 the basis of their differential ion mobilities in high and low electric fields^{29, 30} and offers advantages
21 including improved signal-to-noise ratios, reduced chemical noise³¹ and separation of isomeric
22 analytes^{32, 33}. In the current work, we show that LESA FAIMS mass spectrometry of DBS enables
23 separation of the haemoglobin α - and β - sub units from lipid species. Lipids identified included
24 phosphatidylcholine or phosphatidylethanolamine species and sphingomyelin species. The lipids
25 could not be detected by LESA mass spectrometry in the absence of FAIMS.
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37 **Methods**

38 ***Dried blood spots***

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42 The work was approved by the University of Birmingham STEM Ethical Review Committee (ERN_14-
43 0454). Dried blood spots were acquired from healthy human adult donors via finger prick onto NHS
44 blood spot (Guthrie) cards, Ahlstrom grade 226 filter paper ID Biological Systems, (Greenville, USA)
45 and dried overnight prior to analysis. Reproducibility data were obtained from four technical
46 replicates from each of two biological replicates. DBS were sampled 1 day after collection and 6 days
47 after collection. Samples were stored in a covered dish at room temperature.
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55 ***Surface sampling***

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58 *LESA sampling:* LESA was carried out by use of a Triversa Nanomate nanoelectrospray platform
59 (Advion Biosciences, Ithaca, USA). The dried blood spot samples were mounted onto the LESA
60 universal adaptor plate and an image was acquired using an Epson Perfection V300 photo scanner.

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The LESA Points software (Advion) was used to select the precise location of the substrate to be sampled. The universal adapter plate was placed in the Triversa Nanomate. A solution comprising 1:1 methanol: (HPLC grade) water (both JT Baker, Deventer, The Netherlands) with the addition of 1 % formic acid (Sigma-Aldrich Company Ltd., Dorset, U.K.) was used as the extraction and ionisation solvent. In the extraction process, 10 μ l of solvent was aspirated from the solvent well. The robotic arm relocated to a position above the sample and descended to a height of 1.6mm above the surface. Four microlitres of solvent was dispensed onto the surface. After a delay of 3 seconds, 4.5 μ l of sample was reaspirated into the pipette tip before samples were infused into the mass spectrometer via chip-based electrospray ionisation. Samples were infused with a gas pressure of 0.3 PSI and an ionisation voltage of 1.7 kV.

ultraFAIMS

FAIMS was carried out in positive ion mode using a miniaturized ultra-FAIMS device (Owlstone, Cambridge, UK), using a NC chip with a trench length of 78.1 mm and a gap of 101.3 μ m and a thickness of 700 μ m. The FAIMS device was interfaced to an Orbitrap Velos (Thermo Scientific, UK) mass spectrometer. *2D FAIMS*: separation was performed at eight discrete dispersion fields (DFs) between 130 Td and 270 Td with step size 20 Td. At each DF, the compensation field (CF) was varied between -1 to +4 Td over a 180 s time period. *Static FAIMS*: Optimal DF and CF conditions for the transmission of ions of interest were selected from the 2D FAIMS analysis. For transmission of lipids the conditions were DF = 130 Td and CF = 1.8 Td.

Mass Spectrometry

2D FAIMS experiments were performed on a Thermo Fisher Orbitrap Velos mass spectrometer at a resolution of 100,000 at m/z 400. Static FAIMS and HCD experiments were performed on a Thermo Fisher Orbitrap Elite mass spectrometer at a resolution of 120,000 at m/z 400. Full scan mass spectra were collected in the Orbitrap between m/z 600-2000. The AGC target was 1×10^6 charges with a maximum injection time of 1000 ms. Each scan was composed of 1 microscan. MS/MS: Precursor ions were selected for fragmentation in the linear ion trap (isolation width 2 Th). HCD experiments (NCE 18-20%) were recorded at a resolving power of 120 000 at m/z 400. AGC target was 2×10^4 charges with maximum injection time 1000 ms. Each scan comprised 1 microscan. MS/MS spectra shown comprise 125 co-added scans (3 minutes of data).

