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Lipid Dynamics in Zebrafish Embryonic Development Observed by DESI-MS Imaging and nanoelectrospray-MS †

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Abstract

The zebrafish *Danio rerio* is a model vertebrate organism for understanding biological mechanisms. Recent studies have explored using zebrafish as a model for lipid-related diseases, for *in vivo* fish bioassays, and for embryonic toxicity experiments. Mass spectrometry (MS) and MS imaging are established tools for lipid profiling and spatial mapping of biomolecules and offer rapid, sensitive, and simple analytical protocols for zebrafish analysis. When ambient ionization techniques are used, ions are generated in native environmental conditions, requiring neither sample preparation nor separation of molecules prior to MS. We used two direct MS techniques to describe the dynamics of the lipid profile during zebrafish embryonic development from 0 to 96 hours post-fertilization and to explore these analytical approaches as molecular diagnostic assays. Desorption electrospray ionization (DESI) MS imaging followed by nanoelectrospray (nESI) MS and tandem MS (MS/MS) were used in positive and negative ion modes, allowing the detection of a large variety of phosphatidylglycerols, phosphatidylcholines, phosphatidylinositols, free fatty acids, triacylglycerols, ubiquinone, squalene, and other lipids, and revealed information on the spatial distributions of lipids within the embryo and on lipid molecular structure. Differences were observed in the relative ion abundances of free fatty acids, triacylglycerols, and ubiquinone - essentially localized to the yolk - across developmental stages, whereas no relevant differences were found in the distribution of complex membrane glycerophospholipids, indicating conserved lipid constitution. Embryos exposed to trichloroethylene for 72 hours exhibited an altered lipid profile, indicating the potential utility of this technique for testing the effects of environmental contaminants.

Graphical abstract

†Electronic supplementary information (ESI) available: Supplementary Figures S1-S9.

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Keywords

desorption electrospray ionization; ambient mass spectrometry; lipidomic; metabolomic

Introduction

The zebrafish, *Danio rerio*, is a small tropical fish that is popular as a model in developmental biology, drug discovery, and disease studies^{1,2}. Metabolomic studies of zebrafish embryos and adults allow a systematic and comprehensive overview of metabolites in a specific biological condition, which helps reveal molecular deviations due to genetic modifications, pathological stimuli, environmental exposure to toxic chemicals, etc³. Lipidomic studies are also of interest because lipids play essential roles in cells as signaling molecules, membrane components, and energy stores, and defects in lipid metabolism are associated with a number of diseases including cancer, diabetes, and obesity⁴. Exploring how metabolites and lipids are regulated is key to understanding biological pathways, organogenesis, and developmental processes occurring in a biological system. Several analytical approaches have been described for both metabolomic and lipidomic studies. They commonly rely on chromatography (*e.g.* liquid and gas chromatography, LC and GC respectively) coupled to mass spectrometry (MS), with either a targeted (*e.g.* multiple reaction monitoring) or an untargeted approach (*e.g.* full scan spectra) for MS data collection⁵. In several cases, a multi-analytical approach was used by combining GC-MS, LC-MS and ¹H NMR scans to further broaden the molecular coverage^{2,3,6,7}. However, these approaches require sample preparation to desalt, extract, and derivatize the biomolecules of interest, and they also necessitate pooling of samples to reach sufficient detection limits, thus preventing analysis of individual variation. Alternatively, direct MS analysis and MS imaging can provide both lipid profiling and spatial mapping of biomolecules within individual biological samples with relative ease. Among direct MS techniques, ambient ionization MS utilizes ions generated from a sample in native environmental conditions (*e.g.* pressure, temperature, humidity)⁸ as opposed to techniques in which ionization occurs under vacuum, such as in matrix-assisted laser desorption ionization (MALDI)⁶ and secondary ion mass spectrometry (SIMS).

In ambient ionization MS, minimal to no sample preparation is involved and no separation of molecules is done prior to MS analysis. These ionization techniques have expanded into a variety of forms that differ in desorption and ionization mechanism and the coupling between those two processes in time^{8,9}. Among them, desorption electrospray ionization

mass spectrometry (DESI-MS) was the first ambient ionization technique developed¹⁰. DESI has been widely applied to profile small metabolites and lipids in tissue, biofluids, oocytes and embryos, and can be used to create spatial ion maps of molecular abundance^{11,12}. The capabilities of DESI for tissue and microscopic embryo analysis are increased significantly by the use of non-destructive solvent systems that allow desorption of lipids and small molecules while the intracellular and extracellular proteins are preserved, allowing multi-analytical approaches^{13,14}. To date, DESI has been used to study prostate¹⁵, bladder, kidney¹⁶, gastric¹⁷, and brain cancers¹⁸, as well as lymphoma¹⁹. In each case, the recorded pattern of lipid signals allowed for differentiation of cancer from normal tissue. Bovine, porcine, sheep, mouse, dog and cat oocyte and embryo analysis by DESI revealed differences in lipid profiles related to species or breed, developmental stage, environmental growth conditions, or cryopreservation, and the insights were used to improve *in vitro* culture systems and reproductive technology¹².

Here we present DESI-MS and nanoelectrospray-MS (nESI-MS) as analytical approaches for direct MS analysis of lipids in individual zebrafish embryos. This analytical approach revealed changes in the abundance of individual lipids across embryonic development (0, 24, 48, 72, and 96 hours post-fertilization, hpf). Nanoelectrospray ionization differs from DESI in that it does not rely on the desorption of molecules from a flat surface but rather on the extraction of molecules into a liquid-filled glass capillary and ionization with an electric field created by a high voltage supply, *i.e.* electrosprayed²⁰. Considering the small dimensions of zebrafish embryos (~1 mm in diameter), the insertion of individual samples into the nESI capillaries is feasible, and the extraction of molecules can occur rapidly into a chosen solvent system, making nESI a suitable ionization technique for analysis of individual intact embryos.

