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Examining heat treatment for stabilization of the lipidome

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Aim: To confidently determine lipid-based biomarkers, it is important to minimize variation introduced during preanalytical steps. We evaluated reducing variation associated with lipid measurements in invertebrate sentinel species using a state-of-the-art heat treatment technique. **Materials and Methods:** Earthworms (*Eisenia fetida*), house crickets (*Acheta domestica*) and ghost shrimp (*Palaemonetes paludosus*) were euthanized either by flash freezing or heat treatment. For both experiments, samples were either immediately extracted after removal from -80°C storage or incubated on ice for one hour prior to sample weighing and extraction. Lipidomics was performed on resulting extracts using liquid chromatography high resolution tandem mass spectrometry. LipidMatch and LipidSearch were used for lipid identification. **Results:** Lipid enzymatic products (e.g., phosphatidylmethanols, diglycerides, lysoglycerophospholipids and ether-linked/oxidized lysoglycerophospholipids), were in higher concentrations in flash-frozen samples, when compared with heat-treated samples. Results suggest that heat treatment reduces phospholipase A and phospholipase D activity. **Conclusion:** Heat treatment reduced enzymatic products and increased precursors of these enzymatic products. We believe heat treatment warrants a closer interrogation for improving the robustness of lipid biomarker research, especially in tissue samples, where enzyme stabilizers are difficult to apply, and for use in field studies, where the stabilization of the collected sample is critical.

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Over the last decade, lipidomics has steadily gained status as an established strategy for human health and disease research. Inherently linked to this emergence have been the substantial advancements in the lipidomics workflow, specifically advancements in lipid extraction [1], separation, mass spectrometric detection [2], data processing and biochemical interpretation/analysis [3]. Success of lipidomics research lies in the understanding of the fundamentals and sources of bias/variability at each step of the lipidomics workflow. Perhaps the most overlooked aspects of the lipidomics workflow are the preanalytical steps, those steps or decisions made regarding samples prior to analysis. Despite community-wide awareness of these issues [4–6], the current gap between the perceived gravity of preanalytical influence and the efforts to address these concerns remains large [7].

The preservation of the native omic profile from the time of collection (t_0) is an ongoing preanalytical challenge. Flash freezing samples in liquid nitrogen immediately after collection is considered the gold standard for halting metabolic activity. Sample collection in the natural environment (field studies) poses a difficulty in this regard, as the availability of liquid nitrogen and other methods for maintenance of ultracold environments may be limited, and thus analyzed samples may not exhibit a true t_0 profile. Laboratory studies are not exempt from issues using flash freezing. The integrity of samples is maintained by remaining in a frozen state until chemical extraction; however, it is often necessary to weigh samples prior to extraction where thawing is undoubtedly occurring. At this

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point, certain lipids can be susceptible to changes via enzymatic activity [8] and chemical degradation due to pH and oxidation [9], thus complicating the interpretation of the observed lipid profile. A study by Wang *et al.* analyzed the lipidome in mouse and rat plasma and noted that several lipids significantly increased or decreased after 4 h on a benchtop [8]. To better preserve the lipidome, an enzyme inhibitor (phenylmethylsulfonyl fluoride) was added to the plasma in the study, thus creating a more native profile. For oxidized lipids, chemical stabilizers, such as butylated hydroxytoluene, are commonly used to prevent oxidation [10]. While chemical stabilizers can be applied to matrices such as plasma, stabilizing solid matrices, such as tissues, at the time of collection requires alternative approaches.

Heat treatment is an alternative approach which has been used as early as the 1940s, for example, to reduce lipid byproducts produced by phospholipase C and D in the presence of alcohols used for extraction [11]. Jerne *et al.* examined the application of heat treatment on lipid stability using recent technology that precisely controls temperature and creates a vacuum to reduce oxidation in an effort to study postmortem effects on free fatty acid composition in brain and liver tissues. It was found that heat was effective in reducing phospholipase activity and *ex vivo* lipolysis in comparison to tissues that were not treated with heat [12]. This idea of heat treatment as a means to preserve the lipidome in tissues was expanded upon by Saigusa *et al.* where it was shown that heat treatment was able to retard metabolic changes in certain sphingolipids [13]. While advantages have been shown for specific lipids, a comprehensive study of the heat treatment effect on the lipidome using state-of-the-art heat treatment equipment, UHPLC and high-resolution mass spectrometry (HRMS), is missing. In addition, to our knowledge, true t_0 studies, where heat treatment is used as the method of metabolic arrest, have not been shown.

In this regard, we aimed to evaluate heat treatment as an approach to improve lipid stability using high-resolution tandem mass spectrometric approaches coupled to a UHPLC system. We examined the specific effects of heat treatment on over 40 lipid types including oxidized lipids using LipidMatch and LipidSearch lipid identification software [14,15] in three invertebrate species: the earthworm (*Eisenia fetida*), the house cricket (*Acheta domestica*) and the ghost shrimp (*Palaemonetes paludosus*). The selection of the three invertebrate species was based on the fact that each species has been used previously as bioindicators for environmental monitoring [16–18] and their small size allows them to be directly placed in the heat treatment cartridge for a true t_0 study. Additionally, it has been previously noted that some invertebrate species, like the earthworm, have high enzymatic content [19]. In metabolomics-based studies of earthworms, it was shown that heating whole-body metabolite extracts was beneficial for improving metabolite and total lipid stability [19,20], thus making them ideal candidates for this examination. The current study was performed both to highlight the potential lipid changes that occur immediately following animal sacrifice, and to propose heat treatment as a means to reduce this change. We examined whether heat completely stabilizes the lipid profile by allowing heat-treated samples to incubate for 1 h prior to lipid extraction. Based on trends in lipid profiles, with and without heat treatment, we discuss the underlying enzymatic pathways leading to the largest lipid transformations, and the benefit of heat treatment to prevent these actions.

