Supplementary information

Functional and evolutionary evidence from a widely used molluscan model reveals the absence of ecdysone signaling in lophotrochozoans

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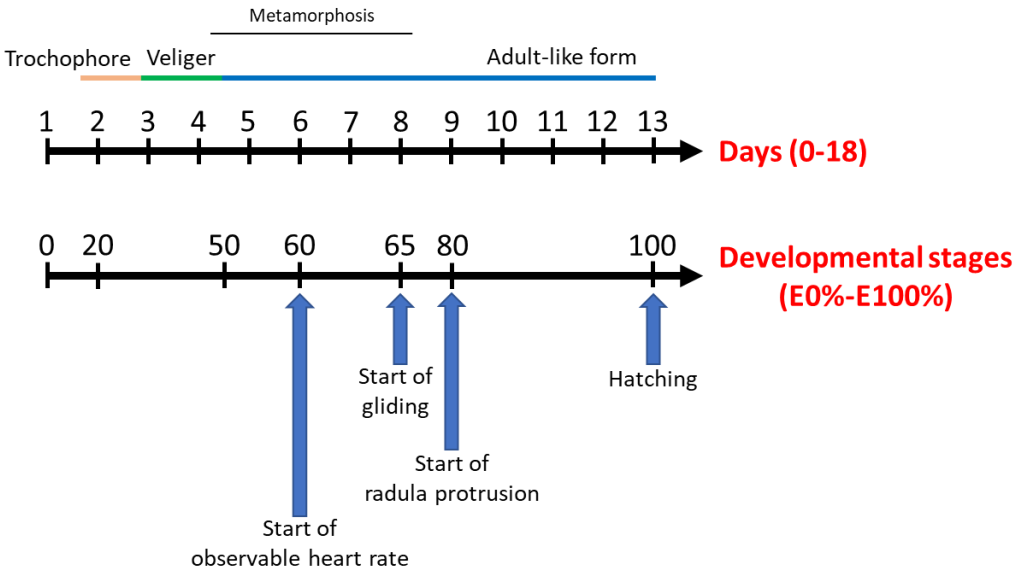
Supplementary Figure 1. Schematic representation of the time course (given in days [0-13] and embryonic stages [percentage of development – E0%-E100%]) of *Lymnaea* embryogenesis in our laboratory. Arrows indicate stages at which an organ (pigmented eyes – E50% [Day 4.5]) or a physiological/behavioural activity (heart rate – E60% [Day 6]; gliding – E65% [Day 8]; feeding – E80% [Day 9]) appears during embryonic development.



Supplementary Table 1. All species of metazoans used in the study for CLANS and phylogenetics

Taxa	Species
Cnidaria	*Hydra vulgaris*
	Nematostella vectensis
Ambulacraria	*Saccoglossus kowalevskii*
	Strongylocentrotus purpuratus
Chordata	*Gallus gallus*
	Homo sapiens
Cephalochordata	*Branchiostoma belcheri*
Lophotrochozoa	*Lottia gigantea*
	Lymnaea stagnalis
	Octopus bimaculoides
Ecdysozoa	*Ascaris suum*
	Caenorhabditis elegans
	Drosophila melanogaster
	Priapulus caudatus
	Epiperipatus broadwayi

Supplementary Table 2. Mortality data during *Experiment 1*

Replicate 1

Time point	C	SC	10 ng	100 ng	1 µg
Living embryos before treatment	10	10	10	10	10
Living embryos after treatment	**10**	**10**	**10**	**10**	**10**

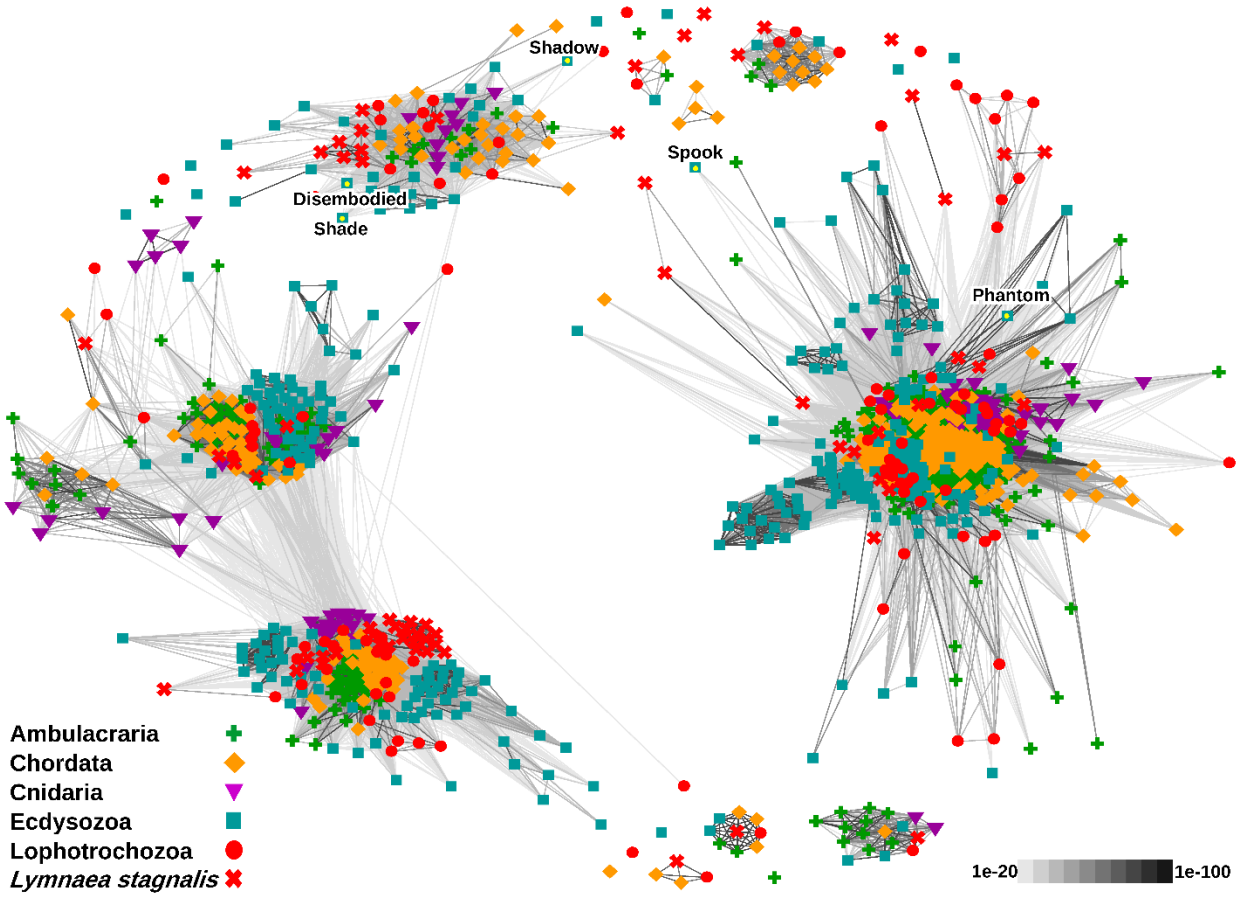
Replicate 2

Time point	C	SC	10 ng	100 ng	1 µg
Living embryos before treatment	10	10	10	10	10
Living embryos after treatment	**10**	**9**	**10**	**10**	**10**

Replicate 3

Time point	C	SC	10 ng	100 ng	1 µg
Living embryos before treatment	10	10	10	10	10
Living embryos after treatment	**10**	**9**	**10**	**10**	**10**

Supplementary Figure 2. Cluster map of cnidarian, protostome, and deuterostome Cytochrome P450 proteins showing the closely related Spook, Phantom, Disembodied, Shadow, and Shade proteins.