In this study, data were acquired in both positive and negative ion mode using different combinations of solvents to detect a large variety of lipids (*e.g.* phosphatidylglycerols, phosphatidylcholines, phosphatidylinositols, free fatty acids, triacylglycerols, ubiquinone, and squalene). DESI-MS imaging in full-scan and nESI in both full-scan and tandem MS (MS/MS) were run in series for the same embryos to provide both spatial relationships and specific structural information on the lipids detected. The intended scope is to present these two MS methods for zebrafish lipidomic studies with remarks on their simplicity, rapidity, and breadth of chemicals detected. The observed differences in lipid profiles through development are integrated with existing information on lipid metabolism and dysregulation.

Materials and Methods

Animal exposure and sample preparation

Adult wild-type zebrafish of the AB laboratory strain were maintained at the Aquatic Ecology Laboratory of Purdue University. Zebrafish were fed twice daily with a combination of hatched *Artemia* nauplii and TetraMin tropical fish flakes. Freshly spawned embryos were obtained daily from the naturally spawning adults as described by Gao *et al.*²¹, and eggs were rinsed in 0.0002% methylene blue. The use of animals was approved by the Purdue Animal Care and Use Committee under protocol number 1308000926. Developing embryos were cultured at 28 °C in Petri dishes under a photoperiod of 14 h light

and 10 h dark until they were collected for analysis. A subset of embryos was cultured in sealed 2 ml glass vials containing trichloroethylene (TCE) at a concentration of 500 parts per billion (ppb) to investigate potential effects of TCE on lipid profiles.

A total of 107 zebrafish embryos between 0 and 96 hpf was analyzed by MS: 22 embryos were analyzed at 0 hpf; 22 embryos at 24 hpf; 19 embryos at 48 hpf; 31 embryos at 72 hpf (15 exposed to TCE); and 13 samples at 96 hpf. The embryos were individually transferred alive to a glass microscope slide (Superfrost Plus, 25×75×1mm, Electron Microscopy Sciences) via careful pipetting. The water surrounding the embryos was removed via pipetting to minimize the movement of the hatched embryos. Then with the aid of fine tweezers, each embryo was placed onto a rectangular-shaped piece of Whatman grade 1 cellulose filter paper (Whatman International Ltd., Maidstone, England), taped onto a glass microscope slide. Four sample arrays containing zebrafish embryos at different developmental times (0, 24, 48, 72 and 96 hpf) were prepared as shown in Supplementary Figure 1. The samples were allowed to dry on the paper at room temperature, and then stored for less than 24 h at -20 °C.

DESI-MS imaging

DESI-MS analyses were performed on a linear ion trap mass spectrometer, model Finnigan LTQ (Thermo Electron Corporation, USA). The instrument was equipped with a custom DESI two-dimensional precision moving stage, which includes a source override adapter, an external high voltage cable, and an extended ion transfer capillary. DESI-MS was performed using the solvent combination dimethylformamide-acetonitrile (DMF-ACN, 1:1 v/v; Mallinckrodt Chemicals, MO, USA) for the detection of negative ions. Acetonitrile (Sigma-Aldrich, MO, USA) doped with silver nitrate (AgNO_3 , Sigma-Aldrich, MO, USA) at a concentration of 5 ppm, was used for the detection of positive ions. The use of AgNO_3 allows the detection of lipid via silver adducts, which are easily recognized by the characteristic 1:1 abundance ratio of ^{107}Ag : ^{109}Ag , as demonstrated by Jackson *et al.*²². Both solvent systems preserve tissue morphology, allowing for subsequent testing on the same samples. DESI-MS parameters in negative ion mode were as follows: solvent flow rate, 2.0 $\mu\text{L}/\text{min}$; pressure of nitrogen gas, 160 PSI; applied high voltage, 5 kV; incident spray angle, 52°; spray-to-surface distance, 2–3 mm; spray-to-MS inlet distance, 5–7 mm; capillary temperature, 257 °C; capillary voltage, -50 V; tube lens potentials, -25 V; full-scan MS range, m/z 200-1000; automatic gain control (AGC), off; and maximum ion injection time, 540 ms. DESI-MS parameters in the positive ion mode were as follows: solvent flow rate, 6.0 $\mu\text{L}/\text{min}$; pressure of nitrogen gas, 160 PSI; applied high voltage, 5 kV; incident spray angle, 52°; spray-to-surface distance, 2-3 mm; spray-to-MS inlet distance, 5-7 mm; capillary temperature, 257 °C; capillary voltage, +15 V; tube lens potentials, +65 V; full-scan MS range, m/z 600-1400; AGC) off; and maximum ion injection time, 337 ms. The glass microscope slide containing the samples was affixed to the custom DESI moving stage and two sequential DESI-MS images were acquired from each sample array. Ion images were collected as rows by coordinated linear motion of the moving stage and MS acquisition rate, defining resolution of 250 μm in “x”; upon completion of a row the moving stage resets to the original “x” position while stepping down 250 μm in “y”. Upon completion of a sufficient number of “y” steps to cover the entire array surface, the moving stage was reset to

the origin to acquire the second chemical image. For this specific study, the DESI-MS image in negative ion mode was acquired first, followed by the image in positive ion mode. A schematic of the analysis workflow is shown in Figure 1. The raw Thermo files were converted with in-house programs into file types compatible with the Biomap software (<http://www.maldi-msi.org>), which was used to display 2D chemical images (*i.e.* spatial distribution of a single m/z ion with its intensity represented in false color scale normalized to the highest value among all pixels).