We provide evidence that there is significant degradation of lipids even under commonly employed sample processing and extraction methods. If lipids are not stabilized, biotransformation of molecules leads to measurement of lipid levels which do not represent endogenous conditions. These changes in lipid signals, which are unrelated to experimental design, can lead to difficulties in the interpretation of biological significance and false negatives and positives in biomarker discovery. We show that heat treatment is a promising technique for reducing variance and shifts in lipid profiles due to enzymatic reactions, especially when employed as the euthanization method.

Materials & methods

Materials

The three invertebrate species investigated in this work included earthworms (*Eisenia fetida*) ($n = 18$) (Haddrell's Point Tackle, SC, USA), house crickets (*Acheta domestica*) ($n = 18$) (Haddrell's Point Tackle) and ghost shrimp (*Palaemonetes paludosus*) ($n = 10$) (PetSmart, North Charleston, SC). Female crickets were excluded from analysis based on identification of the ovipositor, and shrimp containing eggs were excluded. Worms, crickets and shrimp were selected at similar sizes and weights. Optima methanol and HPLC grade chloroform were purchased from Thermo Fisher Scientific (MA, USA). HPLC grade 2-propanol was acquired from Alfa Aesar (MA, USA). Ultrapure water with 18 M Ω cm resistivity (Millipore Milli-Q Gradient A10; EMD Millipore, MA, USA) was used for sample preparation. Ammonium acetate and analytical grade formic acid were purchased from Fisher-Scientific. All mobile phase solvents were Fisher Optima LC/MS grade (acetonitrile, isopropanol and water).

Figure 1. Experimental design. Earthworms and crickets were utilized to study the comparison of euthanization by heat treatment or flash freezing, followed by extraction of the homogenized tissue. Additionally, these organisms were used to assess the stabilization of the lipidome by the euthanization method with 1-h ice incubation. Ghost shrimp were used to test the same euthanization methods but instead incorporated a methanol homogenization step during extraction.

All internal standards used in the mixtures were purchased from either Avanti Polar Lipids (AL, USA) or Nu-Chek Prep (MN, USA). The mixture consisted of the following gravimetrically weighed exogenous internal standards in μg lipid per g solvent: CE(17:0) (45.1 $\mu\text{g/g}$), Cer(d18:1/17:0) (5.8 $\mu\text{g/g}$), DG(20:0/20:0) (43.1 $\mu\text{g/g}$), FA(18:0-d₃₅) (34.1 $\mu\text{g/g}$), GalCer(d18:1/12:0) (7.8 $\mu\text{g/g}$), LPC(17:0) (11.4 $\mu\text{g/g}$), LPE(14:0) (15.5 $\mu\text{g/g}$), PA(14:0/14:0) (45.0 $\mu\text{g/g}$), PC(14:1/14:1) (46.0 $\mu\text{g/g}$), PE(15:0/15:0) (17.1 $\mu\text{g/g}$), PG(15:0/15:0) (20.4 $\mu\text{g/g}$), PS(14:0/14:0) (6.6 $\mu\text{g/g}$), SM(d18:1/17:0) (15.2 $\mu\text{g/g}$), ST(16:0) (3.4 $\mu\text{g/g}$) and TG(17:1/17:1/17:1) (257.5 $\mu\text{g/g}$). About 250 μl of the internal standard mixture was gravimetrically weighed and added to each cricket and worm sample prior to extraction, while 150 μl of the internal standard cocktail was added to each shrimp sample.

Heat treatment

A schematic of the experimental design is shown in Figure 1. Samples were immediately euthanized for heat treatment by placing the whole invertebrate into a Denator Maintainer cartridge (Supplementary Figure 1) which was placed in the Stabilizer T1 (Denator, Uppsala Science Park, Sweden). Of the samples analyzed, nine worms, nine crickets, and five shrimp were euthanized by heat treatment followed by immediate freezing until extraction. Based on internal measurements employed by the Stabilizer T1, the actual heating parameters were as follows: treatment time, 23.9 ± 2.9 s; maximum temperature, $95.1 \pm 0.2^\circ\text{C}$; minimum temperature, $94.0 \pm 0.2^\circ\text{C}$ and pressure, 4.5 ± 0.9 mbar. Treatment parameters were significantly less variable for shrimp, likely owing to smaller body mass than worms and crickets.

Alternatively, samples were euthanized by rapidly freezing in aluminum foil in a cryocart cooled by liquid nitrogen to below -140°C . Of the samples analyzed, nine worms, nine crickets and five shrimp were euthanized by rapid flash freezing.

All common house crickets and earthworms (heat-treated and flash-frozen) were then cryopulverized using a Retsch cryomill (Retsch, Haan, Germany), and the resulting powder was then transferred to a 2-ml cryovial and subsequently placed in a -80°C freezer overnight. Whole shrimp were placed in 2-ml cryovials without cryopulverization because of their small size. All samples were stored for <12 h in a -80°C freezer before extraction.

Lipid extraction

Shrimp

Each whole ghost shrimp was removed from the -80°C freezer and immediately weighed (average mass of 0.11 g, $n = 10$) and homogenized in a Bullet Blender (Next Advance, NY, USA) with 1 ml cold methanol using 0.5 mm zirconium beads (Next Advance) for 4-min cycles at speed 10. The shrimp homogenate was then transferred to a new vial for lipid extraction.

Cricket & earthworm

The cricket and earthworm powdered samples were removed from the -80°C freezer and a portion of the powder was weighed for extraction. The tissue powder (cricket: average mass, 0.089 g [$n = 18$]; earthworm: average mass, 0.10 g [$n = 18$]) were then transferred into new vials. To evaluate lipid stabilization as a result of the euthanization method (heat-treated or flash-frozen), ten cricket and ten earthworm samples (5 heat-treated and 5 flash-frozen per organism) were incubated on ice for 1 h prior to lipid extraction.

