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A Comparison of Tissue Spray and Lipid Extract Direct Injection Electrospray Ionization Mass Spectrometry for the Differentiation of Eutopic and Ectopic Endometrial Tissues

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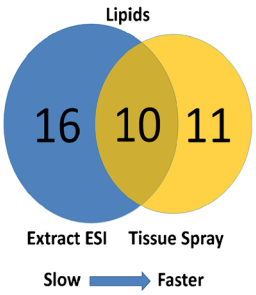

Abstract. Recent research revealed that tissue spray mass spectrometry enables rapid molecular profiling of biological tissues, which is of great importance for the search of disease biomarkers as well as for online surgery control. However, the payback for the high speed of analysis in tissue spray analysis is the generally lower chemical sensitivity compared with the traditional approach based on the offline chemical extraction and electrospray ionization mass spectrometry detection. In this study, high resolution mass spectrometry analysis of endometrium tissues of different localizations obtained using direct tissue spray mass spectrometry in positive ion mode is compared with the results of electrospray ionization analysis of lipid extracts. Identified features in both cases belong to three lipid classes: phosphatidylcholines, phosphoethanolamines, and sphingomyelins. Lipids coverage is validated by hydrophilic interaction liquid chromatography with mass spectrometry of lipid extracts. Multivariate analysis of data from both methods reveals satisfactory differentiation of eutopic and ectopic endometrium tissues. Overall, our results indicate that the chemical information provided by tissue spray ionization is sufficient to allow differentiation of endometrial tissues by localization with similar reliability but higher speed than in the traditional approach relying on offline extraction.

Keywords: Ambient mass spectrometry, Tissue spray, Extractive electrospray ionization, Lipidomics, Endometriosis

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Introduction

Ambient mass spectrometry (MS) enables the analysis of complex samples under atmospheric pressure without or with minimal sample pretreatment. This allows fast screening of molecular composition, which is essential for high-throughput biomarker search, classification of tissue pathologies based on molecular composition, including cancer staging, as well as for the progress in personalized medicine [1–5]. A large selection of ambient methods has been developed since the advent of desorption electrospray ionization (DESI) by Cooks' group in 2004 [1, 6–8]. A sub-group of ambient ionization methods is known as "substrate spray" methods [1]. In substrate-spray methods, chemical extraction of a sample

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occurs online during the analysis inside an ion source. Lipids are readily extracted under such conditions and typically yield the most abundant peaks in mass spectra [9–13], which can be used for fast lipid screening. Substrate-spray methods include paper spray [14], leaf spray [15, 16], a whole organism spray [17], and tissue spray with different modifications [11, 12, 17–20]. The latter method has been shown to be efficient in the analysis of brain and endometrium tissues and various cancer tumors [10, 11, 18]. Although increasing the speed of analysis, the online extraction in substrate-spray methods may also lead to the loss of essential information. This problem has been addressed in the recent publication devoted to the comparison of DESI-MS and ultraperfomance liquid chromatography/electrospray ionization-mass spectrometry (UPLC/ESI-MS) approach [13]. It has been shown that the glycerophospholipid profile detected by DESI-MS is congruent to UPLC/ESI-MS.

There are two main approaches for the comprehensive analysis of global lipidome. The first approach is shotgun lipidomics [21], in which no separation step is used, and the whole lipid extract is analyzed. The second approach is liquid chromatography with mass spectrometry (LC/MS) platform [22, 23]. Shotgun lipidomics accounts for over 90% of lipids directly from the lipid extracts of biological samples, but many of low abundant lipid molecular species remain undetected by this technique [21]. The time-limiting stage in shotgun lipidomics is sample preparation, which takes about 10–20 min. LC/MS lipidomics with reversed-phase, normal-phase, or hydrophilic interaction liquid chromatography (HILIC) allows detection of more lipid species [22–26] because of the separation of isobaric compounds and elimination of suppression effect for different lipid classes, but at the cost of the longest analysis time, which can last for 0.5–1 h. It should be noted that a fast method relying on the separation by supercritical fluid chromatography has been developed that provides high-throughput and comprehensive analysis of 24 lipid classes within 6 min [27]. This brings the LC/MS platform closer to the shotgun platform as far as the duration of the analysis is concerned while keeping all the benefits of chromatographic separation. However, this method is not as widespread as other chromatographic methods. Therefore, a trade-off clearly exists between the speed and chemical depth of lipid profiling.