Identified *Lymnaea* protein sequences showing homology to Phantom and Shade. The conserved domain is presented under the sequences.

Phantom

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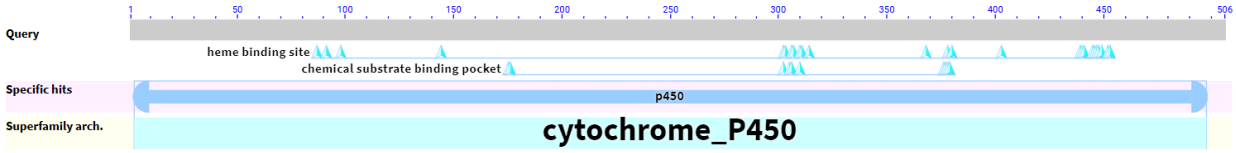

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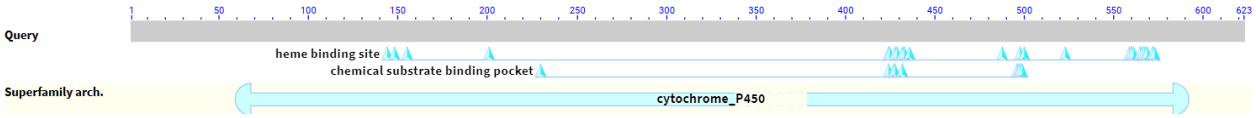

Shade

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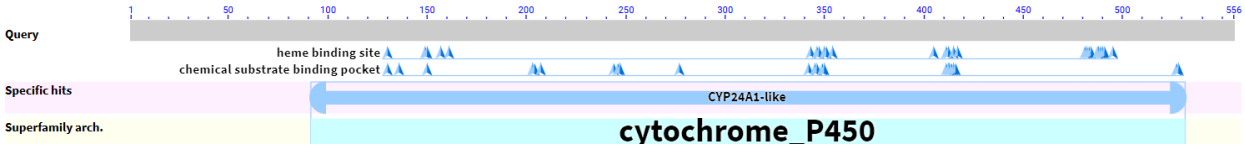

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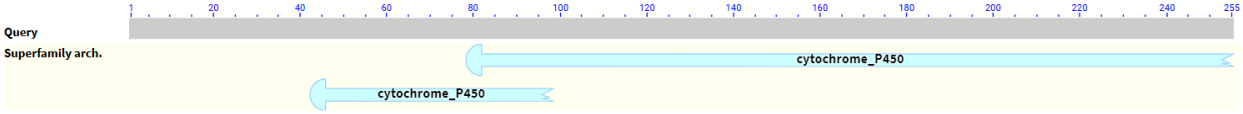

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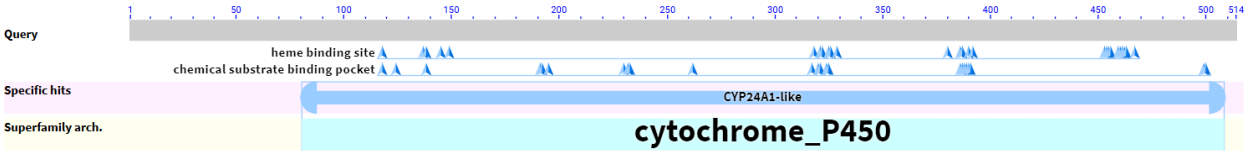

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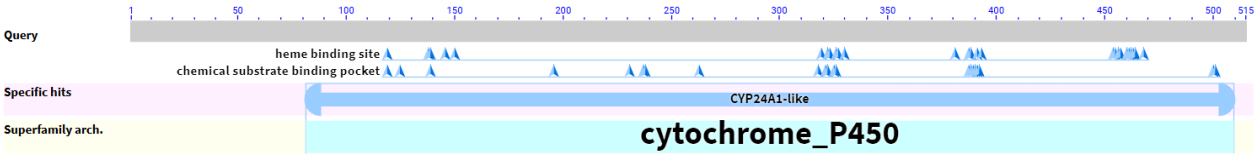

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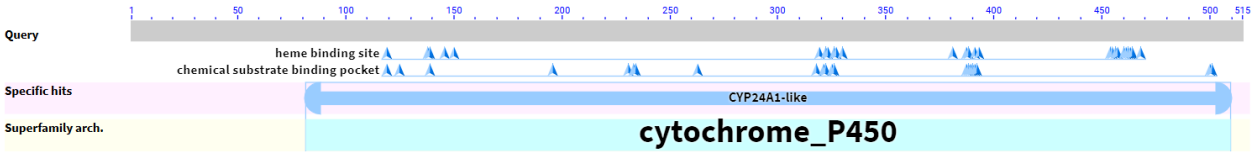

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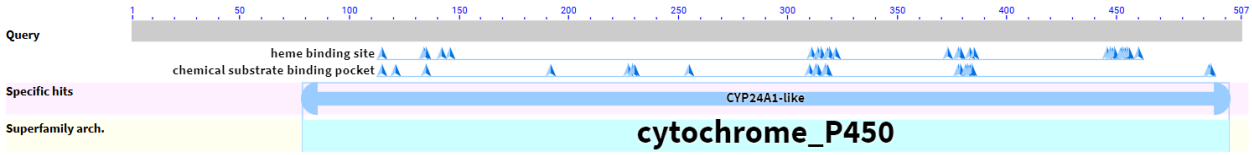