Lipid extraction followed the Bligh–Dyer extraction protocol [21]; 3 ml of a methanol/chloroform mixture (2:1, v/v) was added to all of the samples. Then, 250 μl of the internal standard mixture was added to the cricket and earthworm homogenates, while 150 μl was added to the shrimp homogenates. Following the addition of the internal standard, 1.8 ml of water was added to the cricket and worm homogenates, while 1.7 ml was added to the ghost shrimp homogenates. One milliliter of chloroform was then added to all of the homogenates and vortexed. The samples were subsequently centrifuged (IEC Centra CL3, Thermo Fisher Scientific, MA, USA) at 2000 rpm for 10 min, and the chloroform layers were transferred to new vials. An additional 2 ml of chloroform was added to the homogenate samples for reextraction and samples were vortexed and centrifuged again. The chloroform layers were combined with the previous chloroform layers. Samples were dried (Biotage TurboVap LV, NC, USA) and reconstituted in 1 ml of 2-propanol. Then, samples were transferred to autosampler vials, dried and reconstituted in 200 μl of 2-propanol to concentrate them for mass spectrometric analysis.

Lipidomic analysis via mass spectrometry

Cricket, earthworm and ghost shrimp reconstituted extracts were kept at 4°C in the autosampler. Five microliters were analyzed using reversed-phase liquid chromatography with a Waters Acquity BEH C18 column (50 mm $\times$ 2.1 mm, 1.7 μm , Waters, MA, USA) maintained at 30°C on a Dionex Ultimate 3000 RS UHPLC+ system (Thermo Fisher Scientific, CA, USA). The gradient ramp was performed using mobile phase A (60:40 acetonitrile:water, v:v) and mobile phase B (90:8:2 isopropanol:acetonitrile:water, v:v:v), both with 10 mmol/L ammonium formate and 0.1% (v:v) formic acid (Supplementary Table 1).

Mass spectra were acquired using a Q-Exactive Orbitrap (Thermo Fisher Scientific) equipped with a heated electrospray ionization (HESI II) probe, in polarity switching and data-dependent top 10 (ddMS²-top10) modes. In positive-ion mode, diisooctyl phthalate (m/z 391.2842) and polysiloxanes (m/z 371.1012 and m/z 445.1200) were used as lock masses. No lock masses were used in negative-ion mode. The Q-Exactive was externally calibrated before data-acquisition. The S-lens voltage, skimmer voltage, injection flatapole offset voltage and bent flatapole DC voltage were 35, 25, 15, 8 and 6 V, respectively. All samples were analyzed in batches (each invertebrate constituted a batch) with at least three solvent blanks analyzed per batch. Samples were randomized within batches. Four ddMS²-top10 analyses were conducted for each invertebrate for lipid identification (two in positive polarity and two in negative polarity). Scanning parameters are provided in Supplementary Table 2.

Data processing

A list of features (m/z and retention time) and peak areas were obtained using MZmine 2.2 [22]. Chromatograms were smoothed and deconvoluted using a local minimum search; isotopic peaks were grouped, features were aligned and missing features were gap-filled. Peaks with peak heights below 1×10^5 intensity were removed. An MZmine batch file containing exact parameters can be found in the Supplementary Information. Features were then identified using a proprietary software LipidSearch (Thermo Fisher Scientific) [23] and an open source software LipidMatch [14], which can be accessed online [14,15]. These software have different confirmation criteria and fragmentation libraries, thereby adding additional confidence if features were identified as the same lipid by both software and expanding the total number of identified lipids. Resulting identifications from both LipidSearch and LipidMatch were aligned with code available in LipidMatch to the MZmine outputted feature list. A script was also used to combine duplicate features using a retention time window of 0.1 and an m/z window of 0.006, retaining the maximum value of all the replicates.

Identified lipids were semiquantified using class representative standards, and if no standards were analyzed for the lipid class in question, the standard with the greatest similarity in structure and retention time was used (Supplementary Table 3). Quantified lipids were normalized to tissue weight (microgram lipid per gram of wet tissue). A list of defined acronyms and the observed adducts in LipidMatch are provided in Supplementary Table 4.

After quantification, features in negative and positive polarity were combined, using negative mode data if molecular species were represented in both polarities. Lipids quantified in negative mode were prioritized due to

lower background noise and more accurate annotation due to high intensity fatty acyl fragments [24]. Molecular species represented by multiple adducts were quantified using the adduct with the highest calculated concentration. Tentative identifications, such as identifications by exact mass only or identification of sphingomyelin (SM) or phosphatidylcholine (PC) solely by the m/z 184 fragment ion, were removed. Sodium adducts were also removed from the final dataset, as quantitation on the sodium ion without excess sodium is problematic. For oxidized lipids, which are both present in LipidMatch and LipidSearch databases, only lipids identified by MS/MS in both negative and positive polarity were kept to reduce the probability of false positives. Therefore, oxidized lipids that only occur in positive polarity, for example TGs, were excluded from analysis; oxidized lipidomics is an emerging field without proper guidelines for proper annotation, and therefore only the most confident identifications were retained.

Statistical analysis

After quantification and annotation of features, both univariate and multivariate statistical methods were applied. Mean centering and a generalized log transformation (Glog) was used prior to principal component analysis (PCA) using Metaboanalyst 3.0 [25].