Data analysis

Data were converted from Thermo .raw format to mzML using msconvert as part of ProteoWizard³⁴ and then to imzML using imzMLConverter³⁵. Data in the imzML format were then loaded into MATLAB (version 2013, The MathWorks Inc., Natick, Massachusetts) using imzMLConverter and in-house software. Total ion transmission maps and single ion transmission maps were generated as described in [28], further implemented to allow generation of three dimensional total ion transmission maps. Software for generation of total ion transmission maps and single ion transmission maps, together with a step-by-step guide, is available for download from <http://www.biosciences-labs.bham.ac.uk/cooper/software.html>.

Lipid Assignment

Abundant lipid species detected from DBS samples were identified by a combination of higher energy collision dissociation (HCD) and accurate mass (5ppm tolerance) via cross-referencing with the LIPID MAPS database^{36, 37}. Lower abundance species were assigned solely on the basis of accurate mass (5ppm tolerance) with cross referencing against the LIPID MAPS database. MS/MS spectra were analysed manually in Xcalibur version 3.0.63 (Thermo Fisher).

Results and Discussion

The LESA Nanomate robot was coupled to the Owlstone ultraFAIMS device, which in turn was coupled to a high mass resolution Orbitrap mass spectrometer. LESA sampling of DBS on filter paper was coupled with a 2D FAIMS analysis in which the dispersion field (DF) was varied between 130 and 270 Td and the compensation field (CF) was varied between -1 and 4 Td. **Figure 1 (Panels A-H)** show the total ion transmission maps (m/z versus CF) obtained at each of the dispersion fields (DF 130 to 270 Td in increments of 20 Td). Figure 1 (Panels I-P) show the same data plotted as three dimensional total ion transmission maps. Displaying the data in this way offers insight as to the global changes afforded by changing DF. It is evident from these maps that increasing DF results in transmission of ions across a greater range of compensation fields. Similar behaviour was observed for LESA FAIMS analysis of thin tissue sections²⁸. The striations apparent in the 2D total ion transmission maps correspond to the various charge states of the protein ions. These features are particularly noticeable here due to the high concentration of haemoglobin in these samples. *Supplemental Figure 1* shows the 2D total ion transmission map obtained at 270 Td (Panel H) together with the averaged mass spectrum acquired during this 3 minute experiment overlaid. It is

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3 clear from this figure that the high intensity strata in the total ion transmission map correlate to α -
4 globin and β -globin ions in charge states varying from +14 to +20.
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10 Single ion transmission maps for the various charge states of α - and β -globin, in which the
11 transmission of specific m/z values according to DF and CF, are shown in *Supplemental Figure 2*. It is
12 evident from these maps that the transmission of each globin chain did not differ significantly from
13 the other; hence the use of the FAIMS field did not enable separation of the α - and β -globin chains.
14 Lower charge state ions were transmitted from lower CF values than higher charge state ions, and
15 higher charge state ions were transmitted over a broader CF range as DF increased. Brown *et al.*
16 have described similar results; that multiply charged peptides are transmitted at higher
17 compensation fields when compared to lower charge state peptides³⁸.
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26 Blood constitutes a complex mixture of proteins, many of which have been identified from DBS
27 samples. Martin *et al.* reported identification of 120 different proteins in a single dried blood spot¹⁰
28 following LESA sampling coupled with automated tryptic digestion. Nevertheless, no proteins other
29 than the globins were observed following LESA FAIMS. That is, the inclusion of FAIMS could not
30 mitigate the issue of dynamic range for the analysis of proteins in DBS.
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36 The main benefit in coupling FAIMS with LESA analysis of dried blood spots arises from the ability to
37 separate molecular classes, i.e., lipids and proteins. **Figure 2A** shows the LESA mass spectrum
38 obtained from DBS in the absence of FAIMS. Clearly, the most abundant species are the α - and β -
39 globin protein ions. Peaks observed at m/z 757, 797, 841, 890, 946, 1009 and 1081 correspond to
40 the +20 to +14 charge states of α -globin, and those at m/z 882, 934, 992, 1058 and 1134 correspond
41 to the +18 to +14 charge states of β -globin. By introducing FAIMS into the workflow, it is possible to
42 focus the protein ions. The total ion transmission map obtained at DF = 130 Td (Fig 1A) shows the
43 region of FAIMS DF/CF space populated by globin ions is more confined than when compared with
44 that obtained at DF = 270 Td. This focusing of haemoglobin protein ions at DF = 130 Td enables the
45 separation of lipid species. Figure 2 B shows the averaged mass spectrum obtained in the CF range
46 0.18 to 1.54 Td at DF = 130 Td. The peaks observed correspond to the α -globin and β -globin ions in
47 various charge states. Figure 2C shows the averaged mass spectrum obtained in the CF range 1.78 to
48 3.86 Td at DF = 130 Td. The mass spectrum is dominated by peaks corresponding to singly-charged
49 lipid species (which were not observed in the absence of FAIMS) and peaks corresponding to
50 haemoglobin are observed in the +20 and +19 charge states only.
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4 A two dimensional FAIMS analysis (in which DF and CF are varied) enables identification of optimum
5 FAIMS conditions for transmission of a particular (set of) ions²⁸. A static FAIMS analysis (in which DF
6 and CF are constant) can subsequently be performed. In the present case, lipid species from DBS
7 were optimally transmitted under the following conditions: DF 130 Td and CF 1.8 Td. Figure 2 D
8 shows an example mass spectrum acquired under these experimental conditions. (Mass spectrum
9 comprises 1 minute of data (42 scans)). Lipid species were assigned by a combination of MS/MS and
10 accurate mass cross-referencing against the LIPID MAPS database^{36, 37}. Higher-energy collision
11 dissociation (HCD) of the abundant precursor ions with m/z 758.6, 760.6, 782.6 and 786.6 resulted
12 in detection of one predominant product ion at m/z 184.1, see *Supplemental Figure 3*, which
13 corresponds to the protonated phosphatidylcholine (PC) headgroup³⁹. A number of further species,
14 with insufficient abundance to acquire MS/MS data, were assigned by accurate mass as protonated
15 phospholipid species, either phosphatidylcholine (PC) or phosphatidylethanolamine (PE) species, and
16 sphingomyelin species (SM). Lipid assignments are summarised in **Table 1**.