In order to study the dynamic of lipids through embryonic development, principal component analysis (PCA) was used to explore the DESI-MS data and nESI-MS and visualize grouping of samples based on their chemical similarity^{23,24} and to relate them to different developmental stages of the zebrafish embryos. For each DESI-MS image, both for positive and negative ions, small regions-of-interest (ROIs) corresponding to individual embryos were selected and the full-scan mass spectra of those pixels were averaged and exported as .txt files in Matlab (MathWorks, Inc., Natick, USA). For the biggest embryos, multiple ROIs were selected, which yielded 159 mass spectra, *i.e.* rows of the data matrix. For the negative ions, the entire full-scan mass spectra (m/z 200-1000) were used in the first PCA (corresponding to 9600 m/z datapoints, *i.e.* columns of the data matrix, with an incremental m/z step of 0.083), and then a second PCA was performed on the truncated mass range m/z 700-1000 to focus on complex phospholipids only (corresponding to 3600 m/z datapoints). The entire mass range m/z 600-1400 was considered for the positive ions detected as silver adducts. Note that for nESI-MS, a total of 81 and 75 samples were included in the PCAs, respectively for negative and positive ion mode spectra. All PCAs were performed on mass spectra normalized by the standard normal variate (SNV) transform²⁵, so as to correct for both baseline shifts and global intensity variations, and then column-centered. Neither background subtraction nor smoothing filters nor data binning were applied.

Nanoelectrospray-MS and tandem MS

Nanoelectrospray-MS and tandem MS experiments were performed on all zebrafish embryos after DESI-MS analysis. After completion of the DESI-MS images, the paper arrays were cut with scissors into strips to isolate individual embryos. Each strip was inserted into a borosilicate glass capillary (O.D 1.5 mm, I.D. 0.86 mm, 10 cm length purchased from Sutter Instrument®, CA, USA) pulled into a $< 5 \mu\text{m}$ O.D. sharp tip (P-97 micropipette puller, Sutter Instrument®, CA, USA) to perform nESI-MS and nESI-MS/MS experiments (Figure 1).

Nanoelectrospray-MS and MS/MS experiments were run both in negative and positive ion mode on a linear ion trap mass spectrometer, model Finnigan LTQ (Thermo Electron Corporation, USA). Ten microliters of DMF-ACN (1:1 v/v) was inserted into the nESI capillary containing the paper strip with the sample. A nESI electrode was inserted into the capillary, which was then positioned three to four mm in front of the MS inlet. High voltage (2.0 kV) was applied to the ESI electrode through an external high voltage cable. Additional MS parameters were set as follows: capillary temperature, 275 °C; capillary voltage, -25 V and 0 V, for negative and positive ion mode analysis respectively; tube lens potentials, -50 V

and +55 V, for negative and positive ion mode respectively; AGC, on; and maximum ion injection time, 15 ms. Full-scan mass spectra were collected for all zebrafish embryos over the mass range m/z 200-1000. Full-scan mass spectra were collected for up to 30 s in negative ion mode and then the polarity was switched to acquire the full-scan mass spectra in positive ion mode. For each sample, the MS data acquired within this period were averaged to represent a single full-scan mass spectrum for both negative and positive ions.

For a few embryos, data independent MS/MS experiments were also performed in order to gain more structural information on the complex phospholipids detected and tentatively identify them. A data independent MS/MS experiment was conducted using the total ion map program of the instrument software XCalibur, version 1.0.1.03 (Thermo Electron Corporation, USA). The detailed description of this experiment can be found in Schwartz *et al.*²⁶ and Ferreira *et al.*¹². As lipids share common structural motifs, such as the same fatty acid acyl chains or head groups, the MS/MS data independent experiment is well suited for designed searches of lipid classes. Several studies in the literature list a number of precursor scans and neutral loss scans to search for specific classes of lipids sharing the same head groups and offer guidance in the exploration of the MS data. Numerous studies also describe the MS/MS fragmentation via collision-induced dissociation (CID) of several classes of lipids, which helps in interpreting the product ion scans²⁷⁻²⁹. In this study, the following parameters were used: range of precursor ions, m/z 700-1000; isolation width, m/z 1.5; precursor mass step, m/z 1.0; normalized collision energy, 30; activation Q, 0.250; activation time, 30 s; product ion mass range, m/z 150-1200; and total acquisition time per sample, 3.42 min. The instrument software XCalibur version 1.0.1.03 (Thermo Electron Corporation, USA) was used to visualize the MS/MS data domain. Online software Metlin (<https://metlin.scripps.edu/index.php>) and HMDB (human metabolome database, <http://www.hmdb.ca/>) were used to tentatively identify the detected glycerophospholipids and interpret their fragmentation profiles.

Results and Discussion

In this research, zebrafish embryos were analyzed by DESI-MS imaging followed by nESI-MS and nESI-MS/MS (Figure 1). DESI-MS imaging provided chemical and spatial information for a variety of lipids concurrently with the aim of exploring and establishing profiles characteristic of embryos at different developmental stages. The main lipid classes detected as negative ions were fatty acids (FAs), dimers of fatty acids that are formed as gas-phase ions during the ionization step, and complex membrane glycerophospholipids such as phosphoinositols (PIs), phosphocholines (PCs), and phosphoserines (PSs). Cytosolic lipids were detected as silver adducts in positive ion mode, including squalene, cholesteryl esters (CEs), diacylglycerols (DGs), triacylglycerols (TGs), and ubiquinone. Two-dimensional DESI-MS images revealed spatially distinct areas of different lipid composition. The majority of the lipids were found in the yolk sac and the head region. The tail region consists of little else than muscle and developing bone, while the eyes, brain, and other organs containing significant lipid reserves develop in the far anterior region of the embryo⁴. At the time of fertilization, egg yolks contain mostly phospholipids and other nutrients. At this stage, most lipid moieties are sequestered in the form of vitellogenins, lipovitellins, and other lipoproteins. Until feeding begins around six days post fertilization, all of the energy

and nutrients necessary for development come from the yolk^{30,31}. More detailed inference on the spatial distribution of lipids was limited by the resolution of DESI-MS, usually hundreds of microns, and by the modest degree of morphological differentiation within embryos at such an early stage of development. However, the feasibility of DESI-MS as a molecular imaging technique to study organ-specific lipid production and accumulation was demonstrated by Chramow *et al.* who imaged whole-body sagittal sections of zebrafish adults³².

