1.0 INTRODUCTION – INSECT LIPIDOMICS

Once upon a time the life was born. It is associated with lipids that represent an important class of metabolites essential for every known living entity. There exist a lot of biochemical pathways which are unable to take place without the lipid molecular species. The large-scale study of pathways and networks of cellular lipids in biological systems is called lipidomics. The word "lipidome" is used to describe the complete lipid profile within a cell, tissue or organism. Lipidomics is an integral part of metabolomic and its role in organism is described in Figure 1.

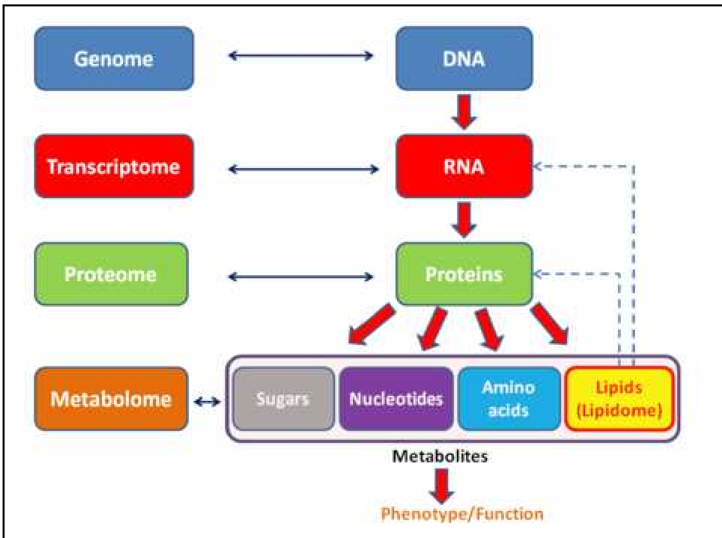

Figure 1: General Wikipedia scheme shows the relationships of the lipidome to the genome, transcriptome, proteome and metabolome. Lipids also regulate protein function and gene transcription.

Han and Gross (2003) first defined the field of lipidomics:

” Lipidomics is focused on identifying alterations in lipid metabolism and lipid-mediated signalling processes that regulate cellular homeostasis during health and disease. Research in lipidomics incorporates multiple techniques to quantify the precise chemical constituents in a cell’s lipidome, identify their cellular organization (subcellular membrane compartments and domains), delineate the biochemical mechanisms through which lipids interact with each other and with the crucial membrane-associated proteins, determine lipid-lipid and lipid-protein conformational space and dynamics, and quantify alterations in lipid constituents after cellular perturbations. Through the detailed quantification of a cell’s lipidome (e.g., lipid classes, subclasses, and individual molecular species), the kinetics of lipid metabolism, and the interactions of lipids with cellular proteins, lipidomics has already provided new insights into health and disease.”

Many modern technologies have been developed to identify, quantify, and understand the structure and function of key metabolic nodes in lipidomics. Mass spectrometric (MS) techniques occupy a leading position in the characterization, identification and quantification of lipids. Two approaches have been used. The first involves a “global” cellular lipidom analysis. The second is focused on a target lipid class of interest. The methodology based on

LC coupled with MS plays an essential role in this area through different enrichment technologies (Han, 2009).

The lipidomic techniques have been extensively used to analyse metabolite pathways and networks associated with lipid metabolism, fluxes and homeostasis, in particular in human and animal samples. Insects represent another useful experimental model in biological research and, thus, insect metabolism has been an attractive research field. Furthermore, many aspects of lipid metabolism in insects remain unclear. This study was dedicated to develop and apply novel LC/MS/MS methodology to investigate some open questions of lipid metabolism in insects.

2.0 GOALS OF THIS STUDY

- To develop proper analytical methodology for characterization of lipid molecular species by LC/MS/MS. This approach involves experimental steps covering lipid extraction from various insect materials, separation, ionization, detection and data interpretation.
- Analysis of phospholipid components occurring in insect cell membranes during overwintering and cold hardening experiments.
- Determination of neutral lipid components from insect haemolymph and tissue during hormonal treatment experiments.

3.0 INSECT LIPID COMPOSITION

Lipids are a large group of heterogeneous compounds characterized by their solubility in solvents of low polarity. Usually are divided to functional lipids (lipids for storage and liberation of metabolic energy) and structural lipids (Tab. 1). Some of lipid types are functional and also structural lipids, for example sterols give birth to important hormones (steroids) and are also essential building stones for parts of cell membrane called rafts.

<table>
<tr><th>Lipid class</th><th>Lipid species</th><th colspan="2">Lipids</th></tr>
<tr><td>Neutral lipids</td><td>MG, DG, TG</td><td rowspan="3">Functional</td><td rowspan="2"></td></tr>
<tr><td>Fatty acid</td><td>Free fatty acids, fatty acid amides, prostanoids</td></tr>
<tr><td>Sterols</td><td>Isoprenoids, cholesterol, steroids, sterols, bile acid</td><td rowspan="3">Structural</td></tr>
<tr><td>Glycerophospholipids</td><td>PC, PE, PG, PS, PI, PA, cardiolipins, Lyso PL, plasmalogens and other ether-linked phospholipids</td><td rowspan="2"></td></tr>
<tr><td>Sphingolipids/
Glycosphingolipids</td><td>Sphingomyelin, glycosphingolipids, ceramides, sphingosine phosphate</td></tr>
</table>

Table 1: Diversity of lipids according the lipidomic core (Murphy et al, 2006) and function.

3.1 FUNCTIONAL LIPIDS

Functional lipids or lipids for storage and liberation of metabolic energy are mostly acylglycerols. Most abundant species in total lipid extract of insect tissues are TGs member of acylglycerol group (Downer, 1978; Canavoso et al, 2001).

3.1.1 Acylglycerols

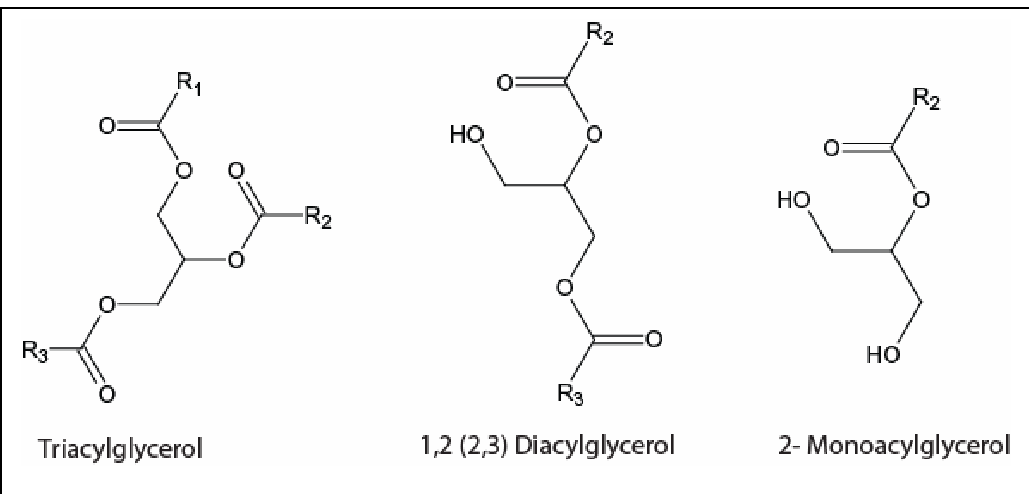

Figure 2: Main acylglycerol representatives, R – fatty acid acyl residuum.

Chemically, acylglycerols consist of the glycerol polar headgroup bound to one, two or three fatty acids designated as mono -, di- and tri- acylglycerols, respectively (Fig.2).

Saturated fatty acids				**Monoenoic fatty acids**		
ethanoic	acetic	2:0		*cis*-9-hexadecenoic	palmitoleic	16:1(n-7)
butanoic	butyric	4:0		*cis*-6-octadecenoic	petroselinic	18:1(n-12)
hexanoic	caproic	6:0		*cis*-9-octadecenoic	oleic	18:1(n-9)
octanoic	caprylic	8:0		*cis*-11-octadecenoic	*cis*-vaccenic	18:1(n-7)
decanoic	capric	10:0		*cis*-13-docosenoic	erucic	22:1(n-9)
dodecanoic	lauric	12:0		*cis*-15-tetracosenoic	nervonic	24:1(n-9)
tetradecanoic	myristic	14:0				
hexadecanoic	palmitic	16:0				
octadecanoic	stearic	18:0				
eicosanoic	arachidic	20:0				
docosanoic	behenic	22:0				
Polyunsaturated fatty acids						
9,12-octadecadienoic	linoleic	18:2(n-6)				
6,9,12-octadecatrienoic	γ-linolenic	18:3(n-6)				
9,12,15-octadecatrienoic	α-linolenic	18:3(n-3)				
5,8,11,14-eicosatetraenoic	arachidonic	20:4(n-6)				
5,8,11,14,17-eicosapentaenoic	EPA	20:5(n-3)				
4,7,10,13,16,19-docosahexaenoic	DHA	22:6(n-3)				

Table 2: Principal fatty acid occurring in insect.

The most of the potential energy available from acylglycerols is contained within the fatty acid component of molecule (Tab. 2). The chemical nature of FAs provides a wide range of combinations of fatty acid structures. Twenty three fatty acids were reported in a single species in 1963. Nowadays, the numbers of described FAs has increased, but only 8 fatty acids represent the major proportion of all FAs in insects. Saturated FAs are myristic acid (C 14:0), palmitic acid (C 16:0), and stearic acid (18:0). Monounsaturated fatty acids are primarily myristoleic (C 14:1), palmitoleic (C 16:1) and oleic acid (18:1) and the polysaturated fatty acids – linoleic acid (C 18:2) and linolenic (C 18:3) (Downer, 1985).

Most insects have to receive polysaturated fatty acid in their diet. The dietary demands differ substantially between species, but many studies proved that either linoleic or linolenic acid adequately satisfy this nutritional need. Many developmental and reproduction deformations are exhibited after non essential fatty acid feeding (Downer, 1978; Canavoso et al, 2001).

TGs serve as a reserve of metabolic energy stored in fat body therefore is not surprising that TGs are the most abundant lipid species in total lipid extract of insect tissues. Fat body is an analogous organ to mammals' liver and adipose tissue. TGs have several advantages with comparison to other source of energy, glycogen, for example a higher caloric content per unit weight, more metabolic water and stored TGs are not so bulky in anhydrous form. These properties determine TGs to be a source of energy for insect which undergo

prolonged periods of metabolic activity without feeding like diapause or migratory flight and also during non-feeding stages like embryogenesis, pupation. On the other hand insects with short burst of metabolic activity demands are primarily carbohydrates users (Downer, 1985).

Diacylglycerols are the most abundant acylglycerols in haemolymph.. DGs are the transport form of acyglycerols and are not present free in haemolymph. DGs are carried by lipoproteins from fat body, where they are released from storage TGs by lipases to fulfil energetical demands, for example in flight muscle (Beenakkers et al., 1984).

3.1.2 Sterols

Sterols are another lipid class essential in insect living with several crucial functions: a fundamental component in subcellular membranes, a precursor of the molting and vitellogenic hormone ecdysone and a constituent of surface wax of insect cuticle and lipoprotein carrier molecules (Downer, 1978; Canavoso et al, 2001).

All insects require sterol in their diets. This is a result of inability of insect tissues to synthesize squalene by reductive dimerization of farnesyl pyrophosphate (Fig. 3).

Cholesterol usually earns in food of predators or blood sucking insects. Among phytophagous insects, phytosterols are often an adequate and, in some cases, better substitute for cholesterol in the diet. These insects have a metabolic capacity for conversion of phytosterols to cholesterol. Cholesterol function as a structure building stone is described in acclimation study chapter 5.1 (Downer, 1978).

Figure 3: Scheme shows synthesis of squalene.

3.2 STRUCTURAL LIPIDS

Phospholipids are lipids containing glycerol phosphate. Their primary function is to serve like building blocks of the most membrans. Diacylglycerol backbone is esterified by the phospho-group in *sn-3*-glycerol position and long chain fatty acids at position *sn-1* and *2* – positions are connected via ester bond (Fig. 4). The diversity of PLs is dependent on a diversity of the long chain fatty acids occurring on a phospholipid headgroup (Figure 5). Fatty acids in the phospholipid structures which form biological membranes of insects are shown in Table 2 (refer to chapter 3.1.1 and Dowhan and Bogdanov, 2002).

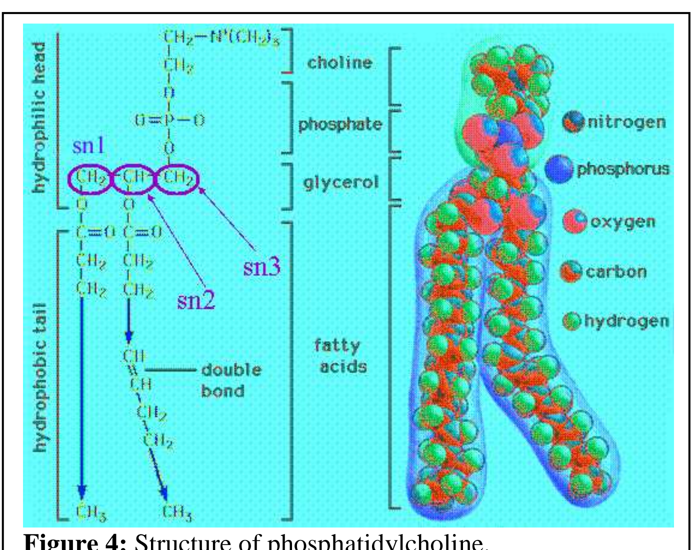

Figure 4: Structure of phosphatidylcholine.