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28 In order to probe the reproducibility of the LESA FAIMS approach for analysis of lipids from DBS,
29 static FAIMS experiments were performed. Two biological replicates were each analysed at four
30 separate locations at two time points (1 day after sample collection and 6 days after sample
31 collection). The results are shown in **Figure 3**. For each set of technical replicates (for each lipid m/z,
32 at each time point from each biological sample), the reproducibility was good. (Error bars indicate
33 one standard deviation). The variation between biological replicates was greater on day 1 than on
34 day 6, although the variation was only significant ($p < 0.05$ as given by a Student's t-test) for m/z
35 813.7 (SMd42:2) on day 1, and for m/z 786.6 (PC 36:2) on day 6. For biological replicate 1, the
36 variation between time points was significant ($p < 0.05$) for m/z 758.6 (PC 34:2) and very significant
37 ($p < 0.01$) for m/z 760.6 (PC 34:1) and m/z 810.6 (PC 38:4 or PE41:4). For biological replicate 2, the
38 variation in relative abundance was significant ($p < 0.05$) for m/z 786.6 (PC 36:2) and very significant
39 ($p < 0.01$) for m/z 810.6 (PC 38:4 or PE 41:4). These results suggest that a) reproducibility between
40 technical replicates is high, b) the (expected) variation between biological replicates is clearer in
41 samples that are 1 day old, c) some lipid degradation occurs after 6 days. Further work to
42 characterise the latter is warranted.