Intraoperative potential of online direct tissue analysis has been demonstrated in rapid evaporative ionization MS (REIMS) and implemented in a commercial product (iKnife) for online routine tissue analysis during surgery [28]. Endometriosis is an abundant gynecological pathology, which is characterized by the extra-uterine presence of endometrial glands and stroma. The only reliable way to diagnose the pathology is surgical laparoscopy for the moment and intraoperative tissue identification methods are of high demand. Information about the molecular composition of eutopic (inside uterine) and ectopic (extra-uterine) endometrium can shed more light on the mechanisms of the disease and be used for tissue type determination [29–31]. Recently we demonstrated application of direct tissue analysis for the rapid differentiation between endometriotic tissues of different foci [17]. A comparison of lipid profile obtained by tissue spray and electrospray ionization mass spectrometry of lipid extracts has not been provided so far for the best of our knowledge.

The present work compares the performance of tissue spray MS and lipid extract ESI-MS for the differentiation between endometriotic tissues of different foci. Endometrial tissues (50 samples) of eutopic endometrium and ovarian endometriotic lesions from 25 patients are studied.

Experimental

Tissue Samples

Endometrial tissues (50 samples) of eutopic endometrium and ovarian endometriotic lesions from 25 patients were obtained from the Department of Surgery, V. Kulakov Research Center for Obstetrics, Gynecology and Perinatology (Moscow, Russia). All patients have read and signed informed consent approved by the Ethical Committee of the V. Kulakov Research Center for Obstetrics, Gynecology, and Perinatology (Moscow, Russia). Part of a sample is studied histologically to confirm tissue type and then separated in two parts for tissue spray MS and lipid extraction, frozen in liquid nitrogen immediately after surgery and stored under –75 °C until the analysis.

Chemicals

Methanol, acetonitrile, 2-propanol, chloroform, and formic acid of HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purchased from Panreac (Barcelona, Spain).

Tissue Lipid Extracts for Direct Injection ESI-MS and HILIC-LC/MS Analysis

Lipid extracts are prepared according to a modified Folch method [32]. Briefly, 40 mg of tissue is homogenized in liquid nitrogen, 4 mL of chloroform–methanol (2:1, v/v) mixture is added to the sample, and the mixture is incubated for 10 min. The homogenate is filtered using coarse filter paper; 800 μL of 1 mol/L NaCl is added, and the mixture is centrifuged at 3000 rpm for 5 min at the ambient temperature. The organic bottom layer containing lipids is evaporated with a stream of nitrogen and redissolved in acetonitrile-2-propanol (1:1, v/v) mixture for analysis.

Tissue Spray MS Conditions

MS analysis of tissue samples is performed on Maxis Impact qTOF (Bruker Daltonics, Bremen, Germany) with the in-lab designed ion source for tissue spray MS [10, 11]. Mixture of H_2O/methanol 1/9 is used for online tissue extraction and following spraying [33]. The solvent is supplied to the tissue with flow rate of 1 mL/min by Dionex binary pump (Thermo Scientific, Germering, Germany). The Taylor cone on the sample tissue and electrospray is formed under 4.2 kV potential applied between tissue and inlet capillary. Distance between a

sample and MS inlet is about 5–10 mm. The distance, high voltage potential, and solvent flow rate are optimized to supply stable spray. The signal of a tissue is saved during 3 min after total ion current (TIC) equilibration. Analysis schedule is as follows: 3 min in positive ion mode following by data dependent analysis (DDA). Mass spectra are registered at a 2 Hz frequency resulting in 360 spectra for 3 min. The mass range is *m/z* 400–1000.

Tandem MS (MS/MS) is done using DDA with the following characteristics. Three most abundant peaks are chosen after full mass scan and subjected to MS/MS analysis. Collision induced dissociation is 35 eV. Mass exclusion time is 1 min.

HILIC-LC Conditions

The lipid extracts are analyzed in triplicate on the Dionex UltiMate 3000 liquid chromatograph (Thermo Scientific, Germering, Germany) coupled to the Maxis Impact qTOF analyzer with ESI (Bruker Daltonics, Bremen, Germany) with a modified method described earlier [24, 34]. Three μL of the sample is injected onto a Spherisorb Si column (150 × 2.1 mm, 5 μm; Waters, Milford, MA, USA). Lipids separation is performed at a flow rate of 50 μL/min using acetonitrile as solvent A and 5 mM aqueous ammonium acetate as solvent B by a linear gradient from 6% to 23% (v/v) of solvent B over 25 min. The column temperature is 40 °C.