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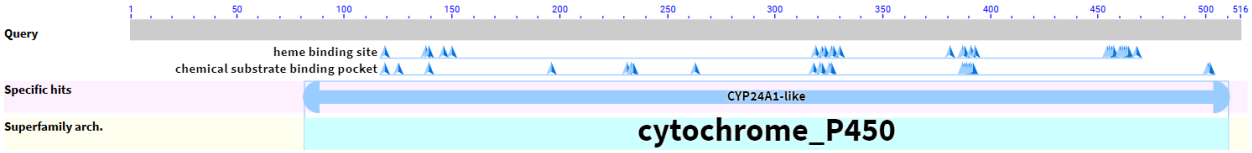
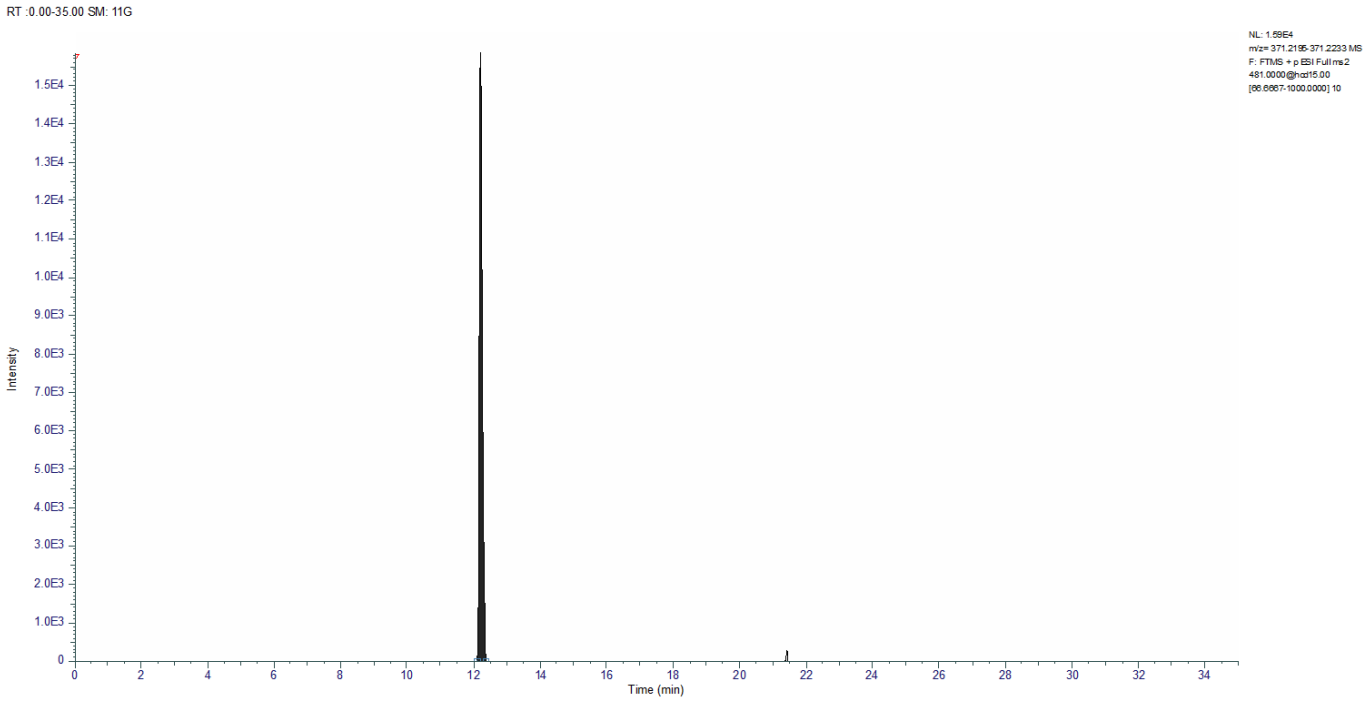
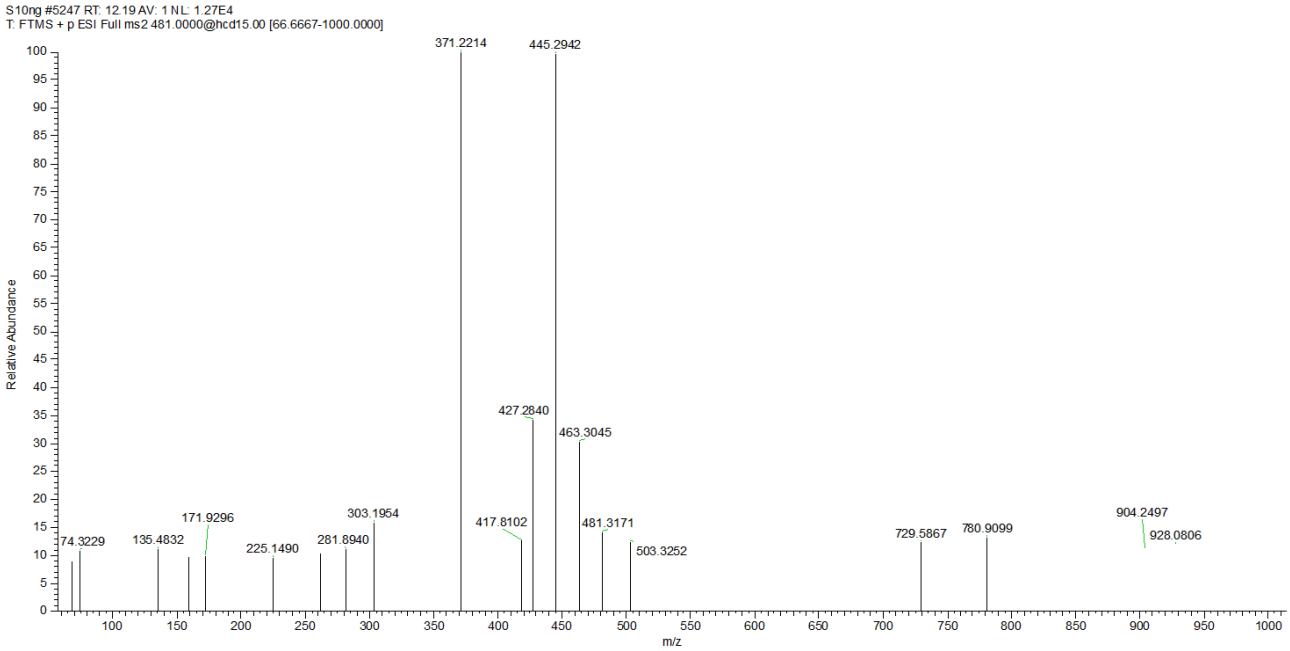


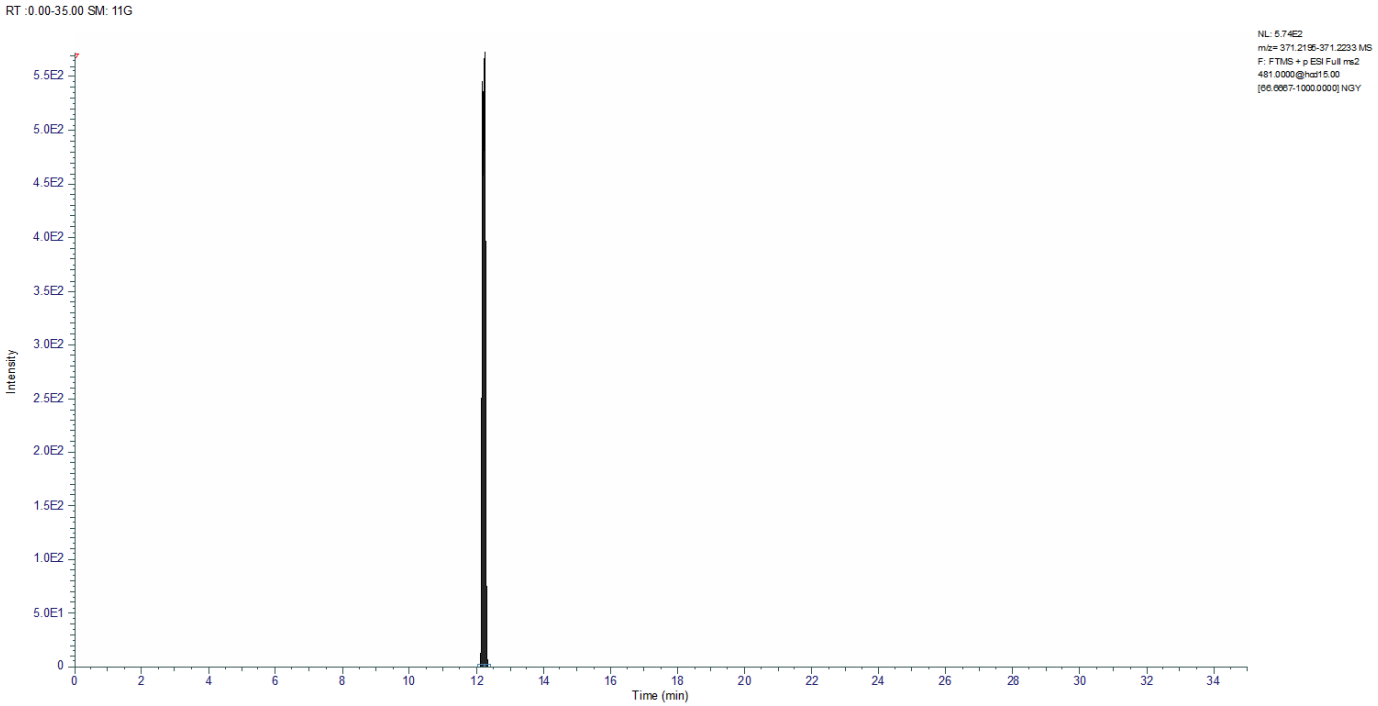
Representative chromatogram and MS/MS spectrum of a 20HE standard solution (10 ng/mL), different *Lymnaea* tissues, and lettuce