Figure 5: Chemical structures of main phosphoglycerols occurred in insect cells.

Phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) are the most abundant phospholipids classes through insects which represent more than 70% of total lipid components (Downer, 1985). The proportion of PEs and PCs and also of particular FAs is species specific and also dependent on the physiological state of insect (overwintering, diapause etc.). For further details, please, refer to the Chapter 5.1.

Other phosphoglycerolipid classes are further important components of biological membrane and their presence and abundance is organism dependent (Fig. 5). Except PC and PE, also PS, PI, PG, CL and Chol (Tab 3.) were detected (Gennis, 1989; Dowhan and Bogdanov, 2002).

Origin of membrane	Lipid / protein proportion	Percentage proportion of majority lipids
Human myelin	3-4	PC 10% PE 20% PS 8,5% SM 8,5% GS 26% Chol 27%
Bovine intervertebrate disc	1	PC 41% PE 39% PS 13% Chol – trace
Human erythrocyte	0,75	PC 25% PE 22% PS 10% SM 18% Chol 25%
Rectal gland of dogfish		PC 50,4% PE 35,5% PS 8,4% PI 0,5% SM 5,7% Chol – trace
Receptor membrane of torpedo	0,7-0,5	PC 24% PE 23% PS 9,6% Chol 40%
Sarcoplasmatic reticulum of rabbit	0,66-0,7	PC 66% PE 12,6% PI 8,1% Chol 10%
Inner membrane of *E.coli*	0,4	PE 74% PG 19% CL 3%

Table 3: Percentual proportion of major membrane lipids originated from different samples (Genis, 1989).

Cardiolipins are found almost exclusively in eukaryotic mitochondria and in bacteria that utilize oxidative phosphorylation for proton pumping across the membrane. CLs are protonated in physiological pH and this property makes them a proton silk or a conduit for proton in transfer processes. The CLs are not absolutely essential metabolites because PGs

seems to be a substitute in many processes. However, the lack of CLs results in reduction of the cell growth dependent on oxidative processes (Dowhan and Bogdanov, 2002).

Cell membrane poses special type of domains called rafts, which are rich in cholesterol, glycosphingolipids (gangliosides), sphingomyelin and proteins (for more details see Chapter 5.1.2.2).

Glycosphingolipids are classified into broad types on the basis of carbohydrate composition (Fig. 6).

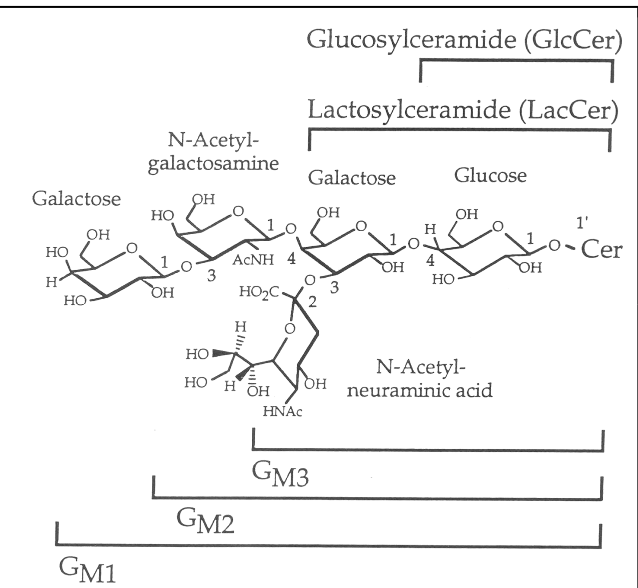

Figure 6: Examples of the carbohydrate components of neutral and acidic glycosphingolipids (Cer – revers to the ceramide backbone) (Merčilo Jr. and Sweeley1996).

The other part of glycosphingolipid molecule is made by sphingolipids and more than 300 different types have been reported.

Sphingolipids are composed from a fatty acid moiety bound to sphingosine base. They are important in number of cellular processes and are involved in essentially all aspects of cellular regulation. Sphingolipids (SLs) serve as ligands for receptors and mediate change in cell behaviour in response to cells environment. SLs are also involved in membrane trafficking for example influence receptor internalization, sorting and recycling (Munoz-Garcia et al, 2006; Merril Jr. and Sandhoff, 2002; Merrill Jr. and Sweeley, 1996).

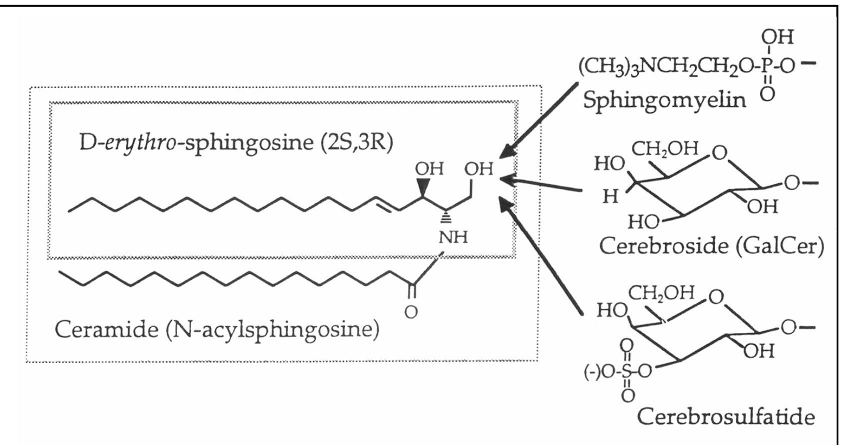

Figure 7: Structures of sphingosine, ceramide, sphingomyelin, cerebroside (galactosylceramide) and cebrosulfatide from human brain (Merril Jr. and Sweeley, 1996).

Sphingomyelin is also a member of this large family (Fig. 7).

4.0 METHODS USED FOR INSECT LIPID ANALYSIS

4.1 EXTRACTION OF INSECT LIPIDS

Lipid extraction has been extensively studied in the fifties of the previous century. Due to the occurrence of long chain alkyls in the lipid structures extraction with non-polar solvents or their mixtures was preferred for lipid enrichment. Lipids share a large proportion in every organism and are concentrated mostly by classical extraction methods described by Folch (1956) or Blight-Dyer (chloroform and methanol). With the advent of the sophisticated LC/MS instrumentation many authors re-examined the lipid extraction methodologies. Honeycut et al (1995) tested three extraction methods for fish tissue. Hexane, acetone, dichloromethane and Blight-Dyer (chloroform and methanol) were tested like extraction solutions. The Blight-Dyer method generally gave higher percent lipid values, yielding significantly higher results for the 1g sample size (Honeycut et al, 1995).

The lipid extraction of insect samples was described in detail by Kostal et al (2003). After the chloroform-methanol-water extraction step the product is dried by nitrogen and kept in – 20°C or lower temperature to prevent lipid oxidation. Samples are solved in 500 µl of chloroform, evaporate, proper dilute in methanol and thus prepared for further LC/MS and GC analysis. Chloroform is used as a primary and storage solution for lipid samples to prevent oxidation of polysaturated fatty acid, which can occurred with other polar solvents contains oxygen.

The *in situ* extraction - transesterification is another approach frequently used in lipid analysis. It involves simultaneous lipid hydrolysis and esterification steps resulting in the formation of fatty acid methyl esters suitable for gas chromatographic analysis with a FID or MS detector (Lewis et al, 2000; Carrapiso and Garcia, 2000).

4.2 SEPARATION TECHNIQUES (HPLC)

A wealth of methods has been developed for separation of lipid classes. According to literature, SPE (solid phase extraction), TLC (thin layer chromatography), HPLC (high performed liquid chromatography) and GC (gas chromatography) have been the most frequent approaches. SPE or TLC have been still applied in practice (Marcato et al, 1996; Silversand et al, 1997; Quin et al, 2000; Adachi et al, 2004; Neron et al, 2004; Persson et al, 2007; Cvačka et al, 2008).

However, HPLC coupled to MS detector is at present the most favourite technique for targeted and non-targeted lipidomics (See Attachment 1). Other types of detectors which are commonly used together with HPLC separation involve UV and ELSD (evaporative light scattering) detection (Patton et al, 1990; McHowad et al, 1996, 1997; Olsson et al, 1996).

Normal phase and reversed phase HPLC separation have been still popular in lipid analysis. The mobile phases usually consist of methanol, 2-propanol or n-hexane. Chromatographic columns with C18 phases still prevail. A few separations were reported on C8 columns. A particular attention is focused on separation of PL and NL lipid classes; only a limited number of studies have been dedicated to separation of lipid molecular species. The analysis time ranges from 20 to 50 minutes for class analyses. The latter approach requires prolonged time to 130 - 260 minutes. For more details, refer to the Attachment 1 which summarizes analytical conditions reported for HPLC of lipids.

4.3 SEPARATION OF INSECT LIPIDS

Two HPLC methods, which differ in application range, mobile phase composition and analysis time, have been developed. The first method was used for separation of phospholipid

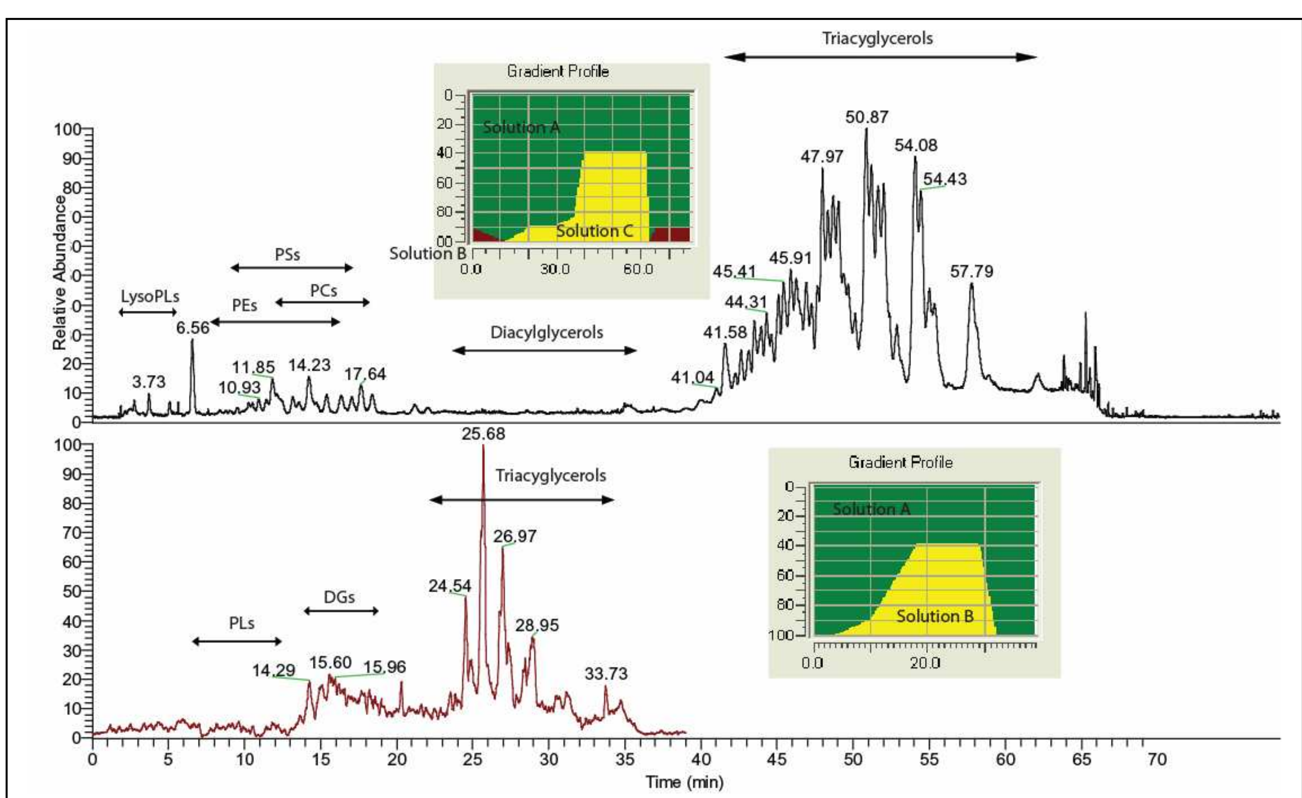

Figure 8: Typical HPLC chromatograms of lipids obtained by the developed HPLC/MS methods. The upper trace: HPLC/MS methodology developed for PL analysis (sample, a whole body extract of *Drosophila melanogaster*). The lower trace: HPLC/MS method for the determination of nonpolar lipids (fat body of *Pyrrhocoris apterus*). Particular mobile phases are described in text below.

molecular species and their separation from nonpolar lipid classes. The latter approach was developed for HPLC/MS analysis of DG and TG molecular species (Figure8).

4.3.1 HPLC separation of phospholipids

Final solvent composition was determined: Solution A – 500 ml of 5mM $AcONH_4$ in MeOH mixed with 5 ml of 25% NH_4OH, B – water, C – 2-propanol mixed with methanol in ratio 80:20. A gradient elution was performed on Gemini 250 x 2.00 mm column at 150 µl/min. Although the method analysis time was 80 minutes, satisfactory separation of phospholipid classes was accomplished enabling in conjunction with MS characterization of particular PL molecular species.