ESI-MS Conditions

Maxis Impact qTOF is used in the direct injection ESI-MS and HILIC-LC/MS methods (Bruker Daltonics, Bremen, Germany). Mass spectra are obtained in positive ion mode in the mass range *m/z* 400–1000 with the following setting: capillary voltage 4.1 kV in positive ion mode (3.0 kV in negative ion mode), pressure of the nebulizing gas 0.7 bar, drying gas flow rate 6 L/min, and temperature of the drying gas 200 °C.

Data Processing

Obtained tissue spray mass spectra from each sample is averaged over 3 min and saved in *m/z* – Intensity tables using DataAnalysis software (Bruker Daltonics, Bremen, Germany). ESI-MS is averaged over the time of a sample injection. Thus

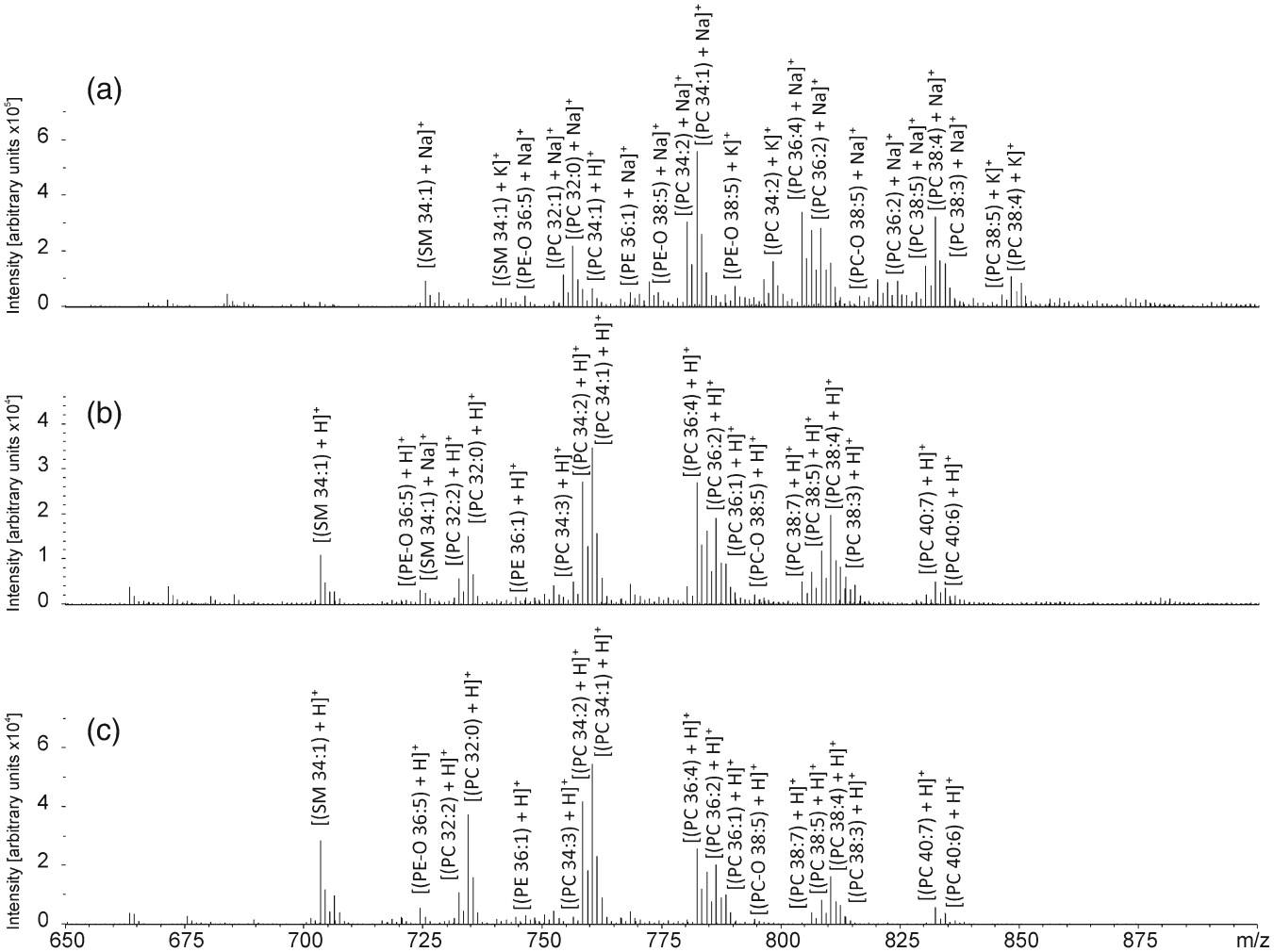

Figure 1. Positive ion mass spectra of one of the eutopic endometrium tissue sample. **(a)** Tissue spray spectrum; **(b)** direct ESI of the tissue lipid extract; **(c)** averaged over time HILIC/MS mass spectrum

obtained data is processed with scaling on TIC and peak alignment by *MALDIquant* R package [35]. Multivariate data analyses is performed using orthogonal projections onto latent structures discriminant analysis (OPLS-DA) method [36] implemented in *ropls* package [37]. The quality of statistical models are estimated by R^2 and Q^2 parameters, where R^2 describes fraction of data that the model can explain using the latent variables, and Q^2 describes part of data predicted by the model according to the cross-validation. Venn diagrams are built with *VennDiagram* package [38].

Lipids Identification

Automated peak annotation for tissue spray and ESI-MS experiments is provided with in-lab created R code, which compares measured accurate *m/z* values with theoretical computer-generated values. The code searches a record within 10 ppm from the experimental *m/z*. Protonated, sodiated, and potassiated ion adducts are considered. More precise identification is done based on the MS/MS data for the peak under consideration, if it undergone MS/MS analysis.