10 ng/mL 20HE standard solution





Stomach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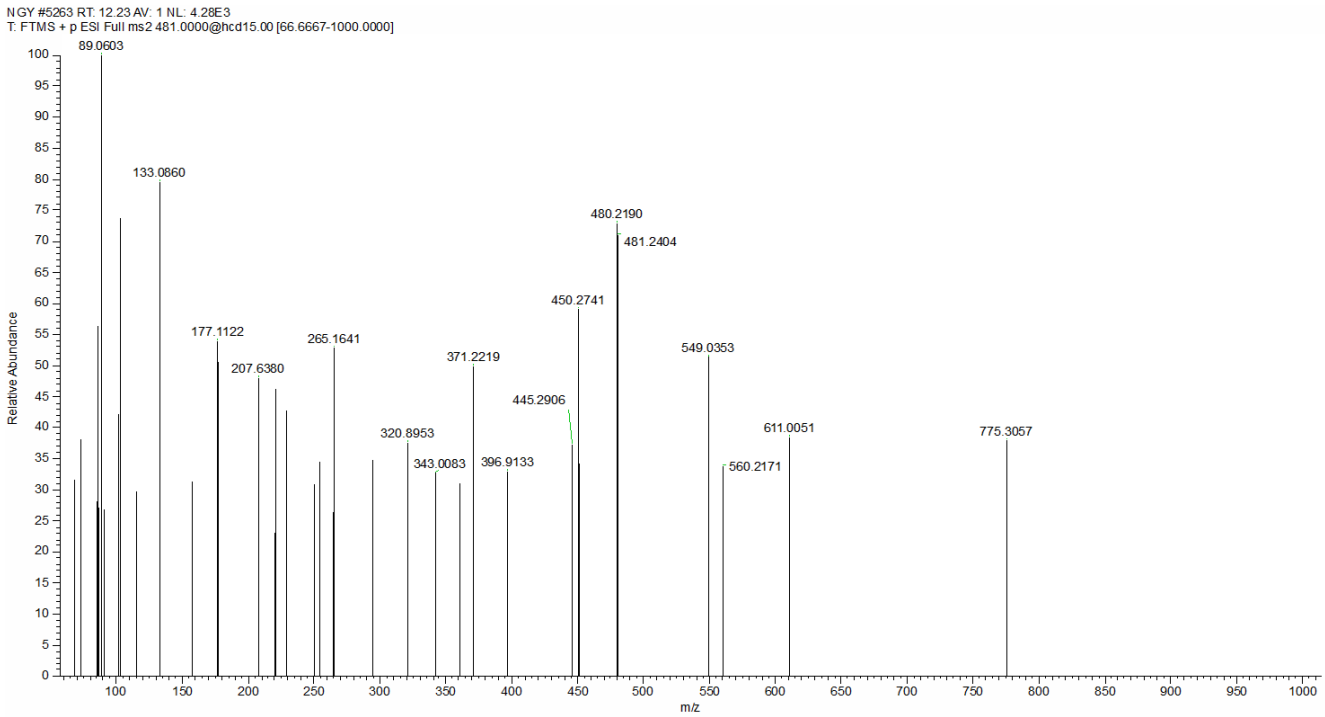
Intensity
Time (min)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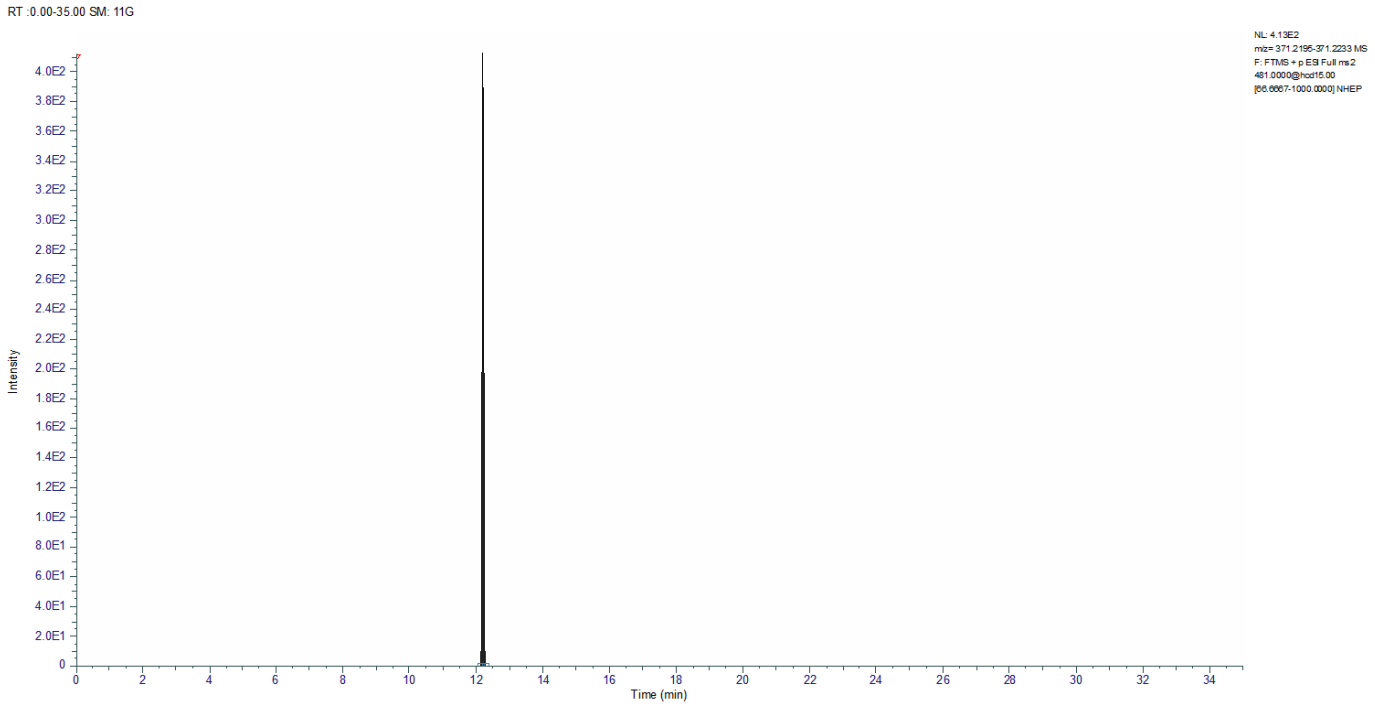
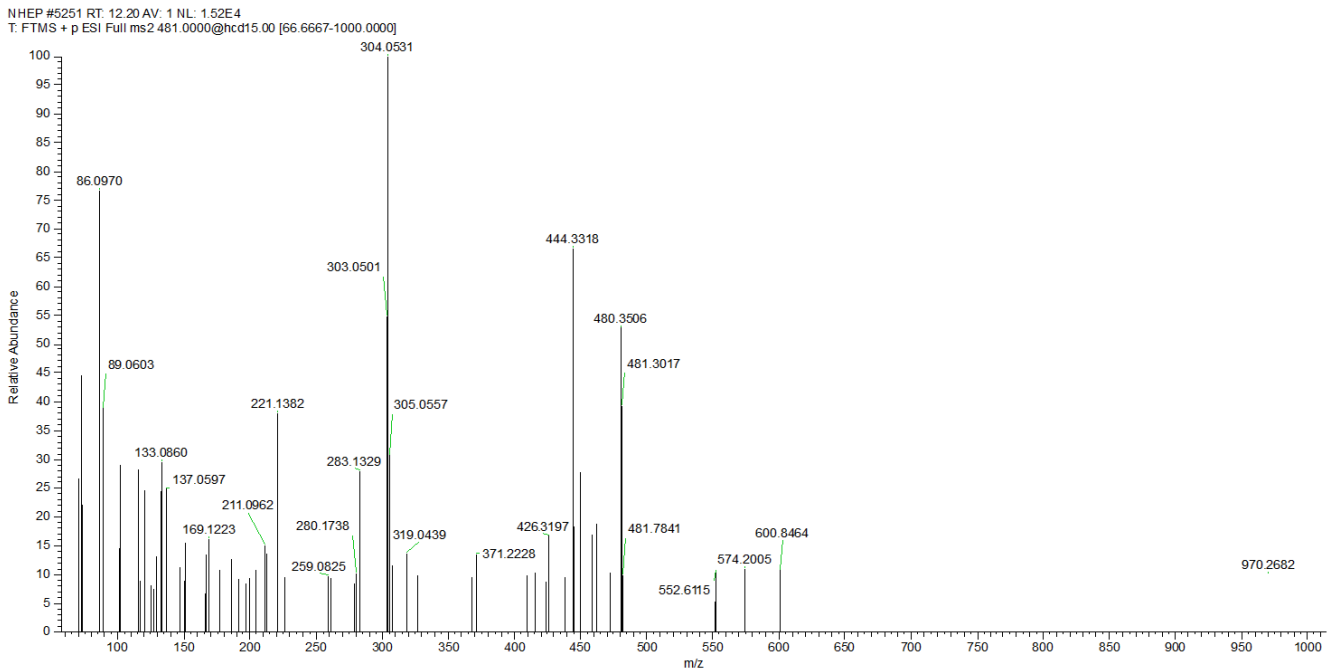
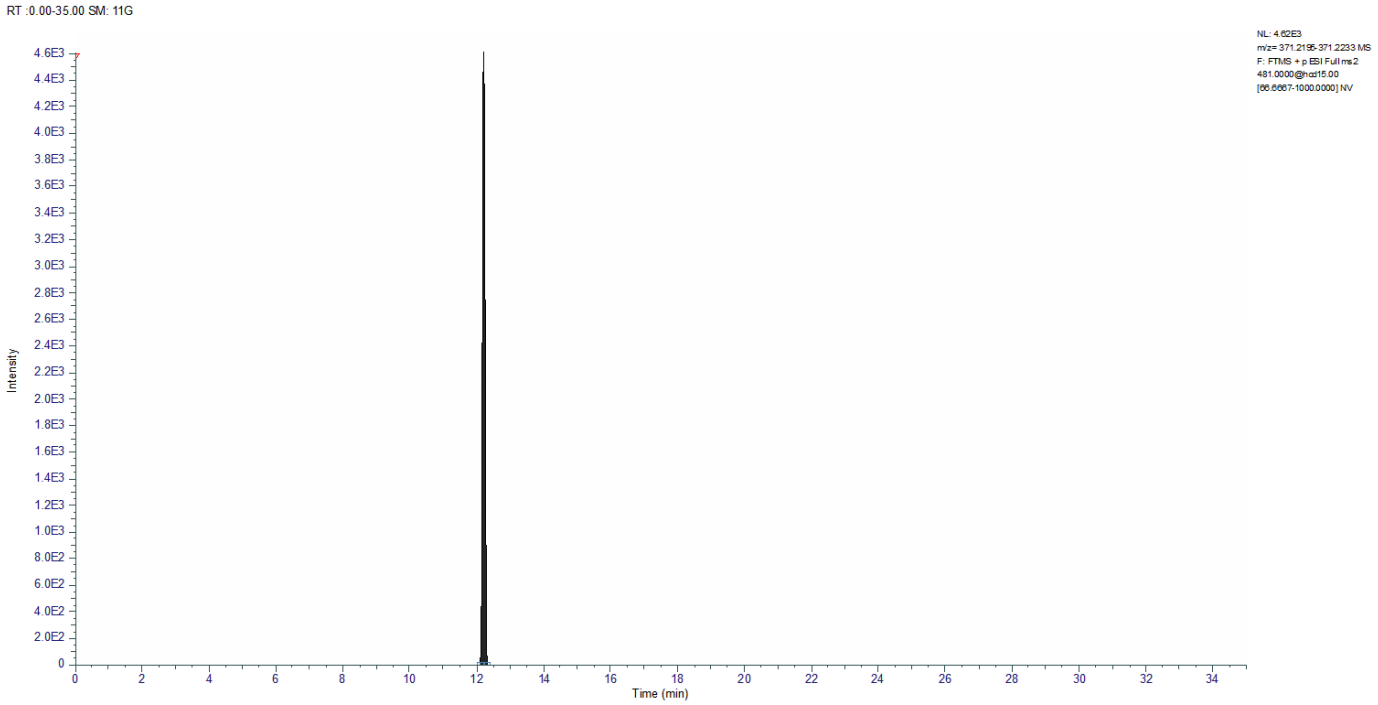
Relative Abundance
m/z