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55 The lipid species identified are major components of cell membranes and therefore play an
56 important structural role. The phosphatidylcholine lipids PC 34:2, 36:2, and 38:4 observed here
57 were also detected directly from dried blood spot samples via paperspray ionisation coupled with a
58 triple quadrupole mass spectrometer; by Espy *et al.*⁴⁰. The results here show a wider range of lipid
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3 species can be detected directly from dried blood spots via LESA FAIMS mass spectrometry. As
4 described above, a number of researchers have used the DBS sample format as a platform for
5 studying phospholipids. In each case, the researchers employed lengthy extraction procedures in
6 order to extract the reported lipid species from the dried blood spot samples. For example, Denes *et*
7 *al.* extracted samples in methanol for 30 minutes and then mixed the sample as a 1:1 mixture with
8 water before ESI mass spectrometry using the Triversa Nanomate robot ²⁷. Our results show direct
9 surface sampling (LESA) coupled with FAIMS leads to the detection of an increased number of
10 different phospholipid species in a much shorter analysis time and using more straightforward
11 experimental methods.
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20 Although a wider range of lipid species have been reported in other investigations (Koulman *et al.*
21 describe the detection of a range of PCs, PEs, SMs, TAGs and CEs in the mass range m/z 640-1000
22 from infant samples ²⁵), the initial results described in this study are promising. Inclusion of the
23 FAIMS device in the mass spectrometry workflow reduces the requirement for lengthy extraction
24 procedures to monitor phospholipid and sphingomyelin species from these samples. Optimisation of
25 the extraction solvent used for LESA-FAIMS experiments could increase the number of lipid classes
26 detected from dried blood spot samples and work in this area is ongoing.
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34 As described above, Edwards *et al.* have shown it is possible unambiguously assign a number of
35 haemoglobin variants via LESA sampling of newborn DBS ^{8, 11, 19}. Furthermore, Koulman *et al.* showed
36 that monitoring of lipid species in newborn DBS is a suitable approach for determining lipid
37 metabolism ²⁵. The method presented here could be tailored for the targeted analysis of a range of
38 analytes, such as proteins and lipid species, in a single analysis. This finding has implications for the
39 clinic: It is feasible that metabolites, for instance phospholipids, could be monitored alongside
40 haemoglobin variants, directly from a single extraction from a dried blood spot sample simply by
41 varying the FAIMS parameters. Moreover, the analysis time is short: the protein mass spectrum
42 shown in Fig 2B was recorded in < 20 seconds and the lipid mass spectrum in Fig 2C in < 60 seconds.
43 Hence it would be possible to acquire both protein and lipid data within 90 seconds of analysis time.
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52 53 54 **Conclusions**

55 LESA FAIMS mass spectrometry is presented as a tool for the analysis of dried blood spots. The
56 approach allows the separation of highly abundant haemoglobin ions from lower abundance lipid
57 ions which could not be detected in the absence of FAIMS. It was not possible to separate the
58 individual globin units (α and β) by use of the ultraFAIMS, nor were any additional proteins
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3 identified. A range of lipids were identified including phosphatidylcholine or
4 phosphatidylethanolamine and sphingomyelin species. These species are biologically relevant and
5 have previously been shown to be potential targets in the screening of diseases in newborns. LESA
6 FAIMS mass spectrometry of DBS could find application in the clinic for multiplexed screening assays,
7 i.e., monitoring of haemoglobin variants and lipids in a single analysis.
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26 Birmingham data archive at <http://findit.bham.ac.uk/>.
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Figures

Figure 1. (A) to (H): 2-dimensional total ion transmission maps (m/z vs. compensation field (CF)) obtained following LESA-FAIMS mass spectrometry of dried blood spots at dispersion fields DF = 130 Td, 150 Td, 170 Td, 190 Td, 210 Td, 230 Td, 250 Td and 270 Td. (I) to (P): 3-dimensional total ion transmission plots of the same data showing signal intensity in the z axis, with a mean mass spectrum projected in red and total ion chromatogram projected in blue.