Lipid assignment for HILIC-LC/ESI-MS experiment is made combining retention time information with the MS data. The HILIC column is used to separate different classes of lipids based on the polar head groups. The HILIC-LC/ESI-MS data is summed across all scans for each lipid class under consideration. Peaks in thus obtained averaged spectra are annotated in a similar way as described previously but, additionally, information about lipid class is taken into account.

Results and Discussion

Comparative Lipid Composition Analysis

Characteristic mass spectra of tissue spray MS and ESI-MS in positive ion detection mode averaged over analysis time are shown for one of the samples of eutopic endometrium tissue in Figure 1a, b, and for the ectopic endometrium in Supplementary Figure 1. Highly similar spectral profiles are obtained, the main difference being the preference to form protonated ions in ESI-MS and sodiated ions in tissue spray. Peak assignment is done by comparison of accurate experimental mass with computationally generated lipid exact mass within 10 ppm error. The possibilities of proton, sodium, and potassium adducts formation are taken into account. The results of lipids identification for the tissue spray and ESI-MS experiments are summarized in the Online Resource 1 and 2. In total, there are 144 candidate lipid species found in the tissue spray mass spectra and 76 species in the ESI-MS spectra. Identified lipids belong to three classes: phosphatidylcholines (PC) including lysophosphatidylcholines (LPC), phosphatidyletnanolamines (PE) including lysophosphatidylethanolamines (LPE), and sphingomyelins (SM). The most abundant peaks correspond to PC and SM. Lipids of these classes contain quaternary ammonium in their structure, which makes them readily ionizable in positive ion mode. Lipid proton adducts prevail in the ESI-MS spectra, whereas sodium and potassium adducts are mainly observed in tissue spray MS. Similar preference for the formation of salt adducts has been reported earlier for DESI-MS [13] of the preferential formation of salt adducts in tissue spray can be explained by the fact that tissue contains significant amounts of sodium and potassium, whereas only trace