The negative ion mode nESI-MS analysis replicated the information collected by DESI-MS without the spatial component, thus providing a direct comparison between the two electrospray approaches. The nESI-MS in positive ion mode allowed further profiling of complex glycerophospholipids as protonated, sodiated or potassiated molecules species. Finally, nESI-MS/MS experiments helped resolve the identification of many of the ions detected in DESI-MS and nESI-MS. The acquisition of the broad untargeted MS/MS data domain²⁶ requires coupling with ionization strategies that yield stable signal over minutes; nESI fulfills this requirement and did not require sample preparation in this instance. For such a protocol of MS analysis, speed and simplicity are of the essence; deconvolution of mixtures of molecules when analyzing complex and intact biological systems relies only upon multidimensional MS scans²⁶. This approach contrasts standard -omics analyses, which are frequently based on chromatographic separation of molecules prior to MS and which generally require pooling of embryos followed by extraction and desalting procedures. Data-independent experiments (*e.g.* SWATH®, Sequential Windowed Acquisition of all Theoretical Fragment ion mass spectra, more generally known as acquisition of full 2D MS/MS data) have been used for MS data collection in LC-MS analyses as well, but in such cases the narrow elution profile of the chromatographic peak limits the MS/MS data acquisition, thus all ions in a large window of m/z values are commonly selected per chromatographic peak, then fragmented together on the fly, reducing analytical specificity³³.

The collection of nESI data after DESI capitalizes on the non-destructive nature of DESI, allowing one to collect independent sources of chemical and morphological information from the same embryos and develop multi-analytical approaches³⁴. The filter paper on top of which the embryos were dried to prepare the arrays for DESI imaging (see supplementary Figure 1) also offered a practical advantage when inserting individual embryos into the nESI capillaries. Alternatively, the embryos could be snap frozen in liquid nitrogen and then inserted into the capillaries.

MS detection of glycerophospholipids in both negative and positive ion modes

Averaged nESI full-scan mass spectra of lipids detected in negative ion mode from zebrafish embryos at different developmental stages are shown in Figure 2. As previously observed for lipid analysis in individual mouse, pig, and bovine oocytes and embryos¹², FA signal dominated the m/z 200-400 region, dimers formed in gas phase were detected in the region of m/z 400-650, and complex glycerophospholipids were detected in the m/z 700-1000 region. Table 1 lists the lipids detected with their tentative attributions. Prominent saturated and unsaturated FAs, detected as deprotonated ions $[M-H]^-$, were myristic acid (m/z 227),

palmitic acid (m/z 255), linoleic acid (m/z 279), oleic acid (m/z 281), stearic acid (m/z 283), eicosapentaenoic acid (m/z 301), arachidonic acid (m/z 303), and docosahexaenoic acid (m/z 327). DESI-MS ion images show the localization of these ions inside the embryos, as depicted in Figure 3. The zebrafish embryos contained several omega-3 polyunsaturated fatty acids (PUFA), which were not observed in previous studies of embryos from mouse, bovine and pig¹². PUFAs play crucial roles in development and functionality of neuronal tissue³⁵, in cell signaling, and adjusting the physico-chemical properties of cellular membranes to regulate fluidity at different temperatures, a process referred to as homeoviscous adaptation³⁶. This finding is consistent with previous reports of zebrafish tissues bearing much higher concentrations of PUFAs than corresponding mammalian tissues³⁷.

Fatty acid dimers were composed of palmitic, linoleic, oleic, and eicosapentaenoic acids (see Table 1). As previously observed during tissue analysis by DESI-MS, the formation of the dimers tends to be more prominent when there is greater abundance of FAs in the sample; however, the intensity of the dimers can change greatly according to the analytical conditions used, especially in DESI-MS by simply adjusting the spray orientation between the sample surface and the MS inlet. In nESI-MS, a time-related increase in the signal intensity of dimers occurred, probably due to incremental extraction of FAs from the embryos, while the FAs themselves and phospholipids ion abundances did not show the same trend (see Supplementary Figure S2). Among the developmental stages studied, higher intensities of oleic and eicosapentaenoic acids relative to the entire MS spectra were found in embryos at 48, 72 and 96 hpf versus embryos at 0 and 24 hpf. PCA score and loading plots visually show this separation, dominated by the contribution of oleic acid (Supplementary Figure S3). This increased relative ion abundance may be due to the high consumption of DGs and TGs to provide FAs for cellular energy supply or *de novo* synthesis of phospholipids. As a note, in both DESI-MS and nESI-MS analysis of intact embryos, differences in the measurement of relative amounts of lipids due to matrix effects cannot be excluded, especially between unhatched (0-24 hpf) and hatched (48-96 hpf) embryos. For example, the spheroid shape of unhatched embryos might lead to bias in ionization efficiency across space. However, the small size ($\sim 700\ \mu\text{m}$ diameter) of zebrafish embryos minimizes this effect. Similarly, care must be taken during the initial positioning of hatched embryos upon the paper support; their delicate nature and small thickness ($\sim 50\text{-}200\ \mu\text{m}$) makes repositioning impossible. The robust signal and reasonable ion maps generated by DESI on these three-dimensional samples emphasizes the usefulness of this technique for small intact biological samples. Comparable lipid patterns were shown by both DESI-MS and nESI-MS, but higher absolute intensity was overall obtained with nESI-MS.

The negative ion of m/z 531, 5 α -cyprinol 27-sulfate (a bile salt), was detected in the majority of the embryos at 72 and 96 hpf, whereas it was low or not detected at earlier stages; no MS/MS data were collected to support the identification of m/z 531 but the same nominal mass was reported for adult zebrafish by Chramow *et al.* and associated to this molecule³². DESI-MS images of the adult zebrafish showed the localization of this molecule in the region of the developing stomach and intestinal system. Independent studies on the evolution of bile salts reported that at least 98% of the bile salts in zebrafish are 5 α -cyprinol 27-sulfate⁴. Cholesterol sulfate was detected as deprotonated ion of m/z 465. Cholesterol

sulfate is a fundamental component of cell membranes. It has regulatory functions and is involved in signal transduction. An unidentified molecule of m/z 203.2 (likely a deprotonated species) was detected in the majority of the samples analyzed, but was particularly intense only in a few samples, regardless of the developmental stage. The DESI-MS ion map confirmed that this ion is located in the embryos. Its MS/MS product ion scan acquired by nESI is shown in Supplementary Figure S4.