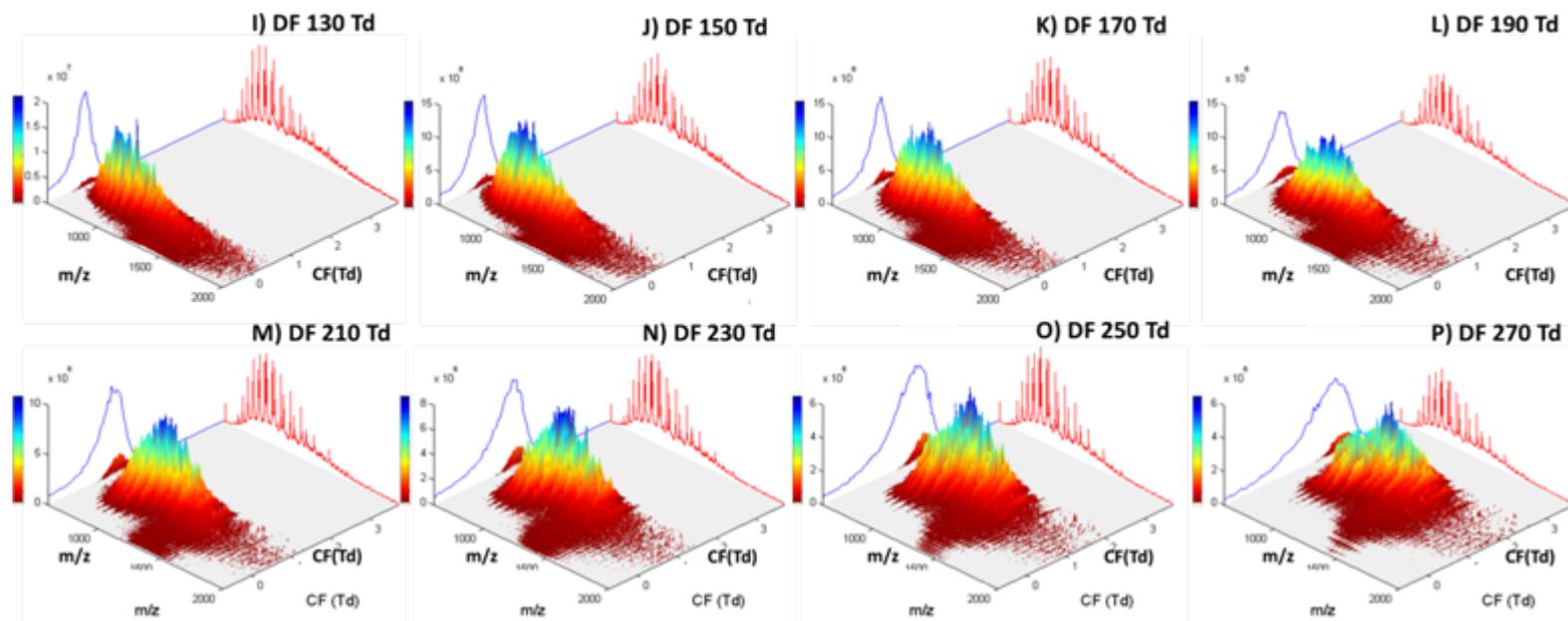
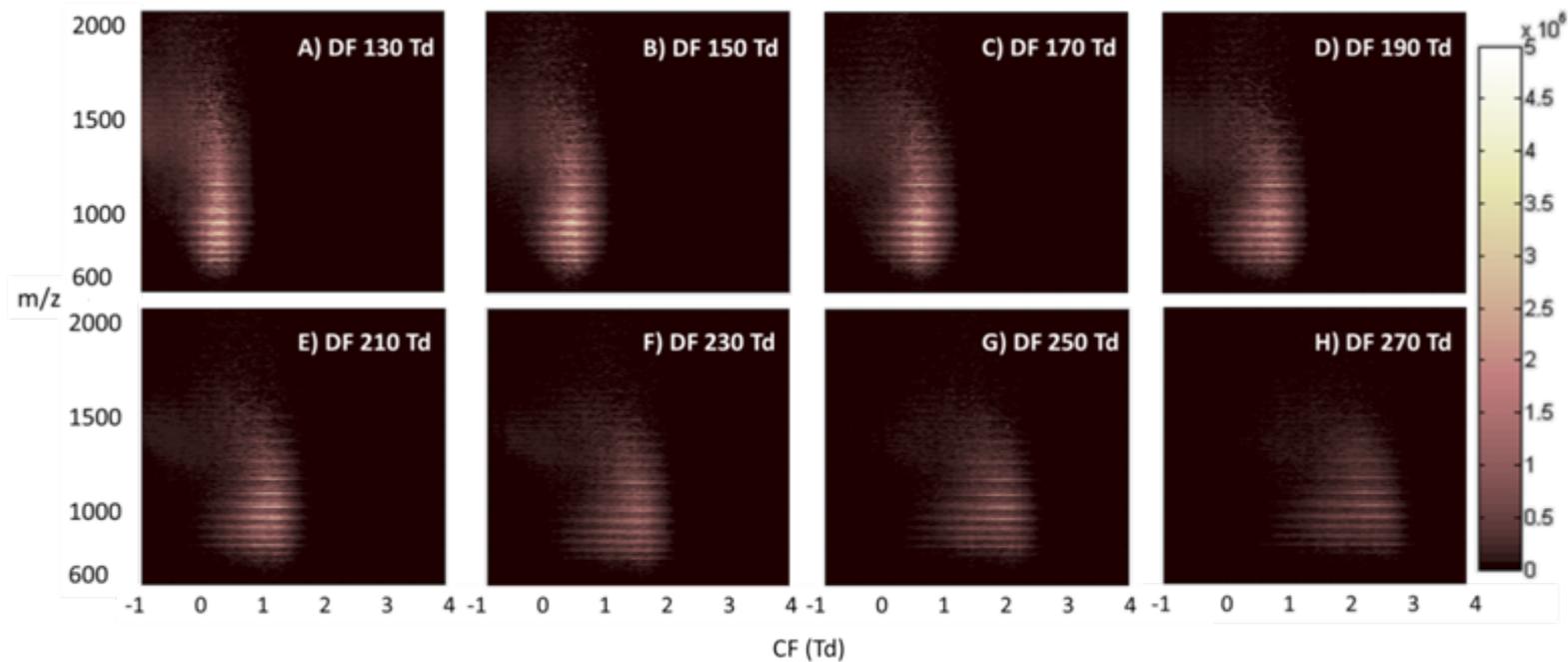
Figure 2. (A) LESA mass spectrum obtained from a DBS in the absence of FAIMS; (B) LESA FAIMS mass spectrum obtained from a DBS (2D FAIMS: DF 130 Td, CF = 0.18-1.54 Td); (C) LESA FAIMS mass spectrum obtained from a DBS (2D FAIMS: DF = 130 Td, CF = 1.78-3.86 Td); (D) LESA FAIMS mass spectrum obtained from a DBS (static FAIMS: DF = 130 Td, CF = 1.8 Td).