PLs with saturated alkyl chains are eluted later than unsaturated acyl homologues (Lee et al, 2007). Separation of PL from TGs is exemplified in Fig. 9, where TIC chromatogram of the *Drosophila* body extract (upper trace) and extracted mass chromatogram of mass m/z 714.4 are depicted. The data indicate the presence of PE C34:3 and TG C41:6 at RT = 9.49 min, 10.14 min and 44.96 min, respectively. In addition, two peaks of isomeric PE were observed with the same m/z value in a different retention time. Determination based on the MS3 analysis in positive mode or MS2 in negative mode reveals that for 714.3 m/z value first peak is responsible molecule PE 16:1/18:2 and for the second PE 16:0/18:3.

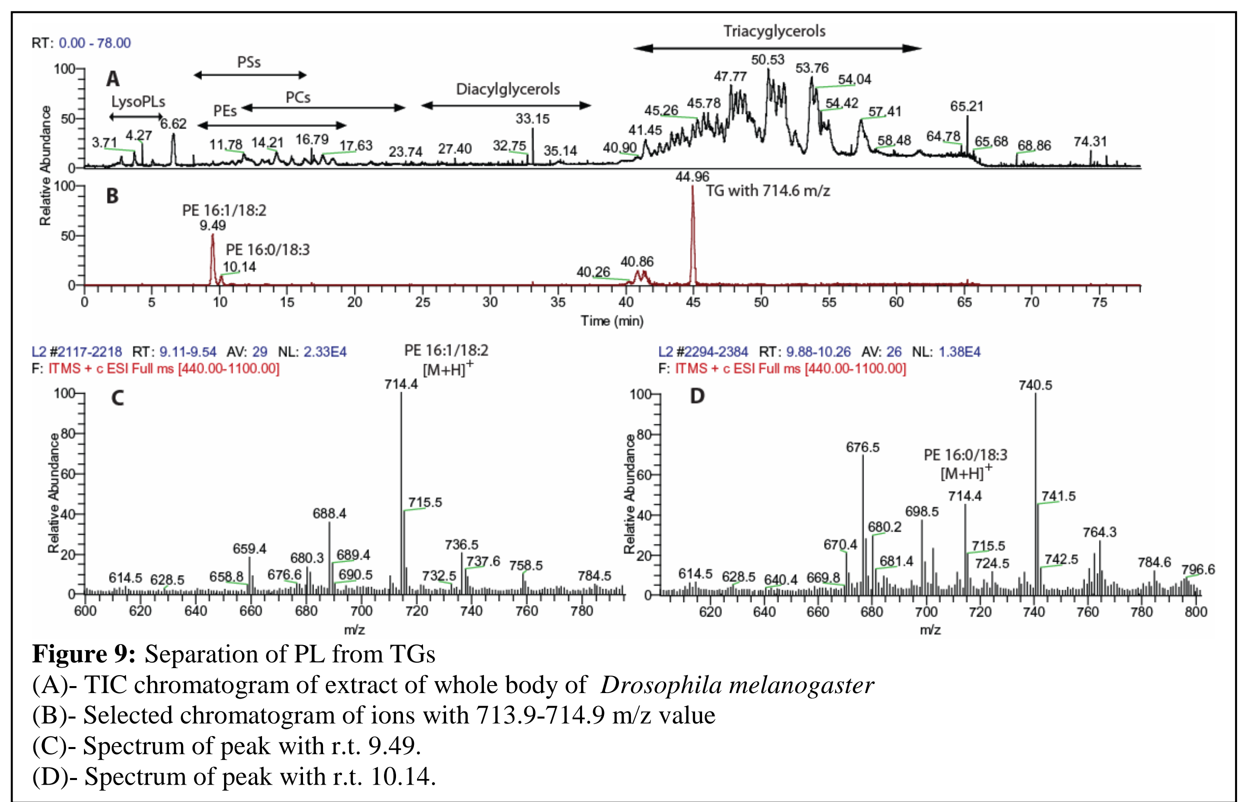

Figure 9: Separation of PL from TGs
(A)- TIC chromatogram of extract of whole body of *Drosophila melanogaster*
(B)- Selected chromatogram of ions with 713.9-714.9 m/z value
(C)- Spectrum of peak with r.t. 9.49.
(D)- Spectrum of peak with r.t. 10.14.

The very similar situation is observable on Fig.10. The supporting information is also obtained by chromatography of synthetic standards. Single standard chromatography followed by chromatography of PC standard mixture to ensure the retention time, responses to extracted PCs with 786.4 m/z values. First of peaks belongs to PC 18:1/18:1 and the second one is PC 18:0/18:2. MS investigation proved the same identification also for PCs from *Drosophila* sample.

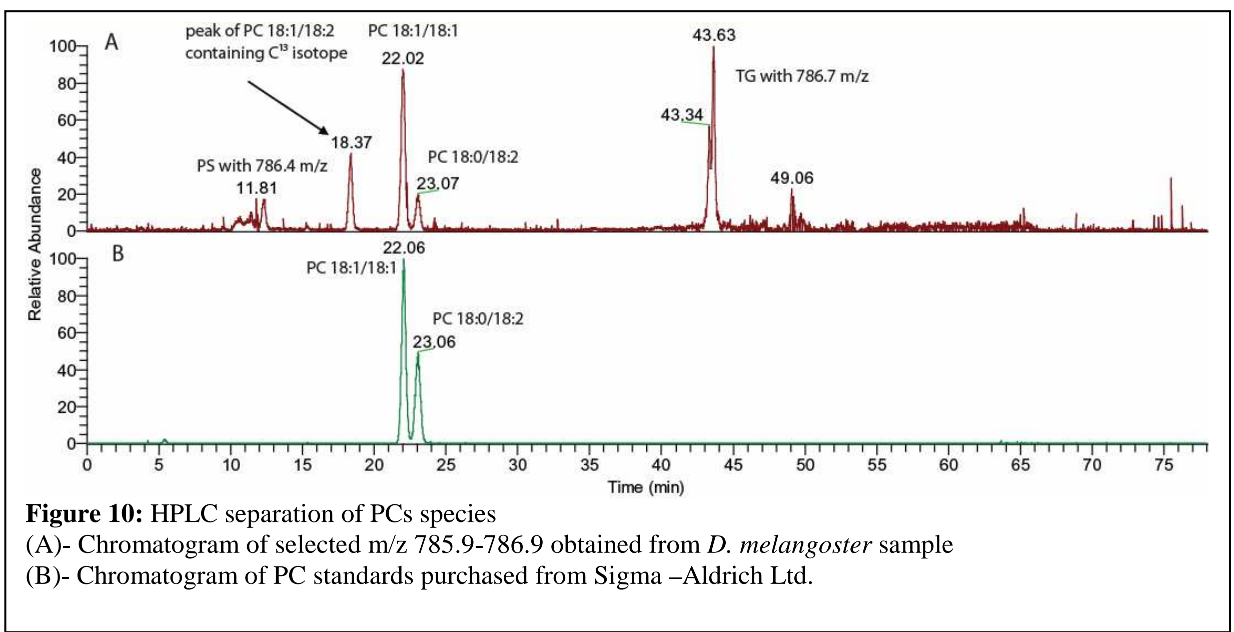

Figure 10: HPLC separation of PCs species
(A)- Chromatogram of selected m/z 785.9-786.9 obtained from *D. melangoster* sample
(B)- Chromatogram of PC standards purchased from Sigma –Aldrich Ltd.

This HPLC method provides also information about DG and TG molecules, but for its investigation presented method is unnecessarily long.

4.3.2 HPLC separation of nonpolar lipids

The mobile phase developed for DG and TG investigation consists of two solutions: A – 500 ml of 5mM $AcONH_4$ methanolic solution mixed with 5 ml of 25% NH_4OH, B –2-propanole mixed with methanol in ratio 80:20. No presence of water provides faster separation of nonpolar lipids. The flow rate, column, column temperature are the same like presented earlier. Time necessary for this analysis was 39 minutes which enabled analysis of principal DGs and TGs detected in insects, Fig. 11.

By using efficient separation and ESI mass spectrometry it was possible to identify lipid isomers. Thus, lipid analysis of haemolymph samples revealed the occurrence of two

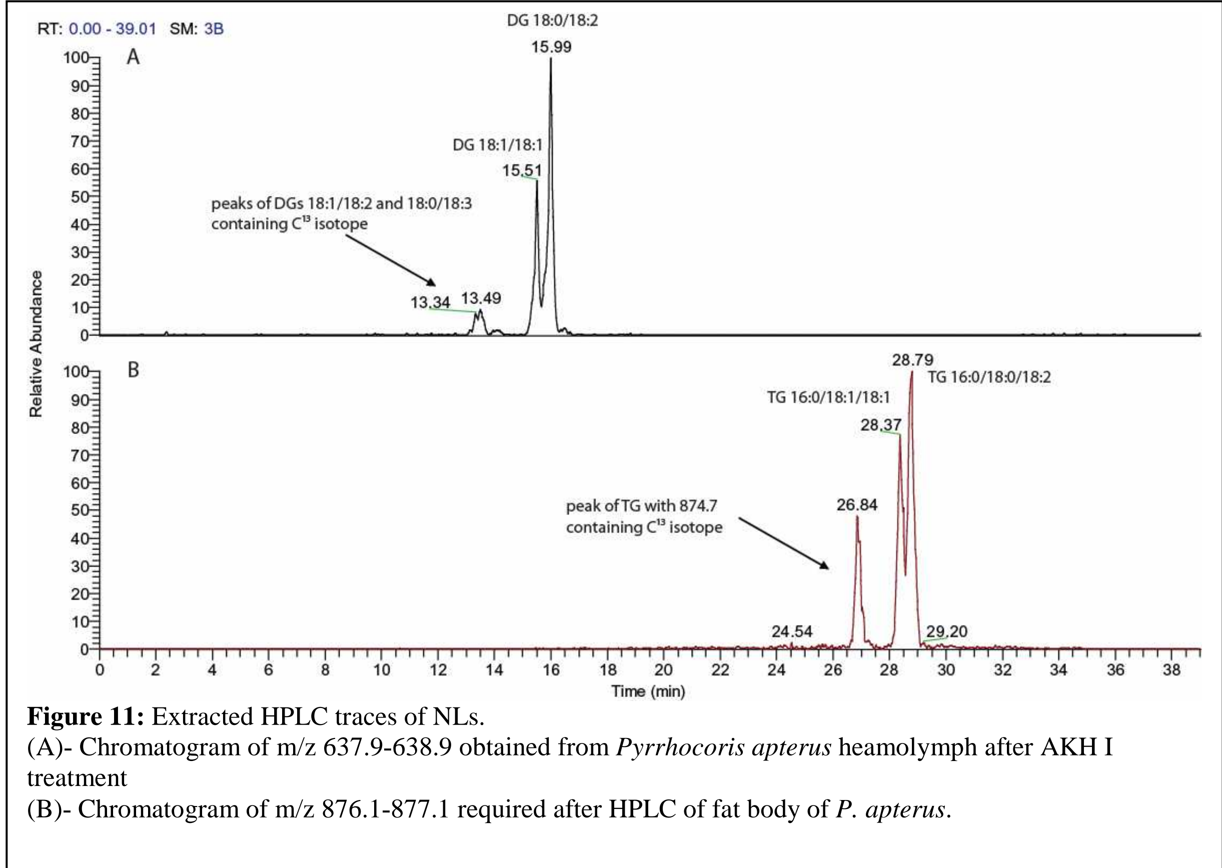

Figure 11: Extracted HPLC traces of NLs.
(A)- Chromatogram of m/z 637.9-638.9 obtained from *Pyrrhocoris apterus* heamolymph after AKH I treatment
(B)- Chromatogram of m/z 876.1-877.1 required after HPLC of fat body of *P. apterus*.

peaks at the mass m/z 638.3, Fig. 11A. First peak agrees with DG 18:1/18:1 molecule and the second with DG 18:0/18:2 according mass spectrometry. Similarly, two peaks in the extracted chromatogram at m/z 876.5 in Fig. 11B, which represents HPLC/MS analysis of fat body extract correspond with TG 16:0/18:1/18:1 and TG 16:0/18:0/18:2 molecular species. The separation data are close the trends presented in literature (Lee, 2007).

4.4 MASS SPECTROMETRY OF INSECT LIPIDS

Van der Klift et al (2008) compared three detectors (UV, ELSD and MS) in HPLC analysis of lipids. UV detection gave the best chromatographic performance but performed poorly in overall detectability and baseline stability. ELSD detector led to severe losses in chromatographic resolution and also suffered from differences in response factor between TGs. The MS detector showed the best overall performance and had the added benefit of structural information (Van der Klift et al, 2008). Also other authors declare that methodology using LC coupled with MS plays an essential role in lipidomics through different enrichment technologies (Han, 2009). Further data related to lipid mass spectrometry including ionization techniques are available in the Attachment 2.

This work was focused on the the following principal lipid classes of TGs, DGs, PCs, PEs, PS, Lyso PCs and LysoPEs which are dominant in insect samples and cover more then 95% of all insect lipids (experimental data). Every data were recorded by LCQ or LTQ spectrometrs (Thermo Finnigen), both acquiring spectra with ESI ionization and linear ion trap analyser.