For univariate methods, analysis of variance was used to investigate the following questions: euthanization method: heat-treated versus flash-frozen (D vs N), heat stabilization: direct extraction versus 1 h ice incubation then extraction (D vs D1hr), flash-frozen stabilization: direct extraction versus 1 h ice incubation then extraction (N vs N1hr) and compare stabilizations: heat-treated versus flash-frozen samples (D1hr vs N1hr). For shrimp, only the comparison between the euthanization methods (D vs. N) was made. The p-values from analysis of variance across all comparisons were adjusted using Benjamini and Hochberg's false discovery rate (FDR) method [26]. Analyses across different organisms were treated as separate experiments, and hence FDR was calculated separately for each of these instances. To determine individual lipids that were significant, an FDR adjusted p-value of 0.05 was used as the cutoff. To determine if there were trends by lipid class, a Fisher's exact test was used with a p-value cutoff of 0.05 using the `Fisher.test` R function. A one-sided Fisher's exact test was used to determine if a significant number of lipids within a class were upregulated or downregulated compared with lipids across all classes. A list of potentially upregulated or downregulated lipids for Fisher's exact test was determined using an FDR adjusted p-value of 0.25. Note that an FDR adjusted p-value of 0.25, means that about one of four significant compounds will be a false positive. Because the Fisher's exact test was used to determine general trends across lipid classes, we are not interested in whether a particular lipid is significant at this FDR rate, but whether lipids increase or decrease within a class compared with across all classes using this FDR rate.

Results & discussion

Lipid identifications from extracted common house crickets, earthworms and ghost shrimp, using LipidSearch and LipidMatch generally agreed (Supplementary Table 5), especially at the level of lipid sum composition (class, total carbons and degrees of unsaturation). There was better agreement between software in negative ion mode than positive ion mode, and therefore when combining polarities, if a molecular species was represented by both polarities, negative ion mode data were retained. Using these software, a total of 426 (common house cricket), 593 (earthworm) and 429 (ghost shrimp) unique lipids were annotated by MS/MS (Supplementary Table 5). The fragment ions m/z , intensity, and annotation for each confirmed lipid ion in LipidSearch and LipidMatch are shown in the supplementary information ('Lipid_Identification_Information.zip'). In addition, confirmation criteria (fragment exact mass matches necessary for confirmation in LipidMatch) are provided in the 'LipidMatch_Criteria.xlsx' file within the 'Lipid_Identification_Information.zip' file. A total of 40 unique lipid types were identified when combining lipids from all sampled organisms. For an overview of the lipidome coverage for each organism, distributions of lipid concentrations and the number of species annotated per class are presented in pie charts in Supplementary Figure 2.

Two identified lipid types were unexpected, and further confirmation is necessary for unequivocal proof that these lipid species exist in the respective invertebrates. The first is the identification of various sphingomyelin (SM) species in crickets, as SM is not expected in insect species, with PE-Cer replacing major functions of SM for insect species. SM has tentatively been identified in previous research on mosquito cell cultures [27]. Multiple fragment types were observed to confirm these species, for example SM(d14:1/20:0) was reported by both LipidSearch, LipidMatch and LipiDex in both positive polarity as $[M+H]^+$ and negative polarity as $[M+HCO_2]^-$. Both the loss of the choline group, loss of $C_5H_{13}O_4PN$, loss of $C_5H_{11}O_3PN$, $So(d14:1)+H-2H_2O$, phosphatidylcholine head group, $C_5H_{14}NO^+$, $C_2H_6O_4P^+$ and $CH_2CHN(CH_3)_3^+$ were observed in positive mode. In negative ion mode,

Figure 2. Principal components analysis of cricket, worm and ghost shrimp features grouped based on (N) flash-frozen, (N1hr) flash-frozen and 1 h ice incubation, (D) heat treatment and (D1hr) heat treatment followed by 1-h ice incubation.

choline-related fragmentation and fragmentation related to fatty acid composition were also observed. The second was triglycerides with unexpected chain lengths identified by LipidSearch. For example, TG(18:2_10:1_12:4) and TG(18:2_11:4_13:2) were identified, but only by fragments representing two of the three fatty acid fragments (score of B; Lipid_Identification_Information.zip), while TG(17:0_9:0_11:2) and TG(21:3_15:3_15:3) were identified by the neutral loss of all three fatty acids (score of A; Lipid_Identification_Information.zip). In addition to possible false positives, false negatives exist (lipids which exist in the invertebrates, but were unidentified) because of the lack of comprehensive *in silico* fragmentation libraries for insect specific lipids such as PE-Cer.

Multivariate statistics were used to determine general changes in the lipid profile across samples and treatments. PCA scores plots showed different patterns of enzymatic degradation or enzymatic transformation of lipids depending on organism (Figure 2). All heat-treated (D) and flash-frozen samples (N) were separated along the first principal component (PC1) with t-test p-values of the scores less than 0.05, and PC1 explained variances of 25% (earthworm), 66% (cricket) and 62% (shrimp) (Figure 2). In crickets, PC2 better separated heat-treated and flash-frozen samples, with a p-value of 0.01 compared with 0.03 for PC1 scores of all heat-treated versus frozen samples.

To determine whether lipid degradation continued following euthanization, select samples were incubated on ice for 1 h prior to sample extraction (D1hr and N1hr) (Figure 1). For crickets and worms, the PCA scores plot suggests the heat-treated samples (D) and flash-frozen samples (N) had the least amount of variance, while samples after 1 h incubation on ice (D1hr and N1hr) exhibited higher variance (Figure 2A & 2B). This suggests lipid changes occurred after 1 h on ice, and that heat treatment did not completely stabilize the lipids. The earthworms had distinct separation between flash-frozen (N and N1hr) and heat-treatment (D and D1hr) (Figure 2B), unlike the crickets (Figure 2A) where the variability of the ice incubated samples (D1hr and N1hr) resulted in less distinct groupings.