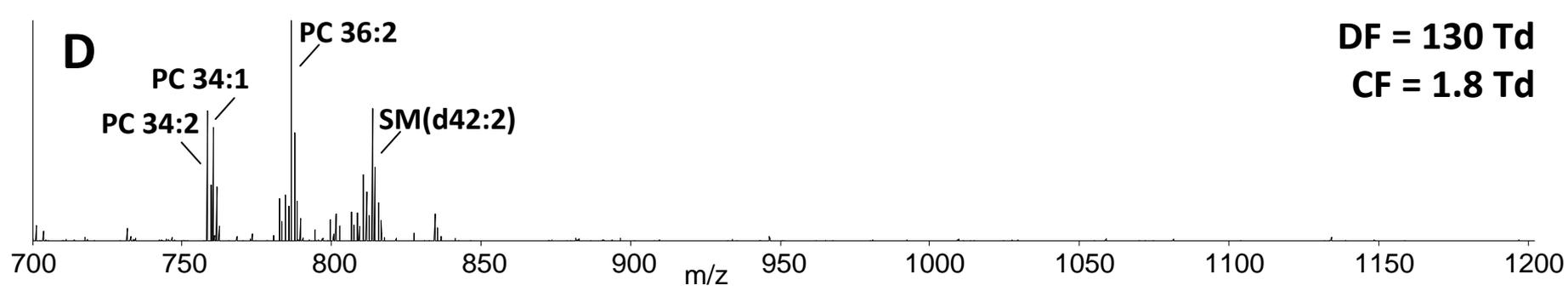
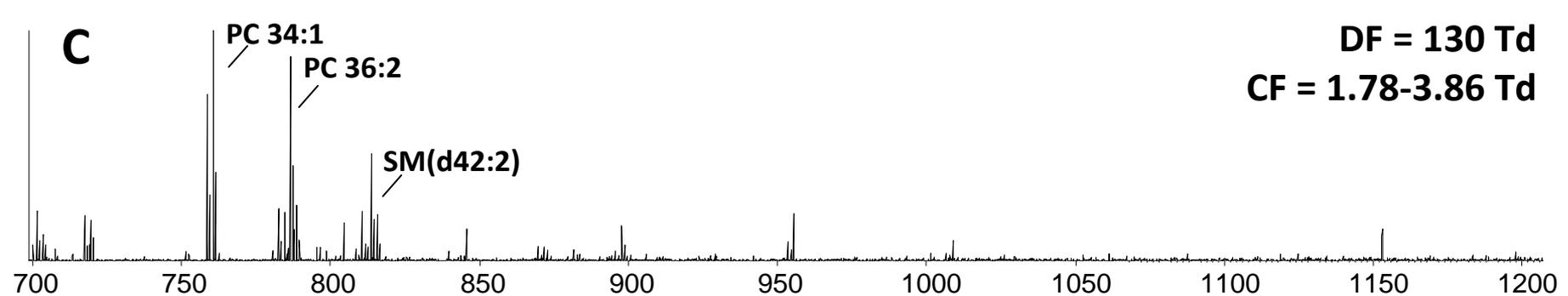
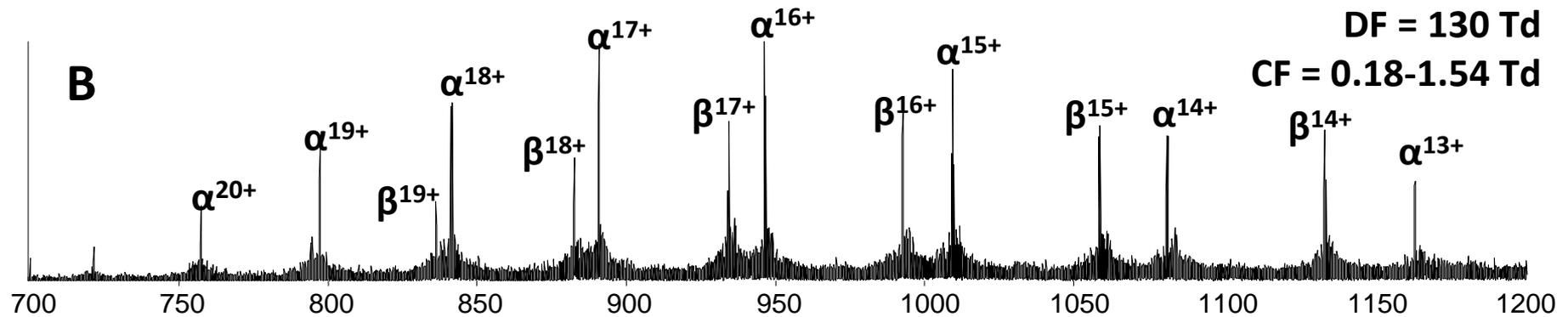
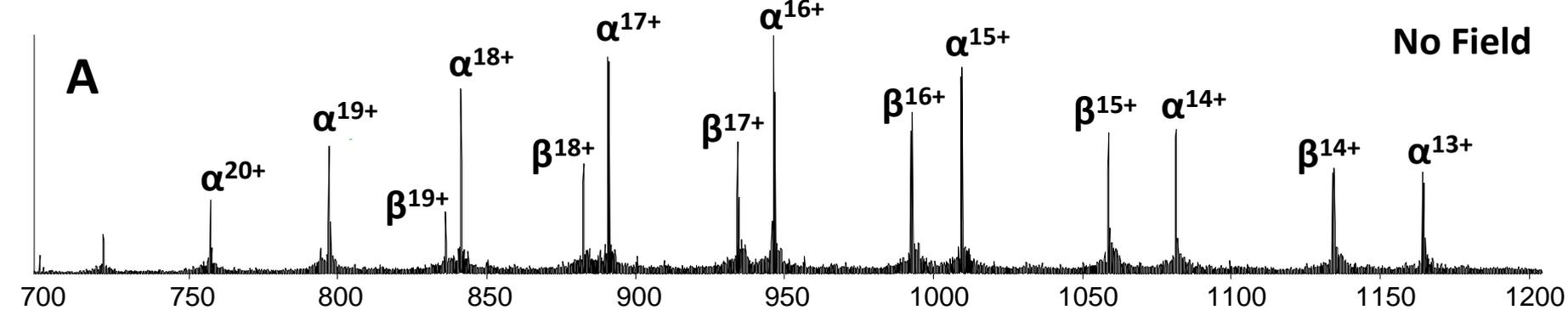
Figure 3. Relative abundances of the 6 most abundant lipid species detected during LESA FAIMS mass spectrometry analysis of DBS (DF = 130 Td; CF = 1.8 Td) at (A) 1 day and (B) 6 days after sample collection. Each biological replicate and time point is represented by 4 technical replicates. Error bars show one standard deviation.