4.4.1 Ionization of Insect Lipids

Each lipid class and even particular molecular species of lipids exhibits different MS ionization efficiency (Kim et al, 1994; Brugger et al, 1997; Koivusalo et al, 2001). When the individual lipid components are ionized together their ionization process is further affected by each other and effects of ion suppression or ion enhancement may distort true signal intensity of particular lipid components in sample extracts (Han and Gross, 2005). In practice, absolute calibration of the MS detector is difficult and data processing on relative scale is a preferred approach.

APCI is definitely the best ionization tool for NLs (refer also to the Attachment 2). For thermally labile PLs is not so fine. ESI being highly sensitive, accurate and reproducible does not cause extensive fragmentation (Forrester et al 2004). Furthermore, ESI is capable to ionize efficiently nearly all lipids except highly nonpolar sterols, their conjugates, waxes and hydrocarbons. In combination with separation efficiency of HPLC matrix effects are minimized and, thus, HPLC/ESI-MS is an efficient methodology for insect lipid analysis.

4.4.2 HPLC ESI-MS lipidomic analysis of insect samples

Similarly to other animal samples, a raw lipid extract of the whole body of *D. melanogasteris* represents a very complex mixture of components. Using metabolite Mapper platform, a home-built software platform developed at the Department of Analytical Biochemistry, Biology Centre of Academy of Sciences of the Czech Republic for automated computer peak detection and data compound processing, almost 2000 of component entities were found. The individual lipid component obtained by HPLC/MS analysis is characterized by three descriptors, i.e. by the retention time, peak area or height and its ESI mass spectrum of both positive and negative ions. A library of the full scans, MS^2 and MS^3 spectra of the available lipid standards and those amenable from the HPLC/MS analysis of insect extracts was created in the course of the thesis. The extracts of *Drosophila melanogaster*, *Pyrhocorris apterus, Locusta migratoria* served as a source of insect lipids which were collected.

4.4.2.1. Adduct ions for characterization of insect lipids

Protonated or deprotonated molecular ions usually complete with the formation of adduct ions with alkali metals like sodium or potassium present or added in the HPLC mobile phase (Kerwin et al, 1994; Kim et al, 1994; Brugger et al, 1997; Koivusalo et al, 2001; Hsu et al, 2003). The presence of the adduct ions is useful indicator for the analyte molecular weight determination. For example, Na^+ adducts are nearly always present in positive ESI mass spectra of the major phospholipid species (PC, PE) (Fig. 12) (Brugger et al, 1997).

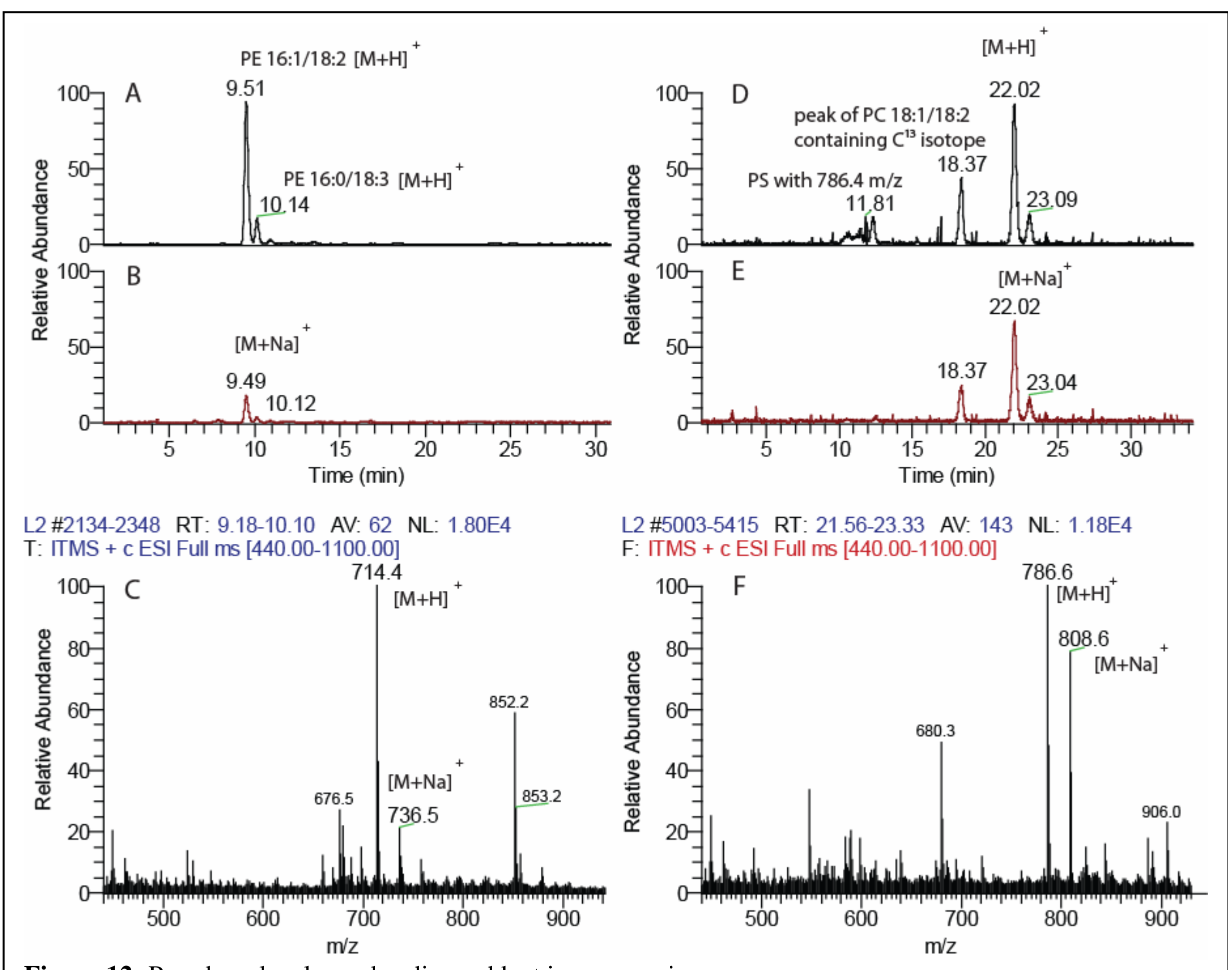

Figure 12: Pseudomolecular and sodium adduct ion comparison
Top: LC/MS TIC chromatograms with the extracted mass chromatograms of the masses m/z 714.4 and 786.4 (A,D) and their sodium adducts (B, E).
Bottom: Raw ESI full scan mass spectra of PE 16:1/18:2 (C) and PC 18:1/18:2 (F) with the respective among $[M+H]^+$ and $[M+Na]^+$ adducts. The sodium adducts are more abundant in choline containing PC than in less basic PE.

4.4.2.2 Positive versus negative ion spectra of insect lipids

In addition to adduct ion formation, the positive and negative ESI spectra are very useful for the identification of insect lipids Nonpolar TGs and DGs appear as ammonium adducts $[M+NH_4]^+$, PEs occur in the $[M+H]^+$ and in $[M-H]^-$ forms, respectively (Fig. 14). The same situation is observed in the PS case (data not shown). PC give $[M+H]^+$ as the principal positive ions, while the $[M+CH_3COO]^-$ adducts dominate in the negative ESI spectra (Fig. 14), if acetate anion is present in the HPLC mobile phase.

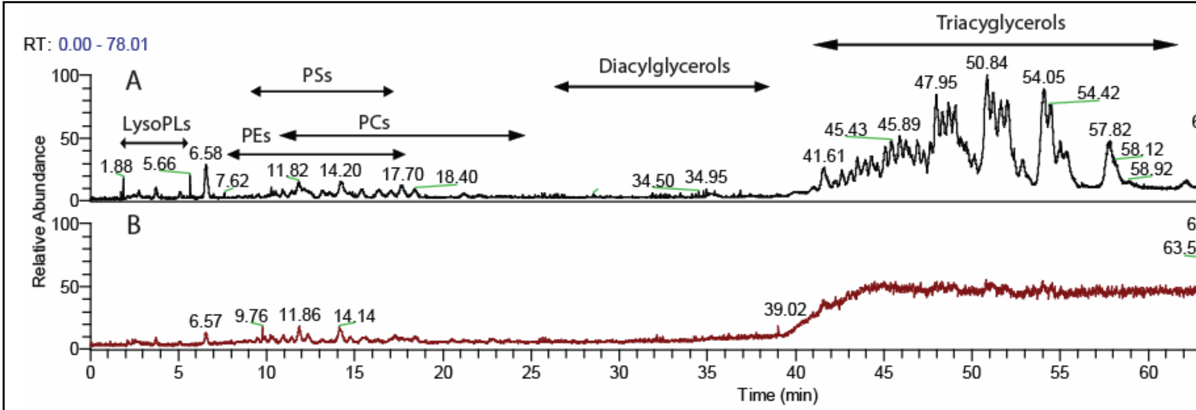

Figure 13: The behaviour of nonpolar acylglyceroles in the positive (A) and negative (B) ionization mode. The DGs and TGs are observed as $[M+NH_4]^+$ adduct ions. Any signal of DGs and TGs is absent in the negative ionization mode. For further detaile refer to Fig .14.

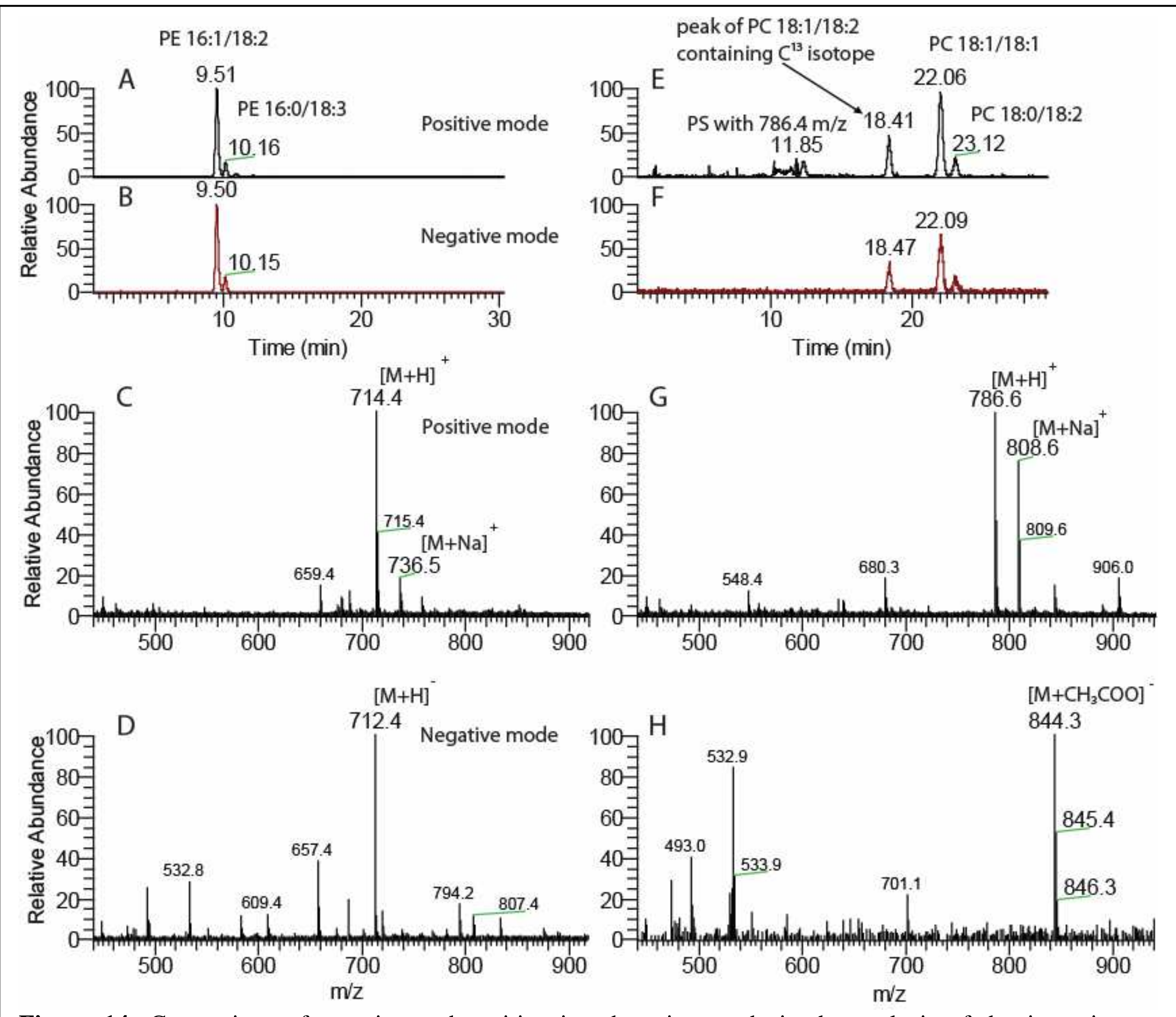

Figure 14: Comparison of negative and positive ion detection mode in the analysis of dominant insect phospholipids.
(A,E)- HPLC ESI MS chromatograms operate in positive mode with the extracted masses m/z 714.4 $[M+H]^+$ and 786.4 $[M+H]^+$.
(B,F)- HPLC ESI MS chromatograms operate in negative mode with the extracted masses m/z 712.4 $[M+H]^-$ and 844.3 $[M+H+58]^-$.
Raw ESI full scan positive (C) and (G) mass spectra of PE 16:1/18:2 and PC 18:1/18:2, respectively. The differences are evident in the negative mode when PEs form dominant pseudomolecular anion m/z 712.4 (D). PCs exhibit much less acidity and, thus by the acetate adduct m/z 844.3 (H) is the principal ion..