Several glycerophospholipids were prominent over the mass range m/z 700-1000 (Figure 2). The most abundant negative ions were of m/z 794, 834, 840, 868, 883, 885, and 909. The complete list of lipids detected is reported in Table 1, with their tentative identification, supported by literature and nESI-MS/MS data. Other glycerophospholipids were detected in positive ion mode by nESI-MS and MS/MS mainly as protonated ($[M+H]^+$), sodiated ($[M+Na]^+$), and potassiated species ($[M+K]^+$). The most abundant of these ions were of m/z 760, 782, 798, 806, 828, 835, 881, 909, 914, and 938. Averaged nESI full-scan mass spectra in positive ion mode are shown in Supplementary Figure S5.

For both negative and positive ions, PCA for the data truncated between m/z 700-1000 showed little separation between embryos at different developmental stages, indicating conserved lipid constitution. The score plot of PC2 vs. PC3 for the glycerophospholipids detected as negative ions showed a weak tendency toward higher relative abundance for the ions of m/z 834, glycerophosphoserine PS(40:6), and m/z 883 and 885 (glycerophosphoinositols PI(38:5) and PI(38:4), respectively), in the embryos at 72 and 96 hpf compared to earlier stages (Supplementary Figure S6). In accordance with our results, Chramow *et al.*³² detected PS(40:6) in the brain of adult zebrafish, and it has been reported that cells in the developing zebrafish start exhibiting clear morphologic characteristics of oligodendrocytes after 54 hpf³⁸. Accordingly, PS(40:6) was detected in numerous studies as a major lipid in mouse and human grey matter of the brain, which is mainly composed of glia and unmyelinated neurons¹⁸. Conversely, glycerophospholipids specifically detected in mouse and human white brain matter and related to the increased myelination of neurons - such as PS(36:1) of m/z 788, (3-sulfo)GalCer 24:1 of m/z 888, m/z 906, and m/z 916 - were not found in the zebrafish embryos. Also absent was the ion of m/z 890, namely (3-sulfo)GalCer 24:0, which was detected in adult zebrafish and associated with white brain matter^{18,34}. Glycerophosphoinositols play crucial role in intracellular signaling, gene transcription, RNA editing, nuclear export, and protein phosphorylation, thus their presence in embryos at early stages of development is not surprising. Hachicho *et al.* estimated that around three quarters of FAs in the zebrafish embryo are in the form of phospholipids, one fifth in neutral lipids, and 3-4% in glycolipids, notwithstanding minor variation across development³⁹. In adult zebrafish, PI(38:4) was found by DESI to be localized in the spinal cord area³⁴. PIs can be searched via a precursor ion scan for the product of m/z 241, corresponding to phosphatidylinositol. The actual fatty acyl residue composition for each PI can also be confirmed via product ion scans, and this information is complementary to that provided by DESI-MS and nESI-MS where only the free portion of FAs is detected. An example is shown in Figure 4 for the ions of m/z 883 and 885, attributed as PI(38:5) and PI(38:4) respectively, where multiple acyl residues are present, suggesting the co-presence of isobaric species. In more detail, ions of m/z 281, 283, 303, and 305 are present for m/z 885 leading to the identification PI(18:1_20:3) and PI(18:0_20:4). Ions of m/z 279, 281,

283, 301, 303, and 305 are detected for m/z 883, suggesting the co-presence of PI(18:2_20:3), PI(18:1_20:4), and PI(18:0_20:5).

Precursor ion scans of specific FAs can be extrapolated from the MS/MS data domain to give an overview of the complex phospholipids that contain particular FAs. Precursor ion scans of linoleic and arachidonic acids are depicted in Supplementary Figure S7. Linoleic acid is an essential fatty acid used in the biosynthesis of prostaglandins and cell membranes. Arachidonic acid can be metabolized to form eicosanoids and other signaling molecules, such as prostaglandin, thromboxane, and leukotrienes³⁶. It acts as a substrate for a number of other lipid modifying enzymes that are critical for cell movements required to pattern the early zebrafish embryo⁴.

Several glycerophosphocholines were also detected as both chlorinated adducts in negative ion mode and sodiated and potassiated adducts in positive ion mode (see Table 1). In particular, PC(38:6) and PC(40:6) appear to be slightly higher in abundance in the full-scan mass spectra for the earlier stages of development (0-48 hpf). Phosphatidylcholines are predominant phospholipids in teleost eggs³ and are key markers for organogenesis³⁶. They have been detected also in other metabolic studies on zebrafish embryos by GC-MS and LC-MS^{3,6}.

MS detection of cytosolic lipids via silver adducts

DESI-MS collected using ACN doped with silver nitrate allows for the detection of cytosolic lipids, such as cholesterol esters, squalene, DGs, TGs, and ubiquinone, as positive ions (Table 2). The adduction to silver confers to such lipids a characteristic isotopic pattern that can be used to ease identification, even in low resolution full-scan MS. The isotopic distribution of squalene and ubiquinone as $[M+Ag_2NO_3]^+$ species is shown in Supplementary Figure S8. Even in low resolution the MS observations closely match the theoretical distribution. Averaged full-scan mass spectra of the embryos per developmental stage are shown in Figure 5. PCA shows that clear differences are present in the cytosolic lipids detected over the first four days of development (0-96 hpf). Strong clustering of the samples based on their developmental stages is visible in the score plot of PC1 vs. PC2 (Figure 5). Between 0 and 48 hpf, an accumulation of DGs and TGs in the yolk sac occurs, indicating activation of metabolism and signaling and *de novo* synthesis of lipids. This observation is in accordance with other metabolic studies reported in the literature³. It may be that the fatty acid moieties initially contained in yolk lipovitellins are liberated and reassembled into DGs and TGs. These may be packaged into very low density lipoprotein particles for distribution throughout the embryo. Previous studies on zebrafish embryos support this model^{38,39}. Moreover, 48 hpf coincides with the development of important organs like liver that allows *de novo* synthesis to occur³. The embryos at 24 hpf expressed lipid profiles intermediate between those at 0 hpf and 48 hpf, reflecting the dynamic of this transition. In the PCA score plot, orthogonal separation is visible for the embryos at 72 and 96 hpf. The content of squalene, ubiquinone, and the relative intensity of the ion of m/z 803.6 is largely increased in comparison to the previous stages, whereas DG and TG content are largely reduced, suggesting depletion of internal nutrient resources⁴. Yolk lipids are the source of TGs, as well as cholesterol, a required component of cell membranes and a