The top 30 features indicative of the lipids influencing the difference between flash-frozen (N) and heat-treated samples (D) and their loadings are shown in Tables 1, 2 & 3. Loadings for all features can be found in Supplementary Table 6. Of the top 15 features influencing heat-treated samples, for crickets, worms and shrimp, 100% consisted of intact glycerophospholipids and oxidized glycerophospholipids, suggesting minimal enzymatic degradation compared with flash-frozen samples. Of the top 15 features indicative of flash-frozen samples, over 60% consisted of lysoglycerophospholipids for crickets and 100% consisted of lysoglycerophospholipids for worms (Tables 1 & 2), indicating enzymatic degradation of intact glycerophospholipids to lysoglycerophospholipids. For shrimp, 87% of the top 15 loadings indicative of flash-frozen samples consisted of phosphatidylmethanol (PMe), an enzymatic product produced when using methanol for extraction (Table 3).

These enzymatic products are likely generated in the preanalytical steps of lipid analysis, such as homogenization or freeze/thawing, which can cause lipid membranes to rupture, resulting in stored calcium in the mitochondria and endoplasmic reticulum lumen to be released. Calcium activates proteases, lipases and kinases, which in

Table 1. Cricket

Flash frozen	PC2	Heat treated	PC2
TG(18:2_10:1_12:4)	-0.12	TG(30:0_16:0_18:1)	0.13281
TG(6:0_12:0_18:2)	-0.12	TG(29:0_18:1_18:1)	0.12347
TG(18:2_11:4_13:2)	-0.11	TG(13:0_16:0_18:1)	0.12344
LPC(20:5)	-0.11	TG(29:1_16:0_18:1)	0.12331
TG(10:0_10:0_18:2)	-0.11	TG(29:1_16:0_18:2)	0.12245
OxLPC(18:2(OOH))	-0.11	PC(20:1_20:3)	0.12162
LPC(20:4)	-0.11	TG(26:2_18:1_23:0)	0.12131
TG(18:1_10:1_12:4)	-0.10	TG(29:0_16:0_18:1)	0.11997
LPC(18:3)	-0.10	TG(13:0_18:2_18:2)	0.1169
LPC(14:0)	-0.10	PC(18:0_20:3)	0.11639
LPE(20:5)	-0.09	TG(29:0_18:1_18:2)	0.11602
LPC(16:1)	-0.09	TG(18:0_18:1_26:0)	0.11523
LPE(18:3)	-0.09	TG(18:2_24:0_24:0)	0.11348
TG(10:0_18:1_18:2)	-0.08	TG(14:1_15:0_18:2)	0.11273
OxLPC(18:2(OH))	-0.08	TG(18:2_22:0_24:0)	0.11171

Table 2. Worm.

Flash frozen	PC1	Heat treated	PC2
LPA(14:0)	-0.25	TG(14:1_14:1_20:4)	0.11607
LPE(15:0)	-0.23	TG(17:2_17:2_17:2)	0.10413
LPC(18:3)	-0.22	plasmalogen-TG(O-18:0_20:3_20:5)	0.1018
LPC(20:2)	-0.22	TG(14:1_17:1_20:4)	0.099155
LPC(22:5)	-0.20	TG(14:1_18:3_22:5)	0.097221
LPC(20:3)	-0.17	TG(14:1_16:0_20:3)	0.094129
LPC(14:0)	-0.16	TG(14:1_18:3_18:3)	0.09167
LPC(20:5)	-0.16	TG(12:0_14:1_18:4)	0.091006
LPC(20:1)	-0.16	TG(14:1_15:0_18:1)	0.08723
LPC(16:1)	-0.15	TG(14:1_20:3_20:4)	0.086726
LPC(22:4)	-0.14	TG(12:0_15:0_18:2)	0.083393
LPC(18:2)	-0.12	TG(18:0_20:2_20:5)	0.08312
LPC(15:0)	-0.12	TG(14:1_14:1_18:3)	0.080973
LPC(18:1)	-0.10	plasmalogen-TG(O-20:0_20:4_20:5)	0.078313
LPC(20:4)	-0.09	TG(14:1_20:1_20:2)	0.076663

turn can lead to degradation of lipid species [28]. For example, glycerophospholipids (GPL) in the presence of phospholipase A, phospholipase C in concert with various kinases and phospholipase D in concert with PA phosphohydrolase are cleaved, producing products including diglycerides (DGs), lysophosphatidylcholines (LPCs), lysophosphatidylethanolamines (LPEs) and LPC and LPE plasmalogen and plasmalogen corollaries, as shown in Figure 3. For a simplified schematic of enzymatic transformation of lipids, which includes the production of phosphatidylmethanol, refer to Supplementary Figure 3.

In a univariate analysis of the euthanization effect on lipids, a Fisher's exact test was used to determine trends across lipid classes. The Fisher's exact test p-values (Supplementary Table 7) show which lipid classes were the most significantly upregulated or downregulated in comparison of euthanization techniques. One-sided Fisher's exact test on heat-treated (D) and flash-frozen (N) shrimp and worm samples showed an overall increase in LPC and LPE species in flash-frozen samples (Supplementary Table 7B & Supplementary Table 7C), with p-values less than 0.005. The general lower concentration of lysoglycerophospholipids (lyso-GPL) in heat-treated samples (Supplementary Table 7), provides evidence that phospholipase A₂ activity [29] (Figure 3) was reduced. For crickets, LPC and LPE species did not differ in heat-treated (D) and flash-frozen samples (N) that were not incubated on ice.

Table 3. Shrimp.