4.4.2.3 ESI CID MS^2 fragmentation of triacylglyceroles

TGs do not posses any ionisable functional group and only adducts with sodium or ammonia are observed in their ESI spectra. In mobile phases containing ammonium ion species only ammonium adducts of TGs, $[M+NH_4]^+$ are observed in positive ESI, which enables the molecular weight of each TG molecular species to be determined. No abundant ions corresponding to $[M+H]^+$ or $[M+Na]^+$ are observed. Number of carbons and double bonds can be calculates from molecular weight of particular triacylglycerol. Collision induced decomposition (CID) of $[M+NH_4]^+$ ions results in the neutral loss of NH_3 (i.e. molecular ion $[M+H]^+$ is observed) and acyl side-chain (as a carboxylic acid $[M-RCOO]^+$) to generate diacyl product ion. This fragmentation is characteristic in all molecular species of TGs.

This feature can be exemplified on the ESI spectra of two isomeric TGs with the same m/z value, but a different retention time (Fig. 15). The ammonium adduct has m/z 876.5, which corresponds to the presence of 52 carbons and two double bounds (C 52:2). The CID MS^2 spectra (Fig. 15 E, G) show together with the precursor m/z 859.5 different diacyl product ions.

TG with retention time 28.4 min gives two major diacyl ions 577.3 and 603.4. First product ion indicates the loss of carboxylic acid 18:1, the second loss of palmitic acid. No other fragment ions are present which clearly indicates the TG structure derived from TG 16:0/18:1/18:1. The *sn* position of particular FA attached to glycerol core is hardly to examine by the MS method used. Additional information may bring silver-ion HPLC (Adlof, 2004).

A TG with retention time 28.77 min gives three diacyl ions 575.3, 579.3 and 603.4 which indicate the loss of C18:0 C18:2 and C16:0, respectively. The proposed TG structure is therefore related to the TG 16:0/18:0/18:2.

TGs ionized by APCI with linear ion trap show very similar features as documented in literature by Laakso et al (1997), McAnoy et al (2005) and Cvačka et al (2006).

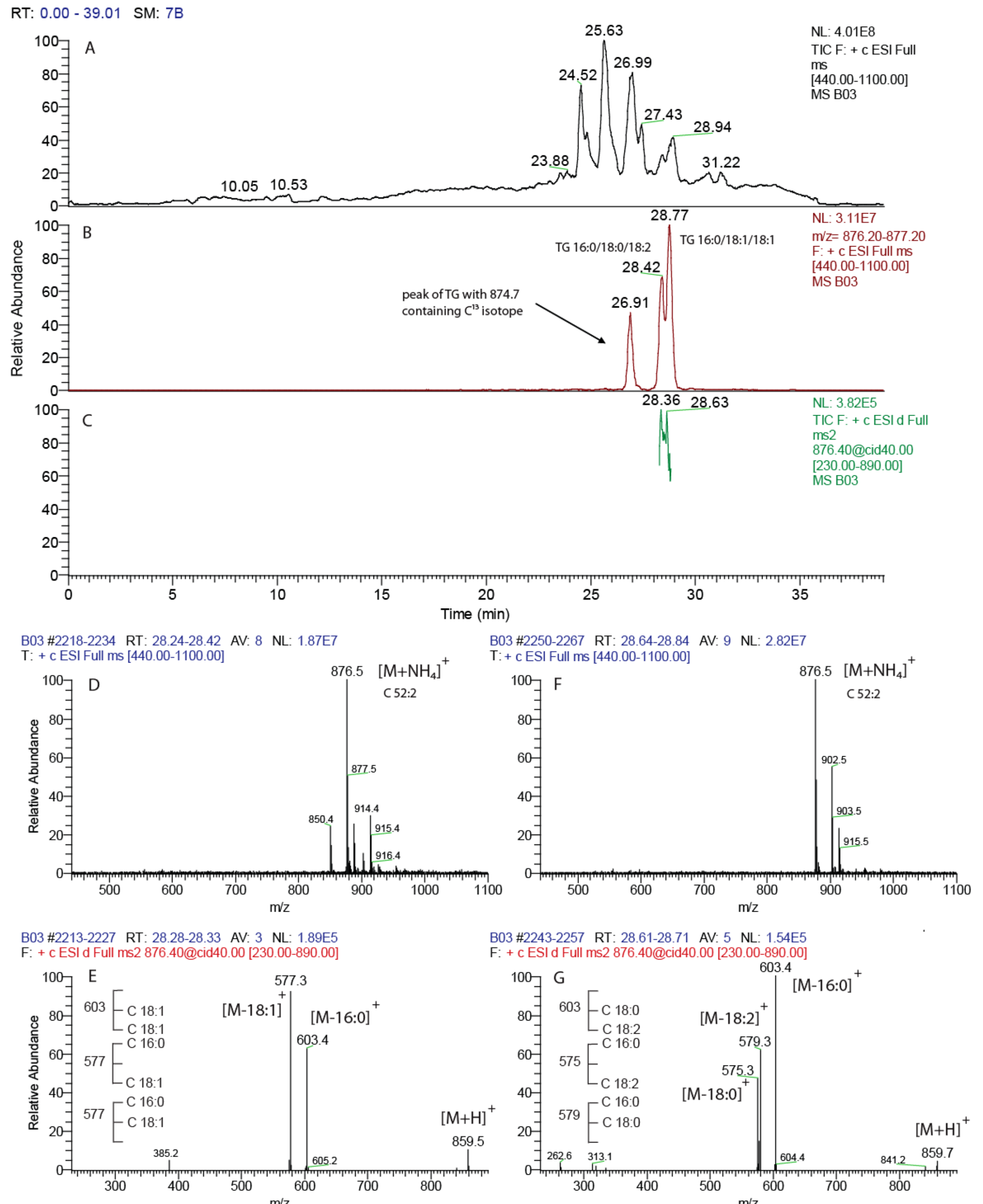

Figure 15: ESI mass spectrometry of triglycerides.
(A)- A TIC chromatogram of the extract from the *Pyrrhocoris apterus* fat body where TGs are dominant.
(B)- An extracted chromatogram of m/z 876.4 showing occurrence 3 iosomeric components in the sample.
(C)- A TIC chromatogram window showing the signal, where CID MS^2 scans of the precursor m/z 876.4 were performed.
(D) and (F)- The full scans ESI spectra of the TG peaks in rt. 28.4 min and 28.77 min with a dominant ammonium adduct. No other adducts or pseudomolecular ions were observed.
(E)– CID MS^2 spectrum of the precursor ion m/z 876.4 in rt. 28.4 minute shows characteristic fragmentation of ammonium adduct and provides pseudomolecular ion $[M+H]^+$and two diacyl product ions allowing identification of the present acyls.
(G)- CID MS^2 spectrum of the TG with m/z 876.4 in 28.7 min., showing characteristic fragmentation of the ammonium adduct ion and providing pseudomolecular ion $[M+H]^+$and three diagnostic diacyl product ions (Attachment 3).

4.4.2.4 ESI CID MS^2 fragmentation of diacylglycerols

The mass spectra of DGs exhibit similar features to TGs and provide $[M+NH_4]^+$ adduct ions and the diagnostic fragment ions arising from the loss of the acyls present in their structures. The spectra enable estimation a number of carbons and double bonds in the structure. In addition to ammonia loss, a consecutive hydroxyl loss as a neutral water species is observed. The features are documented in Fig.16 where two DGs with m/z 638.4 were depicted. Inspection of the CID MS^2 spectra revealed two isomeric DGs, namely DG 18:1/18:1 and DG 18:0/18:2 at 15.7 min and 16.2 min, respectively. The diagnostic fragment ions in the CID ESI MS^2 spectra represent masses m/ 337.0 (loss of C18:0) and m/z 341.1 (loss of C18:2) and are in accord with literature (H. Mu, 2000).

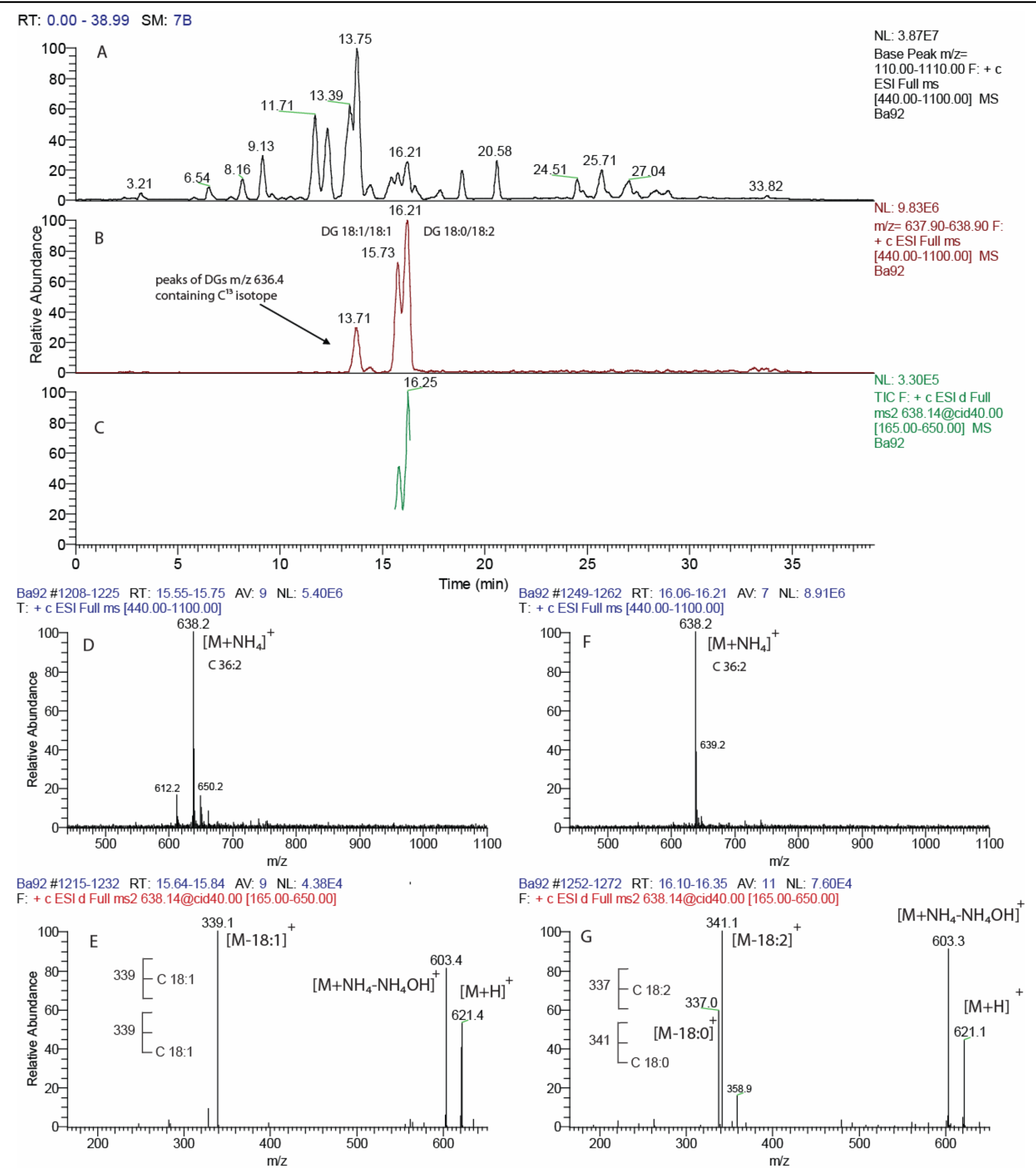

Figure 16: ESI mass spectrometry of diglycerides.
(A)- A TIC chromatogram of the lipid extract from the *Pyrrhocoris apterus* haemolymph, where DGs are dominant.
(B)- An extracted chromatogram of m/z 638.3 indicates two isomeric components in the sample
(C)- A TIC chromatogram window showing the signal where CID MS2 scans of the 638.3 precursor were performed.
(D) and (F)- The full scans ESI spectra of the DG peaks with rt.15.73 min and 16.21 min having a dominant ammonium adduct..
(E)- CID MS2 spectrum of the precursor ion m/z 638.3 in rt.15.73 min shows characteristic fragmentation of the ammonium adduct providing a pseudomolecular ion $[M+H]^+$and fragments allowing identification of the present acyls.
(G)- CID MS2 spectrum of the TG with m/z 638.3 in rt.16.21min., showing two diagnostic acyl product ions (Attachment 4).

4.4.2.5 ESI CID MS^n fragmentation of lysophosphatidylcholines

LysoPC ESI spectra typically show $[M+H]^+$ together with Na^+ adducts $[M+23]^+$ like PCs having the positive charge site on the quartery nitrogen. The CID ESI MS^2 (Fig. 17) spectra contain $[M-18]^+$ typical for water loss. The intensive m/z 184 fragment is derived from the choline headgroup. The diagnostic fragments enable to deduce the lyso-PC identity from its molecular weight. For the confirmation of the acyl residue a CID MS^3 experiment is useful. The loss of $[M-18-N(CH_3)_3]^+$ is accomponied by the acylium C18:1 m/z 265.2 ion confirming the lyso PC structure in accord with literature (Hsu, 2009 and Caprioli, 2008).