precursor for bile acids. As zebrafish embryos grow and organ systems differentiate, mitochondrial activity increases and this can be directly correlated with the increase of ubiquinone over time. DESI-MS images for squalene, m/z 803.6, 913.4, and ubiquinone are depicted in Figure 6 for paper arrays # 1 and 3. They show the specific localization of these ions in the embryos. For the more developed embryos (72 and 96 hpf), these lipids are located in the head whereas no signal is detected in the tail.

Lipid profile disturbed by exposure to trichloroethylene

The lipid profile of embryos exposed to trichloroethylene (TCE) for the first 72 hpf differed from the profile of unexposed embryos of the same age. TCE is a halocarbon commonly used as industrial solvent, and is known to be a hepatotoxin and suspected of being an animal carcinogen⁴¹. When focusing on 72 hpf old embryos only, minor differences were observed in the cytosolic lipids, with the exposed group showing a small increase in ubiquinone, an ion of m/z 914, and squalene, and a decrease in the ion of m/z 1014 compared to the unexposed control group, as shown in Supplementary Figures S5 and S9. Both DESI-MS and PCA qualitatively address such changes relative to the entire profile of cytosolic lipids detected. Gündel *et al.* previously reported that toxic effects on zebrafish embryos can result in changes in the pattern of vitellogenin utilization, with altered abundances of lipovitellins and downstream molecules⁴². Several toxic compounds have been shown to reduce the rate of yolk absorption as well. Our observation, albeit far from complete from an environmental toxicological point of view, stresses the idea that metabolomics and lipidomic studies can be performed with direct MS analyses like DESI-MS and nESI-MS to study and compare biological systems grown under different conditions, and can serve to point out deviations that might be a result of exposure to toxic chemicals.

Conclusions

DESI-MS and nESI-MS(/MS) allowed a large variety of lipids to be detected from individual zebrafish embryos, indicating specific differences related to development and organogenesis. In general, saturated, mono- and poly-unsaturated fatty acids were detected in increasing abundance over development, associated with cell energy demand and *de novo* synthesis of TGs and glycerophospholipids. Phospholipid profiles were conserved across developmental stages, with slight changes in the ratio of PC(40:6), PS(40:6), PI(38:5), and PI(38:4). Drastic differences occurred in cytosolic lipids that are associated with energy production, clearly indicating the initial synthesis and accumulation of a large number of DGs and TGs - differing in the length of the fatty acyl chains and the number of unsaturations - and then the depletion of this store as the developing embryos utilize the FAs for both synthesis and as metabolic fuel. The opposite trend was shown for ubiquinone, suggesting more intense mitochondrial activity over time. Even though we discussed and interpreted the lipid profiles bridging DESI and nESI-MS and nESI-MS/MS data, their combination is not to be considered a unique MS methodology for zebrafish embryo lipid analysis, but rather as alternative approaches that can be pursued, each offering unique benefits, including simplicity together with the richness of the lipid information obtained. The straightforward workflow and the non-destructive nature of DESI and nESI approaches

are both appealing for metabolomics and lipidomics analyses and the type of research question would dictate the optimal strategy to adopt. Also, the coupling of these ionization strategies is optimal with different mass analyzers that can provide different depths of information regarding molecular structure. DESI-MS is commonly used in full-scan mode with ion traps, providing spatial mapping of lipids but with limited chemical specificity. Orbitraps with high mass resolution can be used to increase confidence in identifications via determination of molecular formulae, even though a precise molecular formula does not necessarily lead to unique lipid identification given that many isobaric and isomeric species are possible. Nanoelectrospray contrasts with DESI in that spatial information is lost but greater depth in chemical information is recovered as the long-lasting signal is compatible with multidimensional MSⁿ scans, such as one or more product ion scans, neutral loss scans, and multiple reaction monitoring, suitable for designed searches of lipid classes. Tandem MS of lipids provides complementary information to high mass resolution, as isobaric and isomeric lipids with identical chemical formulae can fragment following different pathways, thus showing unique product ions and allowing for deconvolution of multiple isomers. DESI and nESI offer a powerful means of exploring the spatial and temporal dynamics of lipids in developing embryos, and can also provide evidence of physiological disturbances caused by disease, toxins, or environmental stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1) DESI-MS imaging

2) nESI-MS and nESI-MS/MS

Fig. 1. Analysis workflow

1) For the DESI-MS imaging experiment, each paper array taped on an insulating surface (a glass microscope slide) was individually positioned on the custom DESI moving stage and analyzed. The DESI spray was positioned on top of the paper with optimized angle and distance from both sample surface and MS inlet. With the aid of an inert gas (nitrogen), a solvent stream charged to a high electrical potential is directed at the sample. Gas phase ions are produced in the small area that the solvent strikes via electrospray-like mechanisms¹⁰. For a DESI image acquisition, the sample is moved under the DESI spray to a velocity that is synchronized with the time necessary for data acquisition. The acquired data are subsequently reassembled to display individual DESI ion images and for data analysis²⁴. **2)** After completion of the DESI-MS image, the paper array was cut with scissors into strips to isolate individual embryos. Each strip was inserted into a borosilicate glass capillary pulled into sharp tip to perform nESI-MS and nESI-MS/MS experiments.