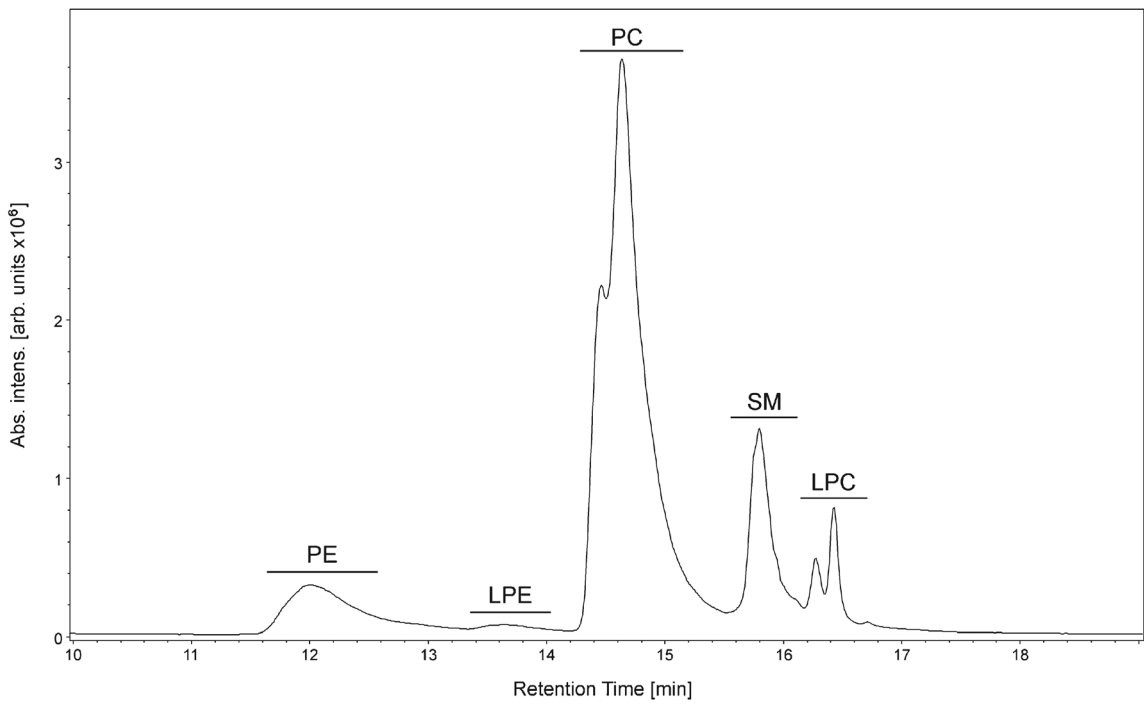

Figure 2. Positive ion TIC of HILIC-LC/MS analysis of lipid extract from eutopic endometrium tissue

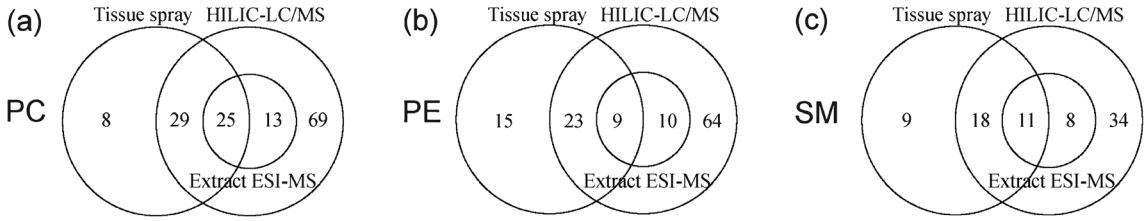

Figure 3. Venn diagrams **(a)** PC and LPC; **(b)** PE and LPE; **(c)** SM

amounts of inorganic salts are present in lipid extracts. The identification of protonated and sodiated PC, PE, and SM is done based on the observation of characteristic fragment signals in tandem mass spectra [39, 40]. Peaks corresponding to both protonated and sodiated lipid species are observed in some of the obtained MS/MS spectra. This observation suggests interference of different ions in positive ions mass spectra of lipids, which hampers their identification and quantitation [10, 40–42]. Another effect that commonly hampers lipid detection and identification in methods without chromatographic separation is ion suppression [43] and insufficient dynamic range for the simultaneous detection of low and high abundant species.