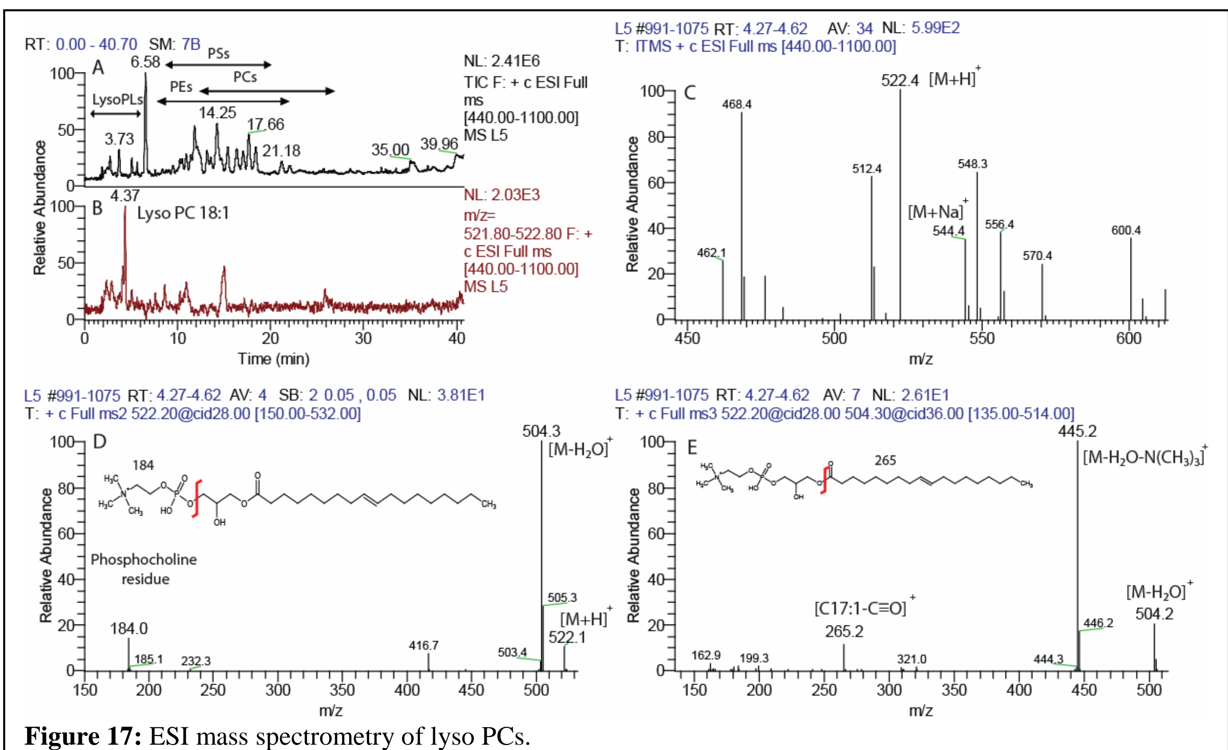

Figure 17: ESI mass spectrometry of lyso PCs.
(A) and (B) A TIC and extracted ESI mass chromatogram of the lipid extract from the Drosophila melanogaster body. The m/z 522.4 trace revealed a lyso-PC peak in RT 4.37 min.
(C)- A background substracted ESI full scan spectrum of the peak in 4.37 min.
(D) CID MS^2 spectrum of the precursor m/z 522.3
(E) CID MS^3 spectrum of the dominant dehydration m/z 504 product ion showing the acylium ion of C18:1

4.4.4.6 ESI CID MS^n fragmentation of lysophosphatidylethanolamines

Lyso-PE contain pseudomolecular ion $[M+H]^+$ and sodium adduct $[M+Na]^+$ as dominant ions in their ESI spectra. Similarly to lyso-PC, the fragment ions arising from the water loss and $[M+H-141]^+$ arising from the loss of phosphatidyethanolamine moiety are diagnostic in their CID MS^2 spectra. The characteristic acyl fragment ions and loss of the ethanolamine moiety $[M-H_2O-(CH_2)_2NH_3]^+$ are obtained in the CID MS^3 spectra if an ion trap mass analyser is used for the MS^n experiments.

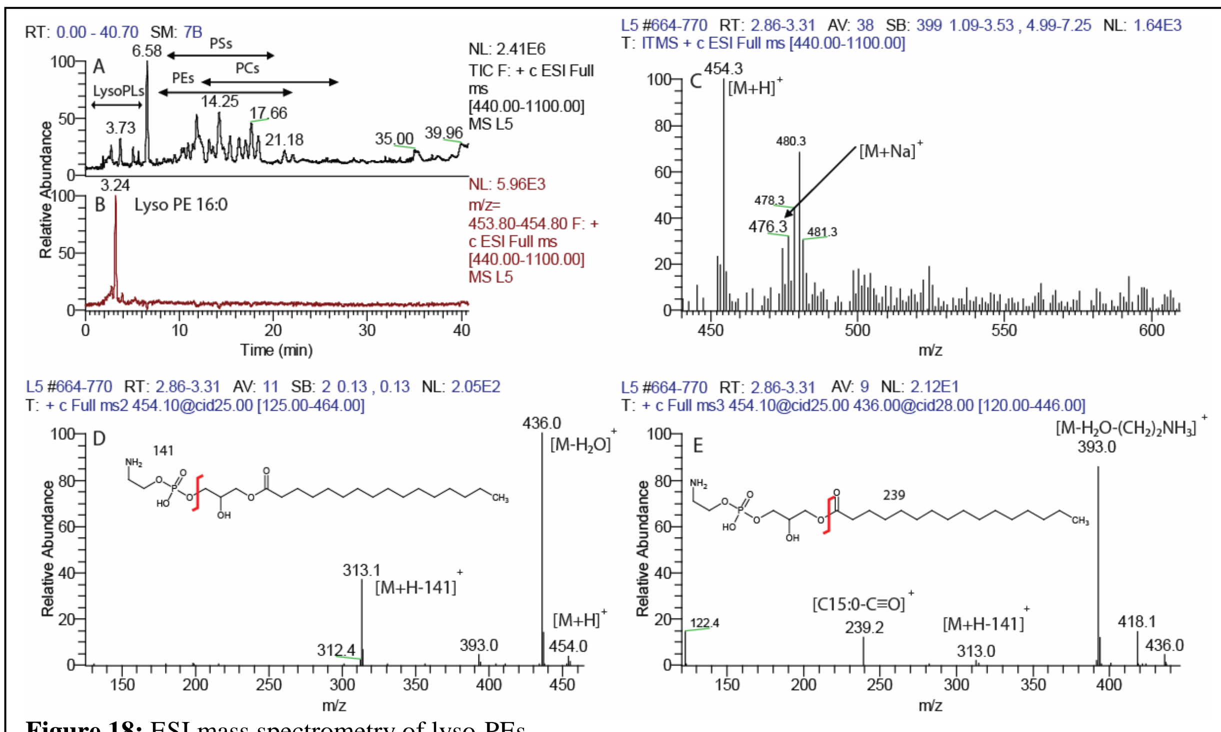

Figure 18: ESI mass spectrometry of lyso-PEs.
(A) and (B) A TIC and extracted ESI mass chromatogram of the lipid extract from the *Drosophila melanogaster* body. The m/z 454.3 trace revealed a lyso-PE peak in RT 3.24 min.
(C) A background substracted ESI full scan spectrum of the peak lyso-PE C16:0 in 3.24 min.
(D) CID MS^2 spectrum of the precursor ion m/z 454.3
(E) CID MS^3 spectrum of the dominant dehydration m/z 504 product ion in the MS2 spectrum (D), showing the acylium ion of C16:0.

Present example deals with m/z value 454.3 (Fig. 18). Presence of molecular ion and sodium adduct points to phospholipids group in molecule and MS^2 investigation prove expected water loose (436.0) and occurrence of ion m/z 313.1 represent neutral loose 141 which is characteristic for phosphatidylethanolamines. MS^3 experiment revealed acylium ion of FA 16:0 m/z 239.2, what proves the identification like LysoPE 16:0.

4.4.2.7 ESI CID MS^n fragmentation of phosphatidylethanolamines

Using the common mobile phase containing ammonium formate or acetate, PEs like lyso-PEs provide $[M+H]^+$ as the most intensive adduct ion which is commonly accompanied by the sodium adduct (Figure 19). Due to the presence of the phosphoethanolamine moiety, the $[M+H-141]^+$ loss is unique fragment ion for PEs (Hsu and Turk, 2009). The fragment ion $[M+H-141]^+$ decomposes to very important ion species $[M+H-141-R_1COOH]^+$ which is preferred at *sn-1* position, in comparison to the acylium ion ($[RC{\equiv}O]^+$ arising largely in position *sn-2*.

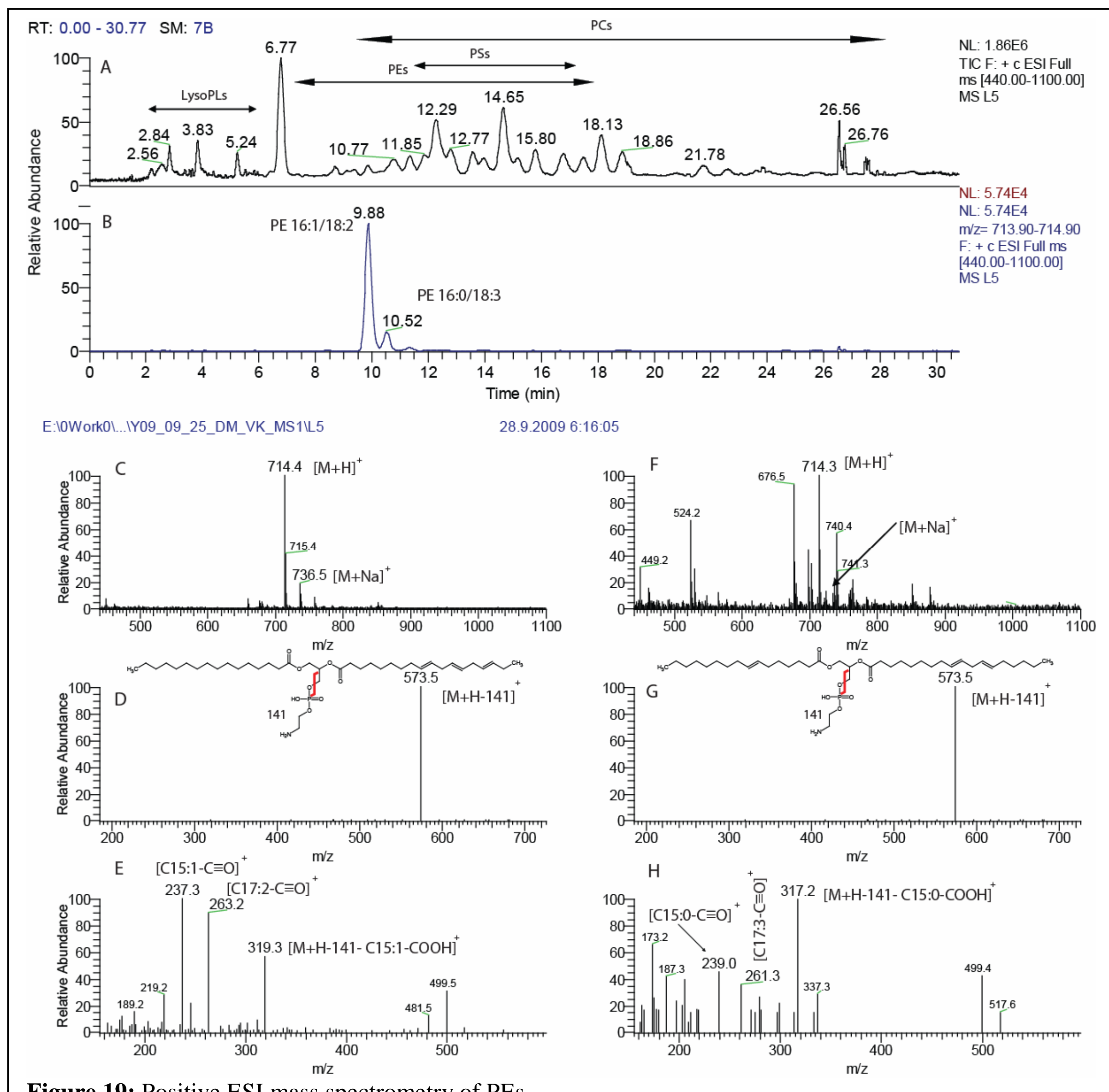

Figure 19: Positive ESI mass spectrometry of PEs.
(A) and (B)- A TIC and extracted ESI mass chromatogram of the lipid sample from the *Drosophila melanogaster*. The m/z 714.4 trace revealed two isomeric PE peaks in RT 9.88 min and 10.52 min.
(C) and (F)- A background subtracted ESI full scan spectra of the PE in RT 9.88 min and 10.52 min
(D) and (G) CID MS^2 spectra of the PE precursor ion $[M+H]^+$ m/z 714.4
(E) and (H) CID MS^3 spectra of the $[M+H-141]^+$ m/z 573.4 product ions dominating in the MS^2 PE spectra. PE in RT 9.88 min (E) shows acylium ions of 16:1 and 18:2 and ketene loss of C16:1, while PE in RT 10.52 min is characteristic by the acylium ions od C16:1 and C18:2 and ketene loss of C16:0. The PE structures can thus be assigned to the molecular species PE C16:1/C18:0 and PE C16:0/C18:3.