Fig. 2.

Averaged nESI-MS spectra in negative ion mode. **A.** 0 hpf (n=16 samples). **B.** 24 hpf (n=13 samples). **C.** 48 hpf (n=16 samples). **D.** 72 hpf, controls (n=10 samples). **E.** 72 hpf, exposed to TCE (n=15 samples). **F.** 96 hpf (n=11 samples).

Fig. 3.

DESI ion images in negative ion mode for paper array # 3 (embryos at 72 and 96 hpf). **A.**

Ion of m/z 279. **B.** Ion of m/z 281. **C.** Ion of m/z 301. **D.** Ion of m/z 303. **E.** Ion of m/z 327.

F. Picture of paper array # 3 displayed to check the position of the embryos.

Fig. 4.

A. Product ion scan for the precursor ions of m/z 883. **B.** Product ion scan for the precursor ion of m/z 885. Average product ion spectra were collected from a representative zebrafish embryo at 72 hdf by nESI-MS/MS in negative ion mode. Identity of the main fragments for PI(18:0_20:4) is reported as insight.

Fig. 5. Averaged DESI-MS spectra in positive ion mode

A. 0 hpf (n=24). **B.** 24 hpf (n=30). **C.** 48 hpf (n=36). **D.** 72 hpf, controls (n=25). **E.** 72 hpf, exposed to TCE (n=23). **F.** 96 hpf (n=21). **Principal component analysis: G.** PC1 and PC2 score plot. Samples are color-coded as follows: 0 hpf, green; 24 hpf, blue; 48 hpf, red; 72 hpf controls, black; 72 hpf exposed to TCE, purple; 96 hpf, magenta. **H.** PC1 and PC2 loading plot; variables are labeled in terms of their m/z ratio.

Figure 6. DESI images of slide # 1 (embryos at 0, 24, and 48 hpf). **A.** Ion of m/z 688 (squalene). **B.** Ion of m/z 803. **C.** Ion of m/z 913. **D.** Ion of m/z 1140 (ubiquinone). DESI images of slide # 3 (embryos at 72 and 96 hpf). **E.** Ion of m/z 688 (squalene). **F.** Ion of m/z 803. **G.** Ion of m/z 913. **H.** Ion of m/z 1140 (ubiquinone).

Table 1

Values of m/z and tentative attribution of lipids detected in negative and positive ion mode by DESI-MS and nESI-MS with DMF:ACN (1:1, v/v) spray solvent system.

Negative ions (<i>m/z</i>)	Ion molecular formula	Ion description	Tentative attribution**
227.3 *	C ₁₄ H ₂₇ O ₂	[M–H] [–]	Myristic acid, FA(14:0)
253.2 *	C ₁₆ H ₂₉ O ₂		Palmitoleic acid, FA(16:1)
255.2 *	C ₁₆ H ₃₁ O ₂		Palmitic acid, FA(16:0)
277.2 *	C ₁₈ H ₂₉ O ₂		Linolenic acid, FA(18:3)
279.2	C ₁₈ H ₃₁ O ₂		Linoleic acid, FA(18:2)
281.2	C ₁₈ H ₃₃ O ₂		Oleic acid, FA(18:1)
283.2 *	C ₁₈ H ₃₅ O ₂		Stearic acid, FA(18:0)
301.2	C ₂₀ H ₂₉ O ₂		Eicosapentaenoic acid, FA(20:5)
303.2	C ₂₀ H ₃₁ O ₂		Arachidonic acid, FA(20:4)
305.2 *	C ₂₀ H ₃₃ O ₂		Dihomo-γ-Linolenic Acid, FA(20:3)
327.2	C ₂₂ H ₃₁ O ₂		Docosahexaenoic acid, FA(22:6)
329.3 *	C ₂₂ H ₃₃ O ₂		Clupanodonic acid, FA(22:5)
355.3	C ₂₄ H ₃₅ O ₂		Tetracosahexaenoic acid, FA(24:6)
465.4	C ₂₇ H ₄₅ O ₄ S	Cholesterol sulfate	
531.4	C ₂₇ H ₄₇ O ₈ S	5α-cyprinol 27-sulfate	
511	Dimer of FA(16:0)		
535	Dimer of FA(16:0) and FA(18:2)		
537	Dimer of FA(16:0) and FA(18:1)		
563	Dimer of FA (18:1)		
603	Dimer of FA (20:5)		
582.8	Dimer of FA(18:1) and FA(20:5)		
608.9	Dimer of FA(22:6) and FA(18:1)		
610.8	Dimer of FA(22:6) and FA(18:0)		
654.8	Dimer of FA(22:6)		
747.4	C ₄₀ H ₇₆ O ₁₀ P	[M–H] [–]	PG(34:1)
766.4	C ₄₃ H ₇₇ NO ₈ P		PE(38:4)
768.2	C ₄₀ H ₈₀ NO ₈ P*** / C ₄₃ H ₇₉ NO ₈ P	[M+Cl] [–] / [M–H] [–]	PC(32:0) / PE(38:3)
794.2	C ₄₂ H ₈₂ NO ₈ P	[M+Cl] [–]	PC(34:1)
818.3	C ₄₄ H ₈₂ NO ₈ P		PC(36:3)
820.3	C ₄₄ H ₈₄ NO ₈ P		PC(36:2)
822.3	C ₄₄ H ₈₆ NO ₈ P		PC(36:1)
834.3	C ₄₆ H ₇₇ NO ₁₀ P	[M–H] [–]	PS(40:6)
840.3	C ₄₆ H ₈₀ NO ₈ P	[M+Cl] [–]	PC(38:6)