HILIC-LC/ESI-MS analysis of the same extracts is carried out to address these problems and to validate lipid identification. Figure 2 shows positive ion TIC of lipid extract from endometriotic tissue. One can observe chromatographic peaks of all classes identified in both tissue spray and ESI-MS spectra. The HILIC-LC/ESI-MS data are summed across the retention time of these classes to compare with previous mass spectra profiles (Figure 1c). The obtained mean mass spectrum demonstrates good correlation with ESI-MS. Lipids identification in the HILIC-LC/ESI-MS is provided for summed mass spectra of each class separately. Venn diagrams are built to compare coverage of lipids in all three methods (Figure 3). The highest amount of identifications is in the HILIC-LC/ESI-MS; 25 PC, 9 PE, and 11 SM lipid species are found in all three experiments. All species from ESI-MS are also entirely found in HILIC-LC/ESI-MS as well. There is some over-identification in the case of tissue spray, probably due to more complex extract formed online that can contain isobars

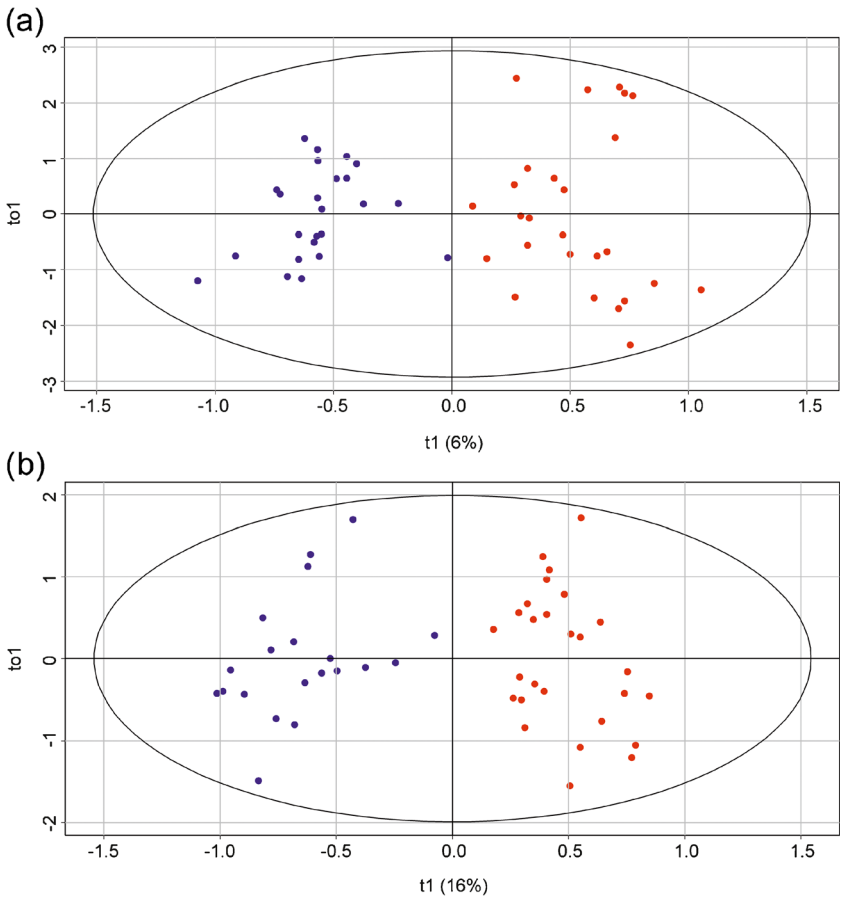

Figure 4. Score plots of multivariate data analysis of relative abundances of ions in positive ion mode using the OPLS-DA method **(a)** based on data obtained by tissue spray MS; **(b)** based on data obtained by ESI-MS of tissue lipid extracts. Blue dots correspond to ectopic and red dots to eutopic endometrium

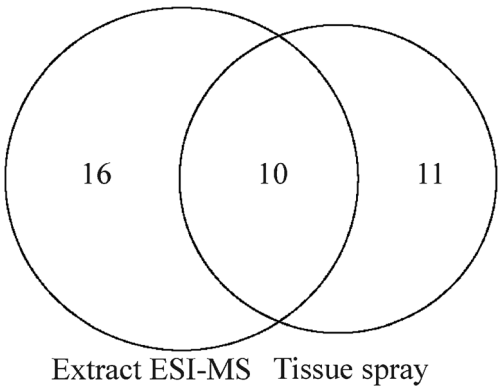

Figure 5. Venn diagram of the most essential for tissue differentiation by tissue spray MS and lipid extract ESI-MS lipids

different from lipids. These are low abundant compounds, and MS/MS is not applicable to validate their identification.