Although the positive ESI mass spectra are an efficient source of structural information about PEs, negative ion mass spectra are even more frequently used for their identification (Brouwers et al, 1999; Khaselev and Murphy, 1999; Kerwin et al, 1994).

The $[M-H]^-$ ions dominate in the PEs negative ESI spectra and yields carboxylate anions from fatty acyl substituents esterified on either *sn-1*or *sn-2* position during the CID MS^2 scan. The loss of neutral ketene from the *sn-2* position is the more abundant process in the ion trap analyser (Caprioli, 2008).

The approach to the determination of the PE structures by means of positive and negative ESI ion trap mass spectrometry is documented on the HPLC/MS elucidation of two PEs having m/z 714.4 in fat the body of *Drosophila*, Fig.19 and Fig.20.

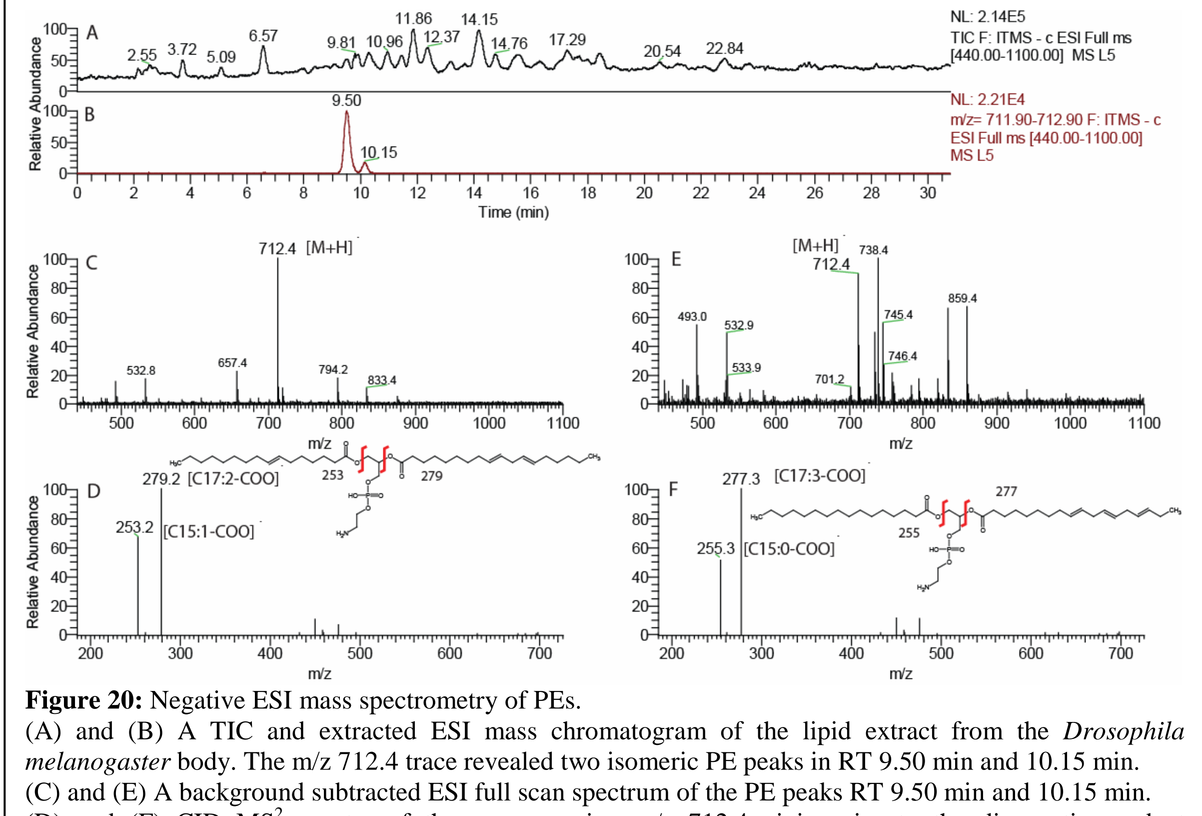

Figure 20: Negative ESI mass spectrometry of PEs.
(A) and (B) A TIC and extracted ESI mass chromatogram of the lipid extract from the *Drosophila melanogaster* body. The m/z 712.4 trace revealed two isomeric PE peaks in RT 9.50 min and 10.15 min.
(C) and (E) A background subtracted ESI full scan spectrum of the PE peaks RT 9.50 min and 10.15 min.
(D) and (F) CID MS^2 spectra of the precursor ion m/z 712.4 giving rise to the diagnostic product carboxylate anions m/z 253, 279 and m/z 255, 277, respectively .

The positive CID MS^2 spectra show the characteristic ethanolamine neutral loss $[MH-141]^+$, represented by the ion m/z 573.3 in both cases. The CID MS^3 scan experiment applied to the m/z 573.3 MS^2 product ion reveals two types of product fragments; the acylium ions corresponding PE C16:1/C18:2 and PE C16:0/C18:3, the peaks with rt. 9.50-9.88 min and 10.15-10.52 min, respectively (Fig. 19,20). The loss of ketene from *sn-1* position is presented by the ion m/z 319.4 , which belongs to the PE residue after the C16:1 cleavage, and by the ion m/z 317.4, arising from the C16:0 cleavage (Fig.19).

The carboxylate anions arising by the cleavage from the *sn-2* position are generally more abundant. Finally, the PE structures can be assigned as PE C16:1/C18:2 and PE C16:0/C18:3.

4.4.2.8 ESI CID MS^n fragmentation of phosphatidylcholines

Positive ESI spectra of PCs are characterized by the intensive positive pseudomolecular ion $[M+H]^+$ accompanied by the sodium adduct $[M+Na]^+$. CID MS^2 scans of the $[M+H]^+$ precursor lead to two cleavage types. The first, involves the loss of fatty acid constituent at *sn-1* and *sn-2* position $[M+H-RCOOH]^+$. The second cleavage type arises from the instability of α-hydrogen in *sn-2* position, resulting in the more favourable formation of the $[M+H-R2-CH=C=O]^+$ ketene ion. Consequently, the position of the fatty acyl moieties on the glycerol backbone can be assigned (Hsu and Turk, 2009).

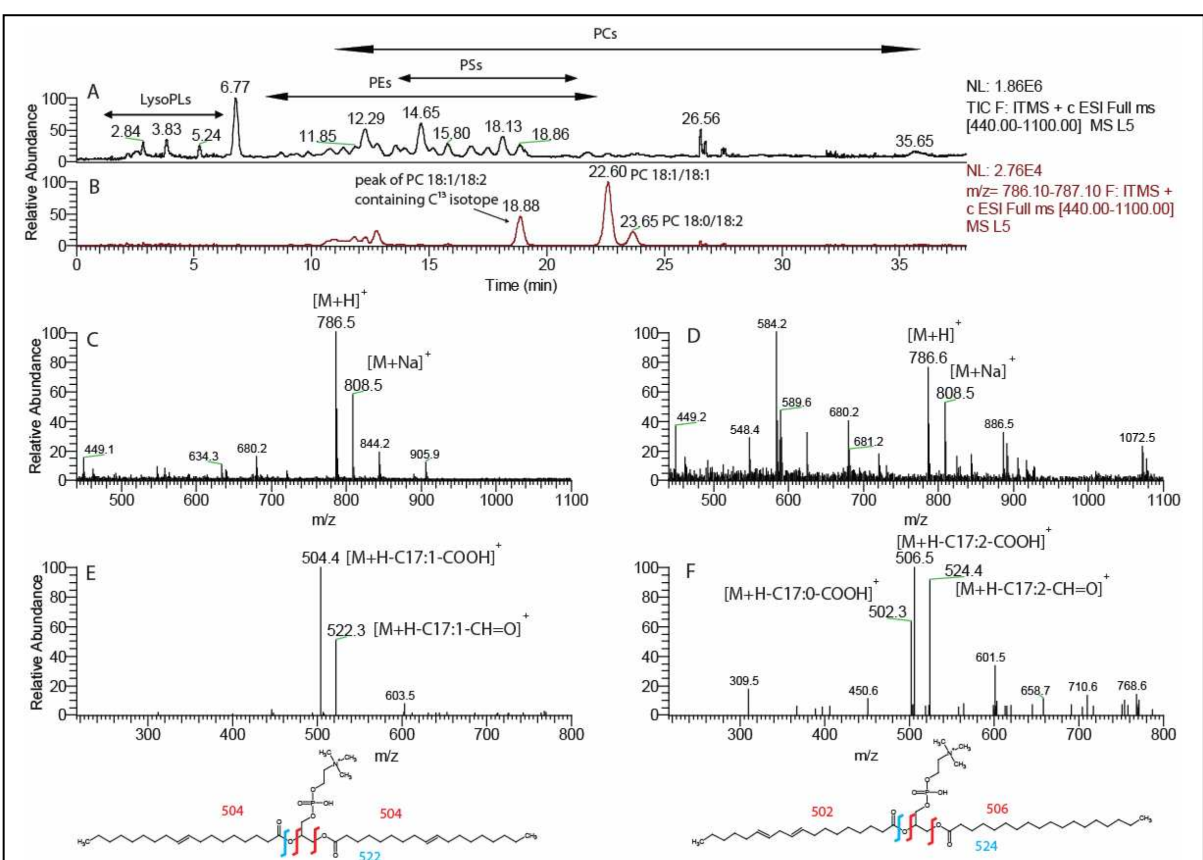

Figure 21: Positive ESI mass spectrometry of PCs.
(A) and (B)- A TIC (A) and extracted ESI mass chromatogram (B) of the lipid extract from the *Drosophila melanogaster* body. The m/z 786.4 trace revealed two isomeric PC peaks in RT 22.60 min and 23.65 min.
(C) and (D) A background subtracted ESI full scan spectra of the PC in RT 22.60 min and 23.65 min.
(E) CID MS^2 spectrum of the PC precursor ion $[M+H]^+$ m/z 786.4, RT 22.60 min shows acylium m/z 504.3 and ketene ion m/z 522.3 related to C18:1 FA residue.
(F) CID MS^2 spectrum of the PC precursor ion $[M+H]^+$ m/z 786.4, RT 23.65 min shows acylium ions m/z 502.3 and 506.3 and ketene ion m/z 524.3.

The structure elucidation, deduced from the ion trap positive ESI mass spectra is illustrated in Fig.21, where PC structural isomers having m/z 786.5 and designated as PC C18:1/C18:1 and PC C18:0 and C18:2 are compared. The ketene fragment ion with m/z 522 and 524 is highly indicative for the *sn-2* fatty acyl position.

Negative spectra of PC fragmentation for its structure determination requires CID MS^3 technique. It disables the possibility of single run analysis for PC species and structure determination, thus no data from negative mode ionization and further fragmentation are not shown.

4.4.2.9 ESI CID MS^n fragmentation of phosphatidylserines

Like other phospholipids, PSs yield abundant $[M+H]^+$. Positive CID MS^2 scans result in a characteristic loss of phosphoserine moiety ($[M+H-185]^+$), which is most abundant. The CID MS^3 spectrum contains acylium ions of the present acyls (Kerwin et al, 1994; Caprioli, 2008, Hsu and Turk, 2009).