Flash frozen	PC1	Heat treated	PC2
PMe(18:0_20:5)	-0.24	PE(20:4_20:5)	0.19807
PMe(20:5_22:6)	-0.24	PE(18:1_20:4)	0.10225
PMe(18:0_20:4)	-0.22	PC(16:1_18:2)	0.07236
PMe(16:0_18:1)	-0.22	PC(24:0_20:4)	0.048626
PMe(16:0_18:2)	-0.21	PE(17:1_20:5)	0.048328
PMe(18:1_18:1)	-0.21	PC(15:0_20:5)	0.043092
PMe(20:5_20:4)	-0.21	PC(24:1_20:4)	0.037523
PMe(16:0_20:5)	-0.21	PC(20:3_20:3)	0.035687
PMe(18:1_20:4)	-0.20	PC(18:2_22:5)	0.030205
PMe(16:0_20:4)	-0.19	OxPC(20:5_22:3(OH))	0.028369
PMe(20:4_20:4)	-0.19	PC(18:4_20:5)	0.027794
PMe(20:4_22:6)	-0.18	TG(16:0_18:1_22:6)	0.024376
PMe(16:0_18:3)	-0.18	Plasmenyl-PC(P-16:1_22:4)	0.024249
PC(16:0_14:2)	-0.16	PS(20:4_22:6)	0.02384
DG(20:5_22:6)	-0.14	PE(20:3_20:4)	0.023266

Figure 3. Schematic of enzymatic degradation of glycerophospholipids and triglycerides. Products and intermediates include fatty acids, lysoglycerophospholipids, phosphatidic acid and diglycerides. Enzymes involved include phospholipase A1 and A2 (PLA1 and PLA2, respectively), phospholipase C, phospholipase D and triglyceride lipase. The fatty acid and LPL products from cleavage via PLA1 are not shown.

While no significant difference in LPC and LPE was observed between heat treated (D) versus flash-frozen (N) cricket samples, which were immediately extracted, after samples were incubated on ice for 1 h, there was a significant difference between heat-treated (D1hr) and flash-frozen (N1hr) cricket samples (p -value <0.005 ; Supplementary Table 7A). This is due to an increase in LPC and LPE concentration only in samples without heat-treatment after 1 h incubation (N1hr vs N). On the contrary, 1-h incubation on ice (N vs N1hr and D vs D1hr) did not increase enzymatic production of LPC and LPE species in worm samples, except ether-LPC and ether-LPE species for flash-frozen samples (N vs N1hr) (Supplementary Table 7B). Ether-linked lipids are more resistant to enzymatic degradation by phospholipase A₁ [30], and therefore slower degradation may explain the continued increase of ether-LPC and ether-LPE after 1 h in samples without heat-treatment. These data indicate that the amount and rate of enzymatic activity are both dependent on lipid class and the sample organism.

LPCs incorporating very long chain fatty acids with over 22 carbons were detected in shrimp and worms by LipidSearch using MS/MS (Supplementary Table 5). For LPC species with 22 or less carbons, 14 out of 16 LPC species in worms and 8 of the 10 LPC species in shrimp, had FDR p -values <0.05 for heat-treated (D) versus

Figure 4. Total PE [M-H]⁻, LPE [M-H]⁻, PC [M+HCO₂]⁻ and LPC [M+HCO₂]⁻ concentrations in all cricket, earthworm and shrimp samples that were flash-frozen (N) and heat treated (D), including samples that were incubated for 1 h on ice. The three significance levels are: $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***).

flash-frozen (N) samples. For species with over 22 carbons, of the 8 LPC species in worms and 6 LPC species in shrimp, none had FDR corrected p -values < 0.05 (Supplementary Table 5). These results suggest that due to the unique enzymatic synthesis and degradation of very long chain fatty acids [31], either heat treatment is ineffective at preventing enzymatic activity, which generates LPC species with over 22 carbons, or significant enzymatic activity generating LPCs for species with over 22 carbons does not occur during sample preparation. Hence, not only is enzymatic activity dependent on the organism and lipid class, but may also be dependent on fatty acyl constituents.

While low concentrations of lyso-GPL in heat-treated organisms (Supplementary Table 5) support that heating reduced phospholipase A activity for species with 22 or less carbons, intact glycerophospholipids would be expected to be higher in heat-treated organisms, as these lipids would not be cleaved by phospholipase A (Figure 3). However, glycerophospholipids were not significant according to the Fisher's exact test (Supplementary Table 7), potentially owing to the small sample size used in this study. In this case, trends in GPL may be less likely to be discerned against biological variation and other sources of noise because of the relatively small percent change in concentration compared with their enzymatic products, lyso-GPLs. For example, the total LPE concentration in shrimp was 417% higher (p -value = 0.0000009) in flash-frozen shrimp (11.8 ± 0.8 $\mu\text{g/g}$) compared with heat-treated shrimp (2.3 ± 1.1 $\mu\text{g/g}$), while the average total PE concentration was 27% higher (p -value = 0.032) in heat-treated shrimp (820.8 ± 123.6 $\mu\text{g/g}$) compared with flash-frozen shrimp (647.4 ± 44.3 $\mu\text{g/g}$) (Figure 4). Using the total sum of PC, PE and respective plasmalogen and plasmalogen species of earthworms (including samples with and without the additional 1 h incubation) suggested that heat treatment reduced degradation of PE to LPE, PC to LPC and ether linked species to their respective lyso-species (Figure 4 & Figure 5). Our results agree with the findings of Wang *et al.* where lyso-GPL were lower and GPL higher in heat-treated rat and mouse plasma samples compared with control samples stored on a benchtop [8].