Tissue Differentiation by Tissue Spray and Tissue Extract ESI-MS

The possibility of tissue spray MS and tissue extract ESI-MS in samples differentiation is tested by the supervised OPLS-DA analysis. The resulting score plots of the analysis are presented in Figure 4. The score plots demonstrated good clustering of dots corresponding to distinct tissue types in both tissue spray and ESI-MS. Quantitative parameters of the models turned out to be somewhat different.

R^2 for the tissue spray data is 0.32 and 0.47 for the ESI-MS of lipids extract. Predictive capability of the models is estimated by Q^2, which is 0.73 for tissue spray and 0.82 for the extracts. This parameter is obtained with leave-one-out cross-validation (LOOCV). Therefore, tissue spray data are quite well described by the model and have weaker but still satisfactory predictive ability [44, 45]. This can be due to the generally noisier and less stable signal in tissue spray as compared to ESI-MS.

Table 1. Lipid Species with high VIP Scores in OPLS-DA Analysis Grouped by Presence in Different Experimental Methods

Tissue spray MS and extract ESI-MS	Extract ESI-MS	Tissue spray MS
PC 38:4	PC 40:7	LPC 16:0
PC 36:4	PC 32:0	PC 36:1
PC 34:2	PC 38:3	PC O-38:5
PC 38:5	PC 32:1	PE O-40:7
PC 36:2	PC 38:7	PE O-34:1
PC 34:1	PC 40:8	PE O-42:10
PC 36:3	PE O-38:5	PE O-44:11
PC 38:6	PE O-40:5	PE 38:4
PE O-40:8	PE 36:2	SM 40:2
SM 34:1	PE O-38:6	SM 42:3
	PE O-36:4	SM 40:0
	PE 38:3	
	PE O-42:2	
	PE 36:3	
	SM 36:1	
	SM 42:1	

Variable influence on projection (VIP) is used to find out detailed information about differential spectra features that allow distinction between different tissues. There are mainly features corresponding to lipids among first 30 VIP with the highest score. These features include different ion adducts and isotopic peaks of the same compounds. Venn diagram comparing these lipids is shown in Figure 5. Ten lipid species are present in the both models. These species can also be found on the periphery of the loading plots (Supplementary Figure 2). There are 16 unique lipids found among the first 30 VIPs for extract ESI-MS and 11 unique lipids for tissue spray (Table 1). The differences between VIPs can be explained by the specificity of different tissue structures and corresponding differences in compounds exposed to the extracting solvent in tissue spray. Some minor inter-cellular species can be subjected to extraction only after cell destruction.

Differential lipid species mainly belong to the class of PC lipids, but several PE and few SM are present as well. PC is an essential source of polyunsaturated fatty acids, which can be transformed into eicosanoids, and the latter are involved in many metabolic pathways. Distortion in PC metabolism may cause various pathologies [46, 47]. Sphingolipids participate in signal transduction and cell fate determination [48]. PE are the most widespread lipids on the cytoplasmic membrane. PE are involved in different cellular activities, e.g., cell cycle, membrane fusion, autophagy, and apoptosis [49]. Several publications have been devoted to the investigation of endometriotic lipidome, but most of these studies are based on plasma, serum, or peritoneal fluid as an object [50–52]. PC, PE, and SM lipid classes were found to be altered in endometriosis patients compared with healthy individuals in those studies. In the investigation of Bi-Cheng Yang and coworkers [52], PC 38:4 and SM 34:1 are also among featured lipids in Vouk's paper [51]. In Dutta's work lipids from endometriosis mice serum and liver were profiled [50]. It was found that PEs were downregulated, whereas SMs, PCs, lysoPCs, lysoPEs, and plasmeny-PEs were upregulated in endometriosis mice [50].

Conclusions

Overall, our results indicate that the tissue spray enables endometrial tissue differentiation as well as extract ESI-MS analysis, but tissue spray is ca. four times faster (5 min per sample) than the classic approach based on offline extraction (20 min per sample). Tissue spray MS and extract ESI-MS yield similar spectra with the essential difference in preferential adducts type. In tissue spray MS the most intensive peaks correspond to sodiated species whereas in extract ESI-MS protonated species are readily formed. The presence of sodiated clusters adds complication to the process of spectral interpretation but is not crucial for the analysis by high-resolution mass spectrometry. Caution should be paid in biological interpretation of tissue differences obtained with tissue spray because correlation between the most essential lipids obtained by tissue spray and ESI-MS is not complete.

Acknowledgements

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