Hepatopancreas





Kidney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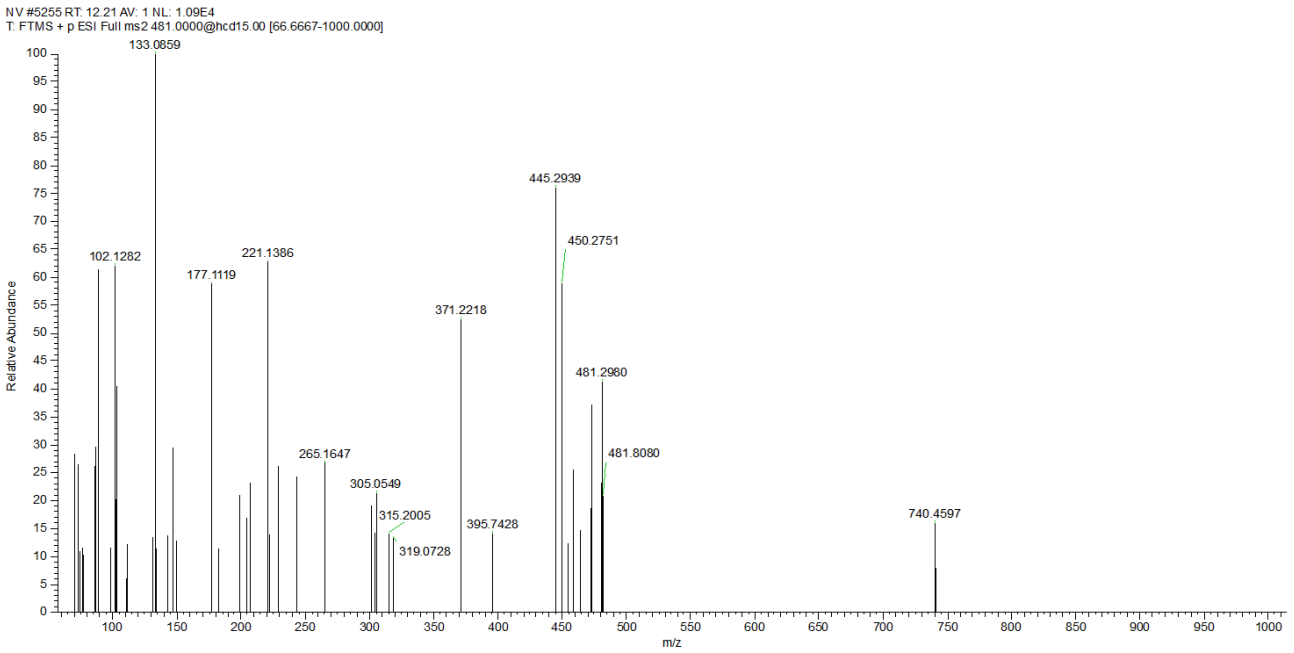
Time (min)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