Glycerophospholipids can also be cleaved by phospholipase D to form phosphatidic acids (PA), which can further be converted into DGs by PA phosphohydrolase (Figure 3). While no PAs were detected in samples, DGs were detected in crickets and shrimp (Supplementary Table 5; Supplementary Table 7). In shrimp, all DGs detected were an order of magnitude lower in heat-treated samples, with FDR corrected p -values < 0.005 , and Fisher's exact test p -values < 0.005 (Supplementary Table 5; Supplementary Table 7C). This trend was not observed for crickets, but, when summing total DG concentration including the samples incubated for 1 h, DGs were 122% greater (p -value = 0.041) in flash-frozen compared with heat-treated crickets. Further evidence that heat treatment denatures, and hence deactivates, phospholipase D is shown by the presence of phosphatidylmethanol (PMe) in frozen shrimp (Figure 6A). It has been shown previously that water can be replaced with alcohols as acceptors in transphosphatidylation via phospholipase D [32] (Supplementary Figure 3), and as shrimp were homogenized in methanol, PMe are expected. In the flash-frozen samples, 14 PMe species were detected as deprotonated ions, while no PMe ions were found in heat-treated samples. It is important to note that PMe was not detected in crickets or earthworms, as these organisms were large enough for cryopulverization, eliminating the need for an additional homogenization step using methanol. Therefore, it is important to note, that in addition to lipid structure and

Figure 5. Total ether-linked (plasmalogen and plasmalogen species) PE [M-H]⁻, LPE [M-H]⁻, PC [M+HCO₂]⁻ and LPC [M+HCO₂]⁻ concentrations in frozen (N) and heat-treated (D). The three significance levels are: $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***).

sample organism affecting enzymatic activity, the sample homogenization and extraction procedure employed also affects enzymatic activity.

Phospholipase D has multiple isoforms that hydrolyze PC, PE, phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), LPC, cardiolipin and plasmalogen head groups. Certain lipid classes and fatty acid chain constituents will preferentially be hydrolyzed depending on the organism [33]. In shrimp, PE(20:5_22:6), PS(20:5_22:6), PC(20:5_22:6) and potential enzymatic products PMe(20:5_22:6), DG(20:5_22:6), LPE(20:5), LPC(20:5), LPE(22:6) and LPC(22:6), suggest PE, PS and PC conversion by phospholipase D into PMe and DG at least for GPLs containing 20:5 and 22:6 (Figure 6A–I). Potential fatty acid preferences for enzymatic reaction of phospholipase D existed. For example, in all 13 DGs generated by enzymatic reaction in shrimp (Supplementary Table 5), each DG contained either a 20:4, 20:5, 22:5, or 22:6 fatty acyl tail, despite high intensity species such as PC(16:0_18:1) and PE(16:0_18:1).

Phosphatidylserine (PS) lipid species can be substrates for a specific lipase: PS-specific PLA₁. For shrimp, a closer investigation of PS(20:5_22:6) (Figure 6B) suggests that the lower concentration in frozen samples is mainly because of phospholipase D and not PS-specific PLA₁ or phospholipase A. The difference in the amount of degradation

Figure 6. Box plots of lipid species concentrations in shrimp flash-frozen (N) and heat-treated (D).

Glycerophospholipids containing 20:5 and 22:6 (A–C) and potential enzymatic products (D–I). Lysolipid (LPC and LPE) concentrations are from protonated ions, glycerophospholipids PE and PMe are deprotonated ions, PC are formate adducts and DG are ammonium adducts. p-values are Hochberg FDR corrected with three significance levels: $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***).

products in frozen shrimp compared with heat treated shrimp was found to be similar to the difference in the product source assuming phospholipase D activity. While PC(20:5_22:6) and PE(20:5_22:6) are only 11.7 µg/g lower in frozen shrimp compared with heat-treated shrimp, their likely degradation products in the presence of phospholipase D, PMe(20:5_22:6) and DG(20:5_22:6), are 74.8 µg/g higher in frozen shrimp. This suggests another source of these degradation products. When PS(20:5_22:6) is included as a source of DG(20:5_22:6) and PMe(20:5_22:6) via phospholipase D activity, the concentration becomes 66.9 µg/g (Figure 6A–C) for sources of the 74.8 µg/g enzymatic products (Figure 6D–K). This suggests that in shrimp, phospholipase D degradation of PS species predominate, versus PS-specific PLA₁, which would lead to lysophosphatidylserines (LPS), which were not detected. Other sources of PMe(20:5_22:6) could be PI(20:5_22:6), PG(20:5_22:6), or PA(20:5_22:6), but none of these species were detected. In addition, DG(20:5_22:6) could be a product of triglyceride lipase activity for TG(16:0_20:5_22:6), TG(18:0_20:5_22:6) and TG(18:1_20:5_22:6) lipids, but together these only accounted for <5 µg/g in concentration, and hence could not be a major factor in the 45.1 µg/g increase of DG(20:5_22:6) observed in flash-frozen shrimp.

This study supports previous evidence that lipase activity depends on fatty acid chain constituents, which has been shown for numerous lipases including neutral lipases [34,35] and phospholipase D [33]. Oxidized lipids have potential to also show various trends depending on acyl chain composition, as they can be produced both via enzymatic and chemical routes owing to exposure to catalysts, electromagnetic radiation and/or oxygen [36]. On average, all nine oxidized PEs (OxPE) and oxidized PCs (OxPC) were higher in concentration when incubated on ice for 1 h compared with those immediately extracted after weighing for both heat-treated (except 1) and frozen crickets, but were not significantly different based on FDR corrected p-values and Fisher's exact test (Supplementary Tables 5A & 7A). The only significant change in oxidized lipids was a general increase in OxPE after 1 h in the

heat treatment samples (Fisher's exact test p -value = 0.016; Supplementary Table 7A). Measurement of oxidative markers such as malondialdehyde could be used in the future to determine if heat treatment increases oxidation.