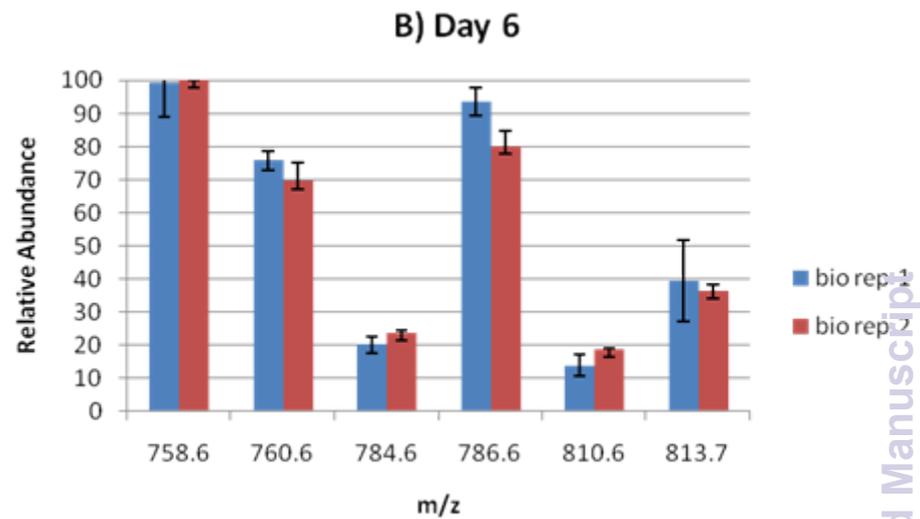
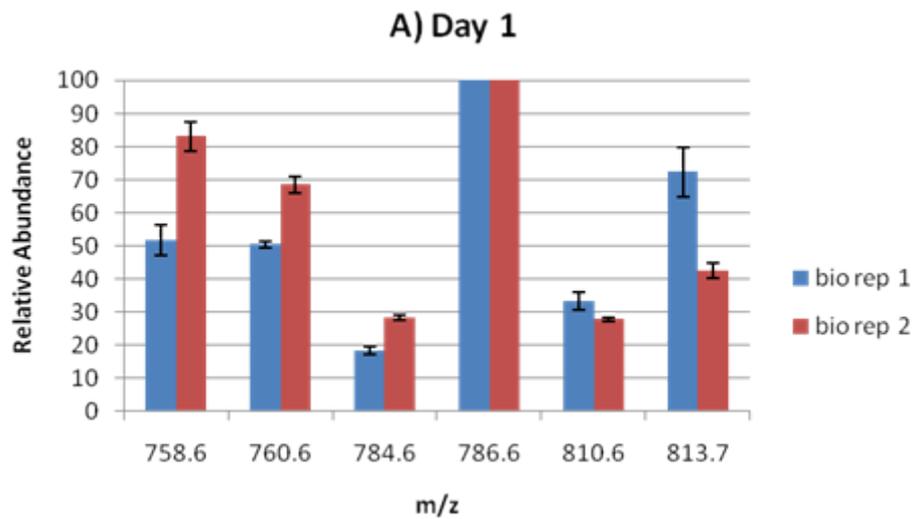
Tables

Table 1. Lipid species identified in LESA FAIMS mass spectrometry analysis of DBS at DF = 130 Td and CF = 1. 8 Td. A number of phospholipid and sphingomyelin species are identified within 5 ppm error.

* indicates lipids identified on the basis of MS/MS.







Measured m/z	Calculated m/z	Δ ppm	Assignment
758.5673	758.5694	2.8	*PC 34:2 [M+H] ⁺
760.5833	760.5851	2.4	*PC 34:1 [M+H] ⁺
782.5668	782.5694	3.3	*PC 36:4 [M+H] ⁺
784.5828	784.5851	2.9	PC 36:3 or PE 39:3 [M+H] ⁺
786.5984	786.6007	2.9	*PC 36:2 [M+H] ⁺
787.6670	787.6687	2.2	SM(d40:1) [M+H] ⁺
788.6132	788.6164	4.1	PC 36:1 or PE 39:1 [M+H] ⁺
810.5987	810.6007	2.5	PC 38:4 or PE 41:4 [M+H] ⁺
811.6661	811.6687	3.2	SM(d42:3) [M+H] ⁺
813.6824	813.6844	2.5	SM(d42:2) [M+H] ⁺
815.6990	815.7000	1.2	SM(d42:1) [M+H] ⁺