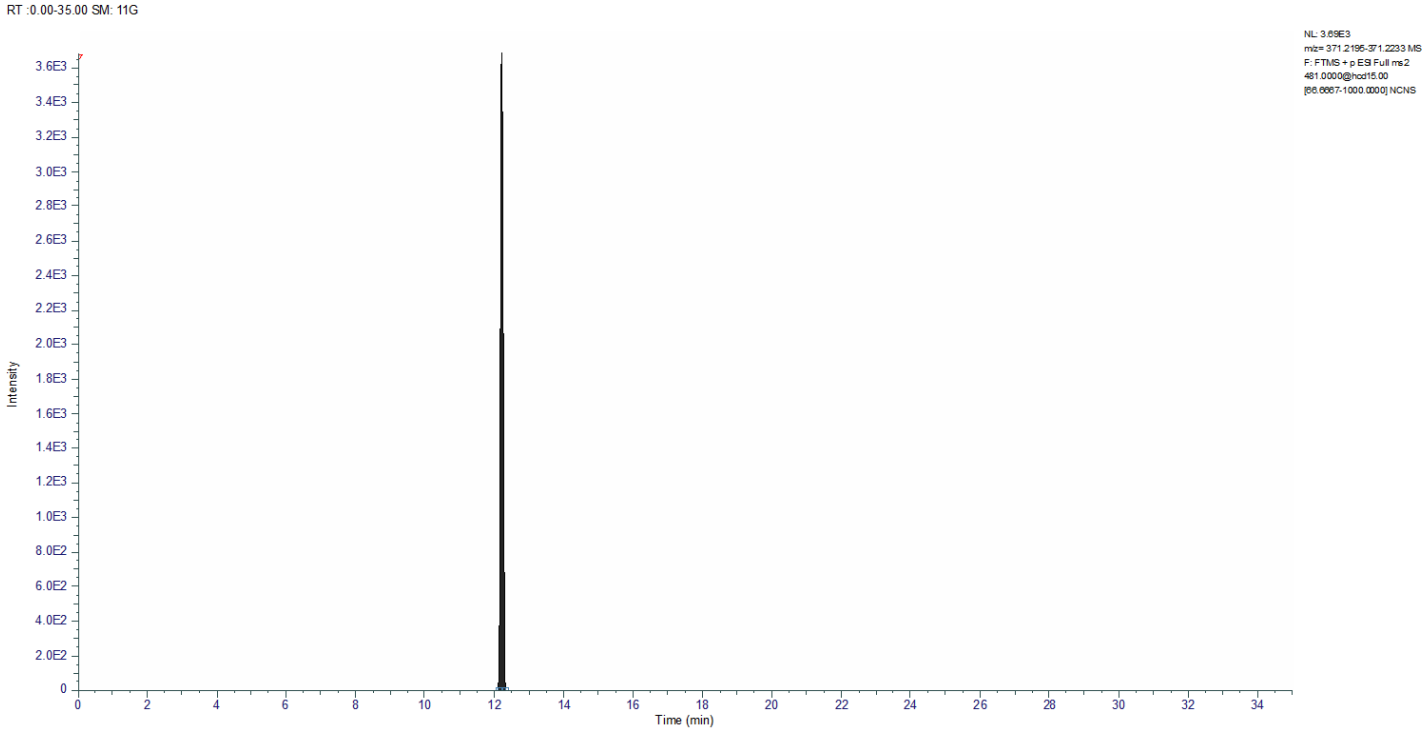
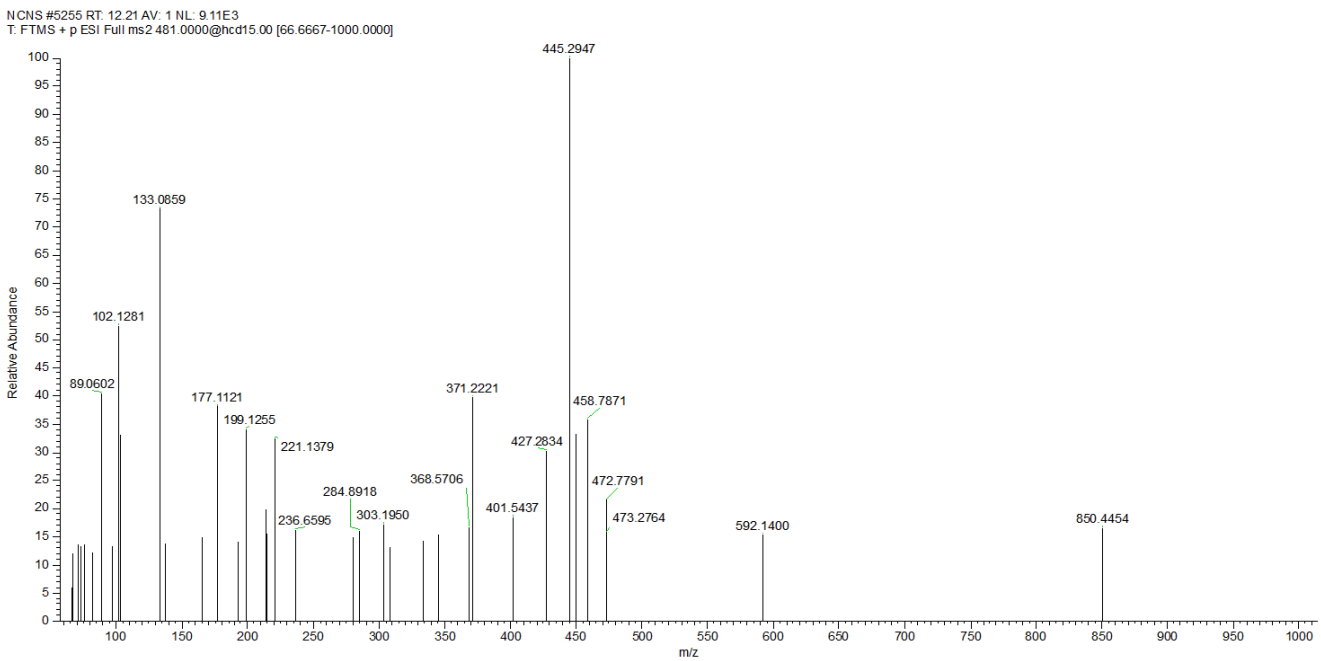
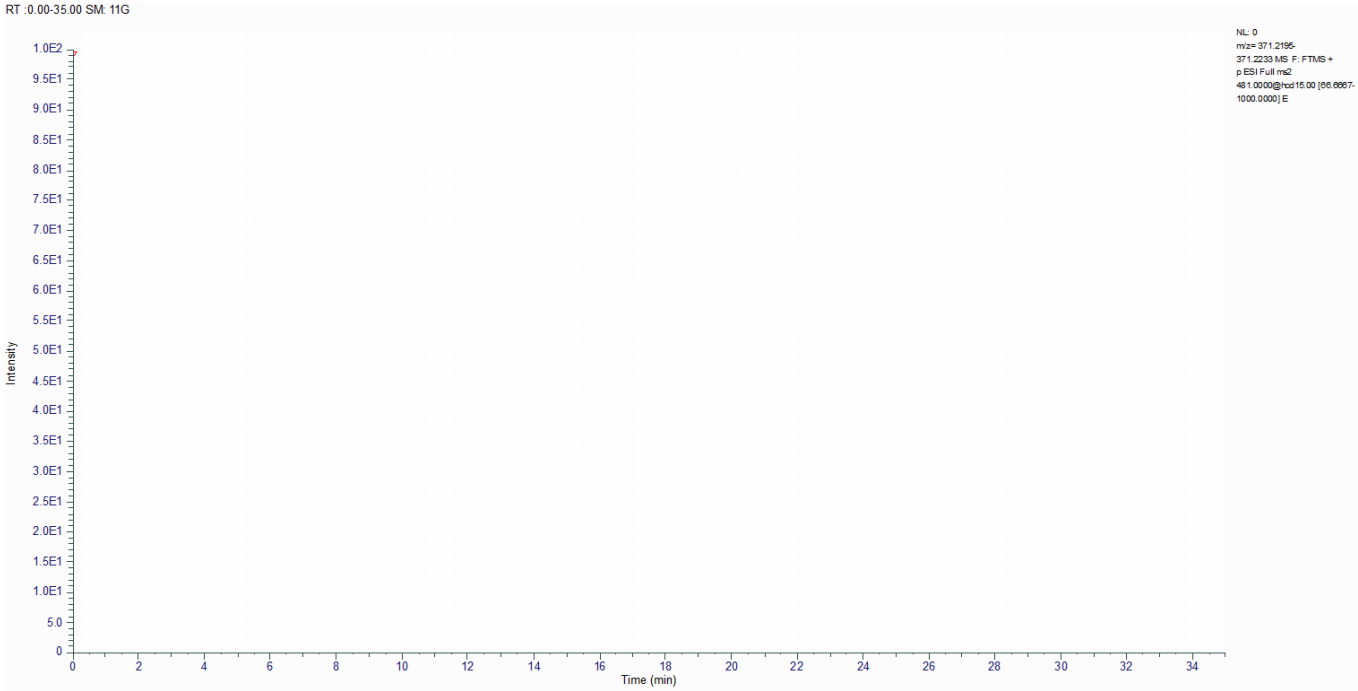
Relative Abundance
m/z