Negative ions (<i>m/z</i>)	Ion molecular formula	Ion description	Tentative attribution**
842.3	C ₄₆ H ₈₂ NO ₈ P	[M-H] ⁻	PC(38:5)
868.3	C ₄₈ H ₈₄ NO ₈ P		PC(40:6)
883.4	C ₄₇ H ₈₀ O ₁₃ P		PI(38:5)
885.4	C ₄₇ H ₈₂ O ₁₃ P		PI(38:4)
887.4	C ₄₇ H ₈₄ O ₁₃ P		PI(38:3)
909.4	C ₄₉ H ₈₂ O ₁₃ P		PI(40:6)
Positive ions (<i>m/z</i>)	Ion molecular formula	Ion description	Tentative attribution**
725.4	C ₃₉ H ₇₉ N ₂ O ₆ P	[M+Na] ⁺	SM(34:1)
741.4	C ₃₈ H ₇₁ O ₁₀ P		PG(32:2)
754.4	C ₄₀ H ₇₈ NO ₈ P		PC(32:1)
756.5	C ₄₀ H ₈₀ NO ₈ P		PC(32:0)
760.5	C ₄₂ H ₈₃ NO ₈ P	[M+H] ⁺	PC(34:1)
780.5	C ₄₂ H ₈₀ NO ₈ P	[M+Na] ⁺	PC(34:2)
782.5	C ₄₂ H ₈₂ NO ₈ P		PC(34:1)
798.4	C ₄₂ H ₈₂ NO ₈ P	[M+K] ⁺	PC(34:1)
806.4	C ₄₄ H ₈₂ NO ₈ P	[M+Na] ⁺	PC(36:3)
828.4	C ₄₆ H ₈₀ NO ₈ P		PC(38:6)
835.2	C ₄₇ H ₉₃ N ₂ O ₆ P		SM(42:2)
844.4	C ₄₈ H ₈₈ NO ₇ P		PC(O-40:5)
846.4	C ₄₈ H ₉₆ NO ₈ P	[M+H] ⁺	PC(40:0)
856.3	C ₄₈ H ₈₄ NO ₈ P	[M+Na] ⁺	PC(40:6)
881.2	C ₄₅ H ₇₉ O ₁₃ P		PI (36:4)
909.2	C ₄₇ H ₈₃ O ₁₃ P		PI(38:4)

* detected as acyl chain residues in MS/MS experiments.

** Abbreviations: FA = fatty acid; PG = phosphatidylglycerol; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol. (C:U) represents the number of carbon atoms (C) and the number of unsaturations (U) of the fatty acid acyl chain.

*** Molecular formula indicated for chlorinated, sodiated or potassiated species does not include the adduct species.

Table 2

Values of m/z , predicted ion molecular formula, ion description, and tentative attribution of lipids detected in positive ion mode by DESI-MS as silver adducts.

Ion m/z	Ion molecular formula	Ion description	Tentative attribution *
686.2	C ₃₀ H ₅₀ O ₃ NAg ₂	[M+Ag ₂ NO ₃] ⁺	Squalene
699.4	C ₃₇ H ₆₈ O ₅ Ag ₂	[M+Ag] ⁺	DG(34:2)
701.4	C ₃₇ H ₇₀ O ₅ Ag ₂		DG(34:1)
725.4	C ₃₉ H ₇₀ O ₅ Ag		DG(36:3)
727.4	C ₃₉ H ₇₂ O ₅ Ag		DG(36:2)
747.5	C ₄₁ H ₆₉ O ₅ Ag		DG(38:6)
755.5	C ₄₅ H ₇₆ O ₂ Ag		CE(18:2)
757.4	C ₄₂ H ₆₆ O ₅ Ag / C ₄₅ H ₇₈ O ₂ Ag		DG(39:8) / CE(18:1)
781.5	C ₄₇ H ₇₈ O ₂ Ag		CE(20:3)
783.5	C ₄₇ H ₈₀ O ₂ Ag		CE(20:2)
803.6	C ₄₉ H ₇₆ O ₂ Ag		CE(22:6)
864.3	C ₃₇ H ₆₄ O ₈ NAg ₂	[M+Ag ₂ NO ₃] ⁺	DG(34:4)
937.6	C ₅₃ H ₉₈ O ₆ Ag	[M+Ag] ⁺	TG(50:2)
939.6	C ₅₃ H ₁₀₀ O ₆ Ag		TG(50:1)
963.6	C ₅₅ H ₁₀₀ O ₆ Ag		TG(52:3)
965.6	C ₅₅ H ₁₀₂ O ₆ Ag		TG(52:2)
967.6	C ₅₅ H ₁₀₄ O ₆ Ag		TG(52:1)
895.7	C ₅₇ H ₉₈ O ₆ Ag		TG(54:6)
897.7	C ₅₇ H ₁₀₀ O ₆ Ag		TG(54:5)
989.7	C ₅₇ H ₁₀₂ O ₆ Ag		TG(54:4)
991.7	C ₅₇ H ₁₀₄ O ₆ Ag		TG(54:3)
993.7	C ₅₇ H ₁₀₆ O ₆ Ag		TG(54:2)
1011.6	C ₅₉ H ₁₀₀ O ₆ Ag	[M+Ag ₂ NO ₃] ⁺	TG(56:7)
1013.6	C ₅₉ H ₁₀₂ O ₆ Ag		TG(56:6)
1035.7	C ₆₁ H ₁₀₀ O ₆ Ag		TG(58:9)
1037.7	C ₆₁ H ₁₀₂ O ₆ Ag		TG(58:8)
1081.8	C ₆₃ H ₁₀₈ O ₆ Ag		TG(60:0)
1107.8	C ₆₅ H ₁₁₀ O ₆ Ag		TG(62:1)
1109.8	C ₆₅ H ₁₁₂ O ₆ Ag		TG(62:0)
1138.4	C ₅₉ H ₉₀ O ₇ NAg ₂	[M+Ag ₂ NO ₃] ⁺	Ubiquinone

* Abbreviations: DG = diacylglycerol; TG = triacylglycerol. (C:U) represents the number of carbon atoms (C) and the number of unsaturations (U) of the fatty acid acyl chain.