This manuscript investigated heat treatment as a mechanism to prevent enzyme degradation of lipids. Untargeted studies often are not concerned with absolute quantitation, but instead investigate profile changes in response to the experimental design. If all samples degrade similarly, and compounds are detectable, the occurrence of degradation (within acceptable experimental design) may not be a hindrance for a biomarker type assessment. Based on this data, it is not conclusive whether heat treatment reduces variance introduced from enzymatic activity. RSDs across all features (Supplementary Table 5) did not follow any trend between heat treatment and flash-frozen samples (t -test). Surveying RSDs of total LPC and LPE concentrations across earthworms and crickets showed higher variability in flash-frozen samples compared with heat-treated samples when incubated and nonincubated samples were combined (Supplementary Table 8). When RSDs for incubated and nonincubated samples were compared without combining euthanization methods (D vs N and D1hr vs N1hr compared separately) there was no clear trend in RSD between heat-treated and flash-frozen samples. This suggests heat treatment reduces variation, when samples are left on a benchtop for varying amounts of time, but may not reduce variance if all samples are left on the benchtop for the same amount of time prior to extraction. If the amount of degradation is consistent across all samples, then for comparative studies where only relative amounts of lipids are of concern, heat treatment may not be necessary. A future study with a larger sample size that minimizes biological variability, such as aliquots of monoclonal cells grown under the same conditions, could provide more conclusive evidence for using heat treatment for stabilization of the lipidome.

Conclusion

Our study provides evidence that heat treatment deactivates enzymes in the early preanalytical phase, which may provide better qualitative and quantitative results for lipids. Based on higher concentrations of PE and PCs, and lower concentrations of LPE, LPC, DG and PMe species in heat-treated organisms, heat treatment reduced or prevented enzymatic degradation of important lipid types in common house cricket, earthworm and ghost shrimp extracts. This evidence supports reduction in the activity of phospholipase A and phospholipase D, specifically. However, oxidation may continue to occur in both heated and frozen samples.

We believe that heat treatment could help minimize variance within treatment groups that can occur as a result of freeze–thaw cycles, variable time between sample collection and freezing or other factors that affect the rate or duration of enzyme activity. To compare and integrate lipidomes characterized across the international community, sample stabilization is extremely important and heat treatment may be a simple solution. This is especially noteworthy for solid matrices, where the inclusion of additives to slow lipid degradation is impractical. Additional studies with larger samples sizes and a homogenous replicated sample would be beneficial in determining whether heat treatment stabilizes the lipid profile during sample preparation. In addition to experiments with larger samples sizes, fundamental studies employing heat treatment to various matrices spiked with lipid standards and enzymes are needed to characterize the extent of enzymatic degradation of lipids with and without heat treatment. While the technology for heat treatment has drastically improved, currently it is not broadly available on the market to handle larger organisms, for example adult mice. In addition, euthanization of vertebrate species, especially mammals, using heat treatment will not meet ethical guidelines for many institutions. Therefore, heat treatment will need to be employed after euthanization and sample collection. Studies describing optimization of methods to reduce lipid degradation during euthanization and sample collection, and prior to heat treatment, are necessary for mammalian systems.

Future perspective

As the lipidomics field matures, the applicability to clinical science has become apparent. Relatively few clinical applications employ lipidomic analyses to determine biomarkers and understand health and disease due to biomarker discovery workflows requiring reproducible lipid measurements. Currently, the effects of preanalytical steps, such as sample handling, storage and extraction on the final measured lipids and their respective concentrations, are not heavily researched or understood. Literature has shown that preanalytical steps can lead to drastic changes in lipid profiles, especially due to enzymatically catalyzed transformation of lipid profiles.

With the emergence of lipidomics on the clinical market, it will be necessary to understand variance introduced from preanalytical steps and introduce technologies to reduce or eliminate these effects. In order for the clinical field to more broadly adopt lipidomics, we predict there will be increased discussion, research and conferences

focused on lipid stabilization. This will most likely lead to specific lipidomics protocols and guidelines accepted and implemented by various sectors of the lipidomics community and applicable fields. Either heat-treatment technologies, or technologies that maintain cryotemperatures throughout the preanalytical procedures, may play significant roles in maintaining sample stabilization.

Summary points

Results & discussion

- By integrating an open-source UHPLC–HRMS/MS workflow with LipidSearch, 426 (common house cricket), 593 (earthworm) and 429 (ghost shrimp) unique lipids were annotated by MS/MS and quantified.
- Earthworms were found with exceptionally high levels of endogenous plasmalogen and plasmalogen lipids, which can serve to prevent oxidative damage to lipids, while crickets were found to have higher levels of oxidized lipids compared with the other species analyzed.
- An experimental design to determine if enzymatic degradation occurs in realistic benchtop extraction scenarios showed that the concentration of enzymatically produced lipid products depended on lipid class, lipid species, organism, homogenization method and time on benchtop prior to extraction, thus suggesting that sample preparation has a dramatic effect on lipid concentrations.
- Concentrations of lysoglycerophospholipid species including lysophosphatidylcholines (LPCs), lysophosphatidylethanolamines (LPEs) and ether-linked lysoglycerophospholipids, significantly decreased when heat-treatment was employed, suggesting the reduction or inhibition of phospholipase A₁ and A₂ activity.
- Exceptionally long-chained LPCs, with over 22 carbons, were observed in shrimp and worms in relatively high concentrations and did not decrease with heat-treatment, which was contrary to the trend for LPC species with 22 carbons or less.
- After applying heat-treatment to shrimp, only diglyceride (DG) species containing 20:4, 20:5, 22:5, or 22:6 were significantly reduced in concentration, which further suggested reduction or inhibition of phospholipase D activity.
- DG, PMe and LPC results suggest that lipase activity is often not only dependent on lipid class, but highly dependent on fatty acid constituents of specific lipid species.
- Ether-linked LPCs and LPEs were the only lysoglycerophospholipids that continued to increase in concentration after 1 h incubation on ice prior to extraction, as compared with immediate extraction in worms. This increase was not observed after heat-treatment.
- Phosphatidylmethanol (PMe) formation was eliminated by heat-treatment of shrimp prior to homogenization in methanol, suggesting reduction or elimination of phospholipase D activity when employing heat-treatment.

Conclusion

- More community-wide effort is needed to establish guidelines on the best practices to identify, quantify and ameliorate lipid instability during sample preparation.

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