Central nervous system

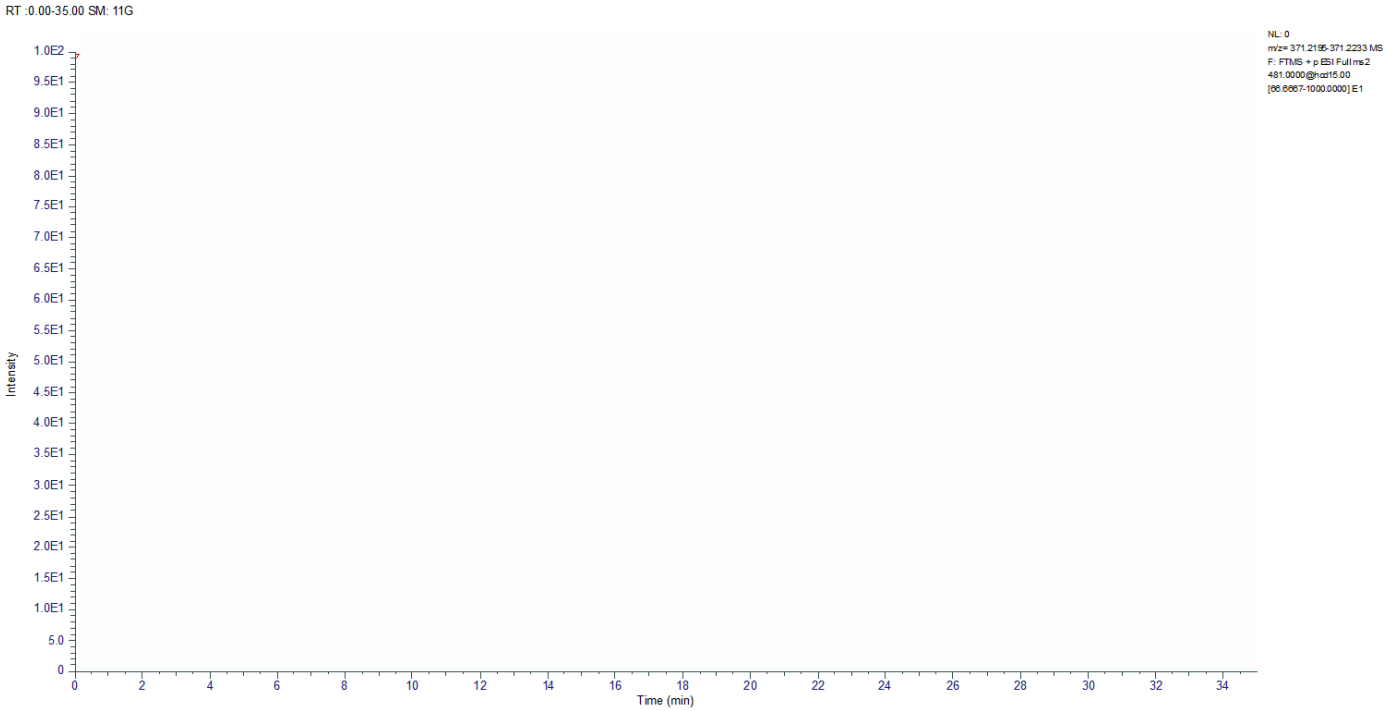




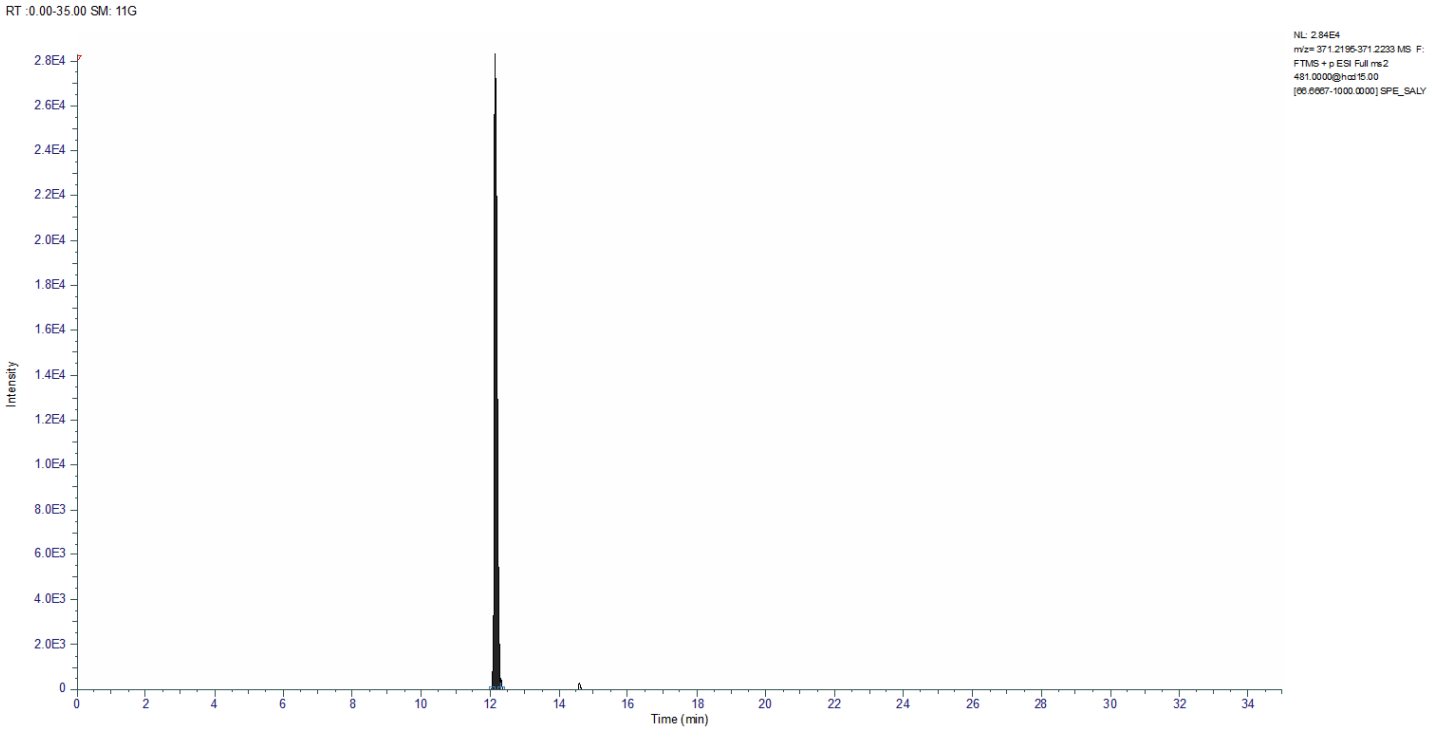
Embryo (early stage)



Embryo (hatching stage)



Lettuce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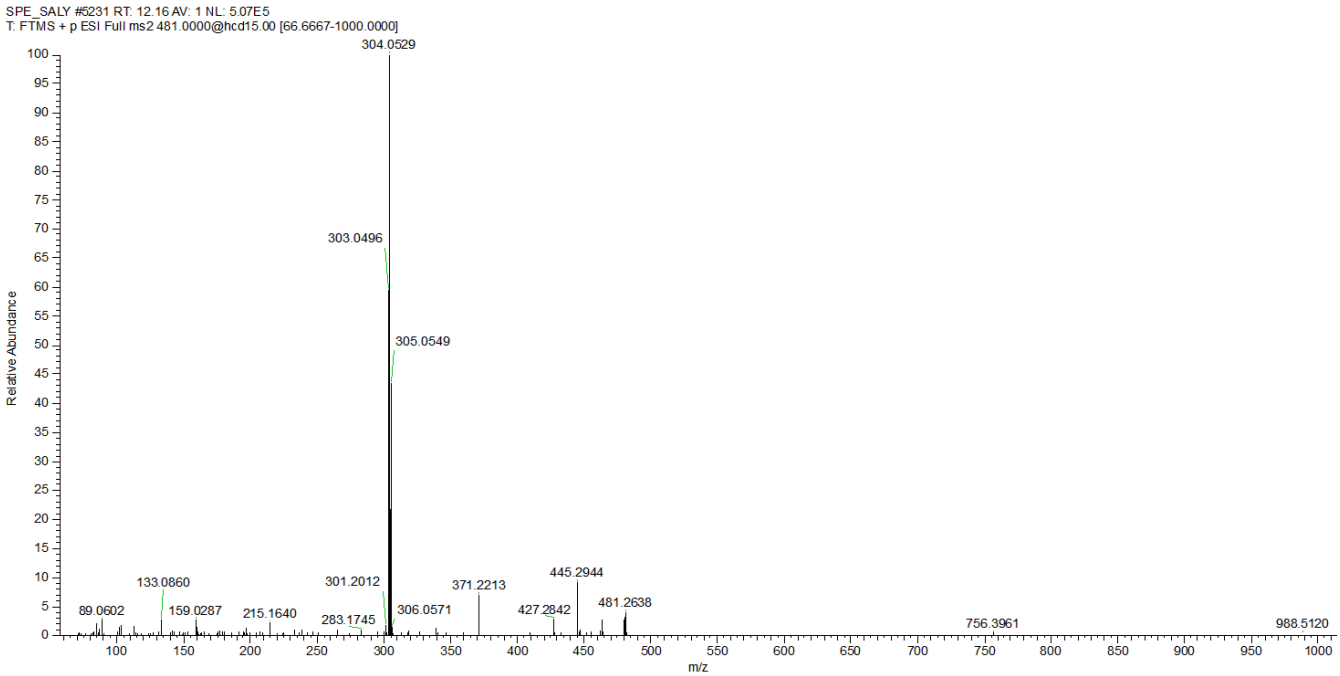
Intensity
Time (min)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Relative Abundance
m/z
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305.0549
133.0860
301.2012
371.2213
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481.2638
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159.0287
215.1640
283.1745
306.0571
427.2842
756.3961
988.5120

Supplementary Figure S3. Phylogenetic analysis was performed using proteins that were grouped into the CLANS of disembodied and shadow proteins. The analysis included proteins from 14 taxa. The midpoint-rooted phylogenetic tree was constructed using the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT) and ultrafast bootstrap with 1000 replicates. The LG+F+R10 model was implemented. Each taxon is presented by a color. Lymnaea stagnalis proteins begin with the code "CAL15". The disembodied and shadow proteins of Drosophila melanogaster are highlighted in yellow. The scale bar indicates amino acid substitutions per site.

Supplementary Figure 1a: The phylogenetic cladogram was generated using publicly available data from ortholog-based phylogenomics. The analysis included protein-coding sequences from 1,000 orthologous Metazoan genes. The phylogenetic tree was constructed using the Maximum Likelihood method using the IQ-TREE software. The tree is rooted using the Ctenophora lineage. The cladogram is drawn with proportional branch lengths. The scale bar represents 0.1 substitutions per site. The phylogenetic tree was constructed using the Maximum Likelihood method using the IQ-TREE software. The tree is rooted using the Ctenophora lineage. The cladogram is drawn with proportional branch lengths. The scale bar represents 0.1 substitutions per site. Cnidaria, Chordata, Ambulacraria, Ecdysozoa, Lophotrochozoa.