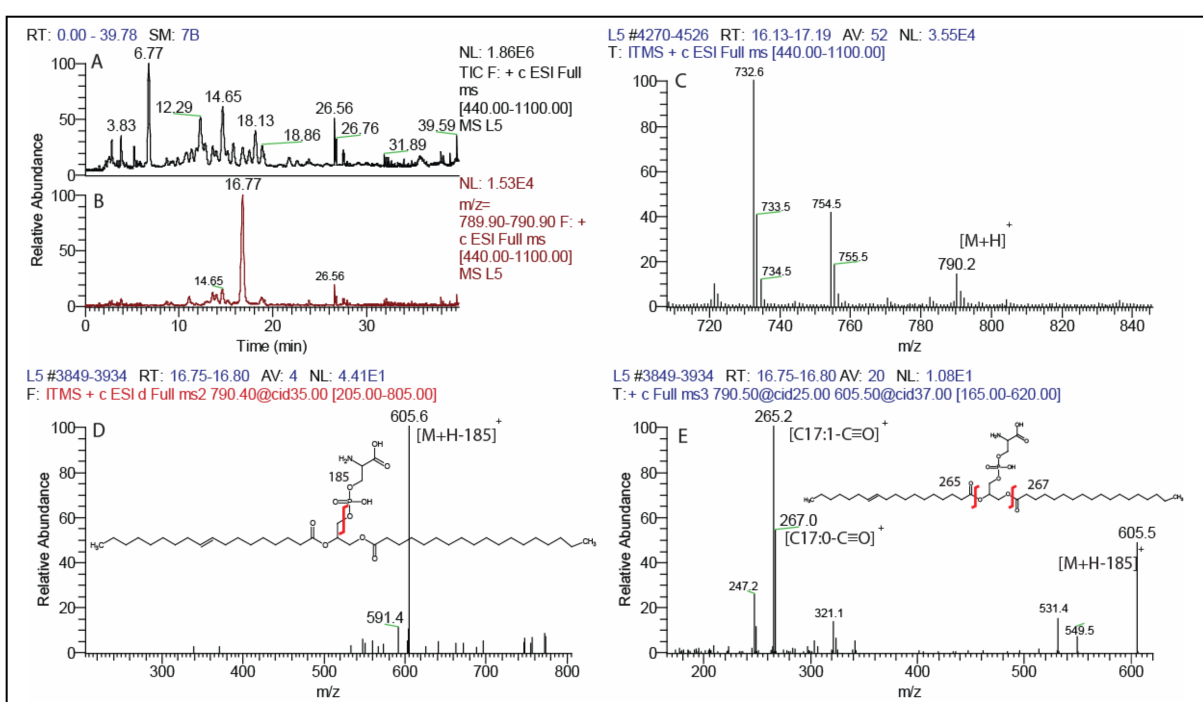

Figure 22: Positive ESI mass spectrometry of PSs.
(A) and (B)A TIC (A) and extracted ESI mass chromatogram (B) of the lipid extract from the *Drosophila melanogaster* body. The m/z 790.2 trace revealed a PS peak in RT 16.77 min.
(C) A background subtracted ESI full scan spectrum of the PS.
(D) CID MS^2 spectrum of the PC precursor ion $[M+H]^+$ m/z 790.5 showing a characteristic serine moiety loss [M+H-185]+ resulting here in the product ion m/z 605.5.
(E) CID MS^3 spectrum of the PC precursor ion $[M+H]^+$ m/z 786.4, RT 23.65 min exhibits characteristic acylium ions m/z 265.3 and 267.3 and ketene ions m/z 321.2 and 323.2.

Phosphatidylserines do not represent an abundant PL class in total lipid extract of insects. Identification of one PS class member having $[M+H]^+$ adduct m/z 790.5 is demonstrated in Fig. 22. The CID MS^2 spectrum gives a characteristic serine moiety neutral loss with m/z 605.6. The CID MS^3 scan experiment revealed that acylium ions of FA 18:0 and 18:1 are present together with the ketene ion m/z 265.2 and 267.0. Our PS reference standard was PS C18:0/18:1. The more intensive *sn-2* acyl in the MS^3 spectrum may be correlated with its *sn-2* position.

4.5 Data processing of lipid analysis in insects

The developed LC/MS brings a wealth of information about lipid composition in insect biological materials collected in the course of this work. Thus, thousands of lipid species may occur in the insect lipid extracts and it is very hard to process or visualize them. Tables can be used for data presentation but do not provide an easy survey and complex view to the problem. Also simple statistic methods like t-test or ANOVA are not able to cover all data and factors in physiological problem with so huge result data pool. One of the statistically and also graphically solution provide the multivariate principal component analysis (PCA). This method is specially designed to deal with many variables and samples. The numeric values of particular lipids enter to the analysis as the response variable. Application of this statistic method was demonstrated in chapters 5.1.4, 5.1.5 and 5.2.5.

4.6 Insect lipid study No.1: Application: Adaptation of HPTLC, GC and HPLC/ESI/MS methods for phospholipid analyses. The study of seasonal changes of phospholipid composition in *Pyrrhocoris apterus.* (manuscript).

Abstract

High performance thin layer chromatography (HPTLC) for phospholipid (PL) class separation, GC (gas chromatography) for quantitation of PL fatty acid (FA) composition, HPLC ESI/MS (high performance liquid chromatography electrospray ionization mass spectrometry) and spectrophotometry for quantitation of individual PL techniques have been adapted for identification and analysis of individual phospholipids (PLs) obtained from thoracic muscles and fat bodies of *Pyrrhocoris apterus*. HPTLC served as a separation tool, aiming at removal of non-polar PLs from insect extracts and to separation of polar lipids, sphingomyelin (SP), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cardiolipin (CL). The most abundant PLs merely, i.e. PE and PC, were processed for GC. Fatty acids (FAs) were quantified by GC as methyl esters, prepared by direct transmethylation of phospholipids scrapped from HPTLC spots with sodium methoxide. High performance liquid chromatography (HPLC) was used like preseparation technique for ESI/MS method. PC and PE were identified by HPLC retention time and both positive and negative ion mass spectra. Positive ESI was used for quantification and also provided information about types of FA in the structure regarding the chain length and numbers of double bonds. A good correlation between FA composition determined by GC and the data obtained with the developed ESI/MS method was found. Individual PLs were quantified using spectrophotometry after conversion into inorganic phosphate (as phosphomolybdate complex). The approach was applied for the study of seasonal changes of PL composition in *Pyrrhocoris apterus.* Considerable differences were found between summer and winter samples both in the proportion of PL classes and individual PL.

5.0 INSECT PHYSIOLOGY APPLICATION

5.1 OVERWINTERING AND REMODELING OF MEMBRANE LIPID COMPONENTS – DETERMINATION OF STRUCTURAL LIPIDS

5.1.1 Membrane lipid characterization

Biological membranes are assemblies of lipids and proteins that separate inside from outside and are responsible for the distinction between compartment and environment. Membrane provides a barrier to diffusion which is a base for the establishment, maintenance, and regulated utilization of transmembrane solute gradients, which in turn are used for acquiring metabolic substrates and for energy production. Membranes are also responsible for uptake and release of material into and out of cell by endo- and exocytosis. Membranes actively participate in transmembrane signal transduction and store pool of precursors for lipid-derived second messengers. All membrane functions are critically dependent on membrane physical properties which are dictated by lipid molecules making the membrane lipid bilayer (Williams, 1998). The bilayer is a matrix for a wide spectrum of proteins involved in many crucial cellular processes. In fact, more than half of total proteins in a typical eukaryotic cell are associated with membrane; either as membrane integral proteins or as proteins functioning at or near of membrane surface. Thus, it is obvious that the physical and chemical properties of membrane directly affect most of cellular processes. Membrane should be considered as a dynamic part of the cell rather than simply as a static barrier (Dowhan and Bogdanov, 2002).

Primary role of lipids is to form a lipid bilayer. Although several types of membranes can be found through the tree of life, glycerol-based phospholipids are probably the most abundant lipids used for construction of membranes. Of course, many other lipid species are also important components, and their presence and abundance is organism- and cell type-dependent. The huge variability of membrane lipids is easily exemplified by *Escherichia coli.* This simple bacteria possesses only three major phospholipid classes, just a few different fatty acids and some precursors and modified products. Despite such a limited selection of basic components the number of their mutual combinations, that is the number of individual phospholipid species, ranges in hundreds! No surprise then, that the number of individual phospholipid species reaches to thousands in more complex eukaryotic organisms (Dowhan and Bogdanov, 2002).

The primary building blocks of most membranes are glycerolphosphate-containing lipids known as phospholipids. In eubacteria and eukaryots, diacylglycerol backbone is esterified in *sn*-3-glycerol position and at position *sn*-1 and 2 – positions are by esteric binding connected long chain fatty acids. Diversity of lipids is dependent on a diversity of these long chain fatty acids, the length and level of saturation of fatty acids provide a many combinations of fatty acids and also many combination of lipid properties.

In archea the situation is different. Long chain fatty acids are replaced by saturated isoprenyls, and also ether linkages are presented. Similar ether linkages are found in the plasmogens of eukaryotes (Dowhan and Bogdanov 2002).

5.1.2 Membrane lipid organization – phase behaviour

Membranes of higher organisms are very complex mixtures of hundreds of different protein and lipids. They held together by relatively weak forces – Van der Walls, electrostatic and hydrophobic interaction. The lack of rigid connections like covalent bonds is very important for the dynamics of membrane because without these strong forces the membrane components are free enough to spin, wobble, and diffused laterally. Dynamic organization of membrane is essential for its proper functioning (Hazel, 1995).

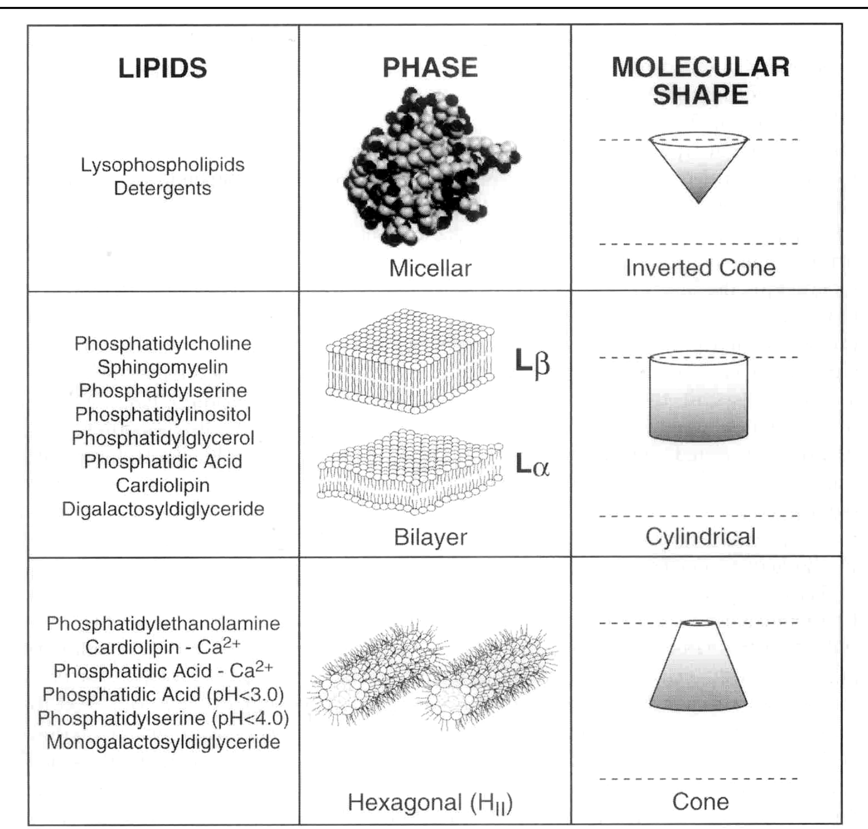

Figure 23: Polymorphic phases and molecular shapes exhibited by lipids. Inverted cone shapes molecules form micelles. Polar lipids with two long alkyl chains adopt a bilayer or non-bilayer structure depending on the geometry of molecule and environmental conditions. The L_{β} (order gel) and L_{α}(liquid crystalline) bilayer phases differ in the order within the hydrophobic domain and in mobility of individual molecules (Dowhan and Bogdanov 2002).

The phase behaviour and physical properties of lipids in biological membrane are highly sensitive to changes in temperature. Perturbation of membrane organization, when cell or body temperature changes, is one consequence of poikilothermy. At physiological temperatures, rotations about carbon-carbon single bonds are freely propagate up and down the length of fatty acyl chains, which results in a relatively fluid, disordered liquid crystalline phase (L_{α}) (Figure 23). When temperature drops below the physiological range, acyl chains adopt the all-*trans* conformation and pack efficiently to form a highly ordered gel phase (L_{β}) (Figure 25). It happens at some defined point – the gel/fluid or chain-melting transition temperature (T_m). The process requires a pronounced incubation time at temperatures considerably below T_m. (Huang at al, 1997). The viscosity or fluidity of the hydrophobic domains of the lipids, which is a function of temperature and an alkyl chain structure, causes the difference between the ordered gel and liquid crystalline phases (Dowhan and Bogdanov 2002). Transition from the fluid to gel phase induces clustering of integral membrane proteins, reduces activity of many membrane-associated enzymes, slows the lateral protein diffusion, thereby reducing the efficiency of ability of diffusion-couple processes, and markedly increases the permeability for cations and water, because of packing defect that form at boundaries between micro domains of gel and fluid phase lipid. In biological membranes may region of phase separation (consisting or coexisting domains of fluid and gel phase lipids) extend over temperature range of 10-15°C due to diversity of present lipid species. When temperature exceeds the physiological range some lipids assume the inverted hexagonal (H_{II}) phase (see Figure 24), which results in a loss of bilayer integrity. The transition to H_{II} phase (occurring at T_h) is driven, in part, by a temperature induced change in phospholipid molecular geometry from a cylindrical to a conical shape (Fig. 23)(Hazel, 1995).

Biological membrane is a mixture of several types of lipid. The physical property of a lipid mixture is a collective property determined by each of the component lipids. A large number of studies show that the L_{α} state of the membrane bilayer is required for cell viability and cells adjust their lipid composition in response to many environmental factors so that the collective property of the membrane exhibits the L_{α} state. Mixture of lipids with different phase properties can also generate phase separations with local domains formation. Such discontinuities in the bilayer structure may be required for many structural organizations and cellular processes such as accommodation of proteins into the bilayer, movement of macromolecules across the bilayer, cell division, and membrane fusion and fission events. The need for bilayer discontinuity may be the reason that all natural membranes contain a

significant proportion of non-bilayer-forming lipids (lipids with tendency to change their shape to conical, Fig. 23) even thought the membrane under physiological conditions is in the L_α phase.

Addition of cholesterol to lipid mixture has a profound effect on the physical properties of a bilayer. Increasing amount of cholesterol inhibit the organization of lipid into the L_β phase and favour a less fluid but more ordered structure than L_α phase resulting in the lack of a phase transition normally observed in the absence of cholesterol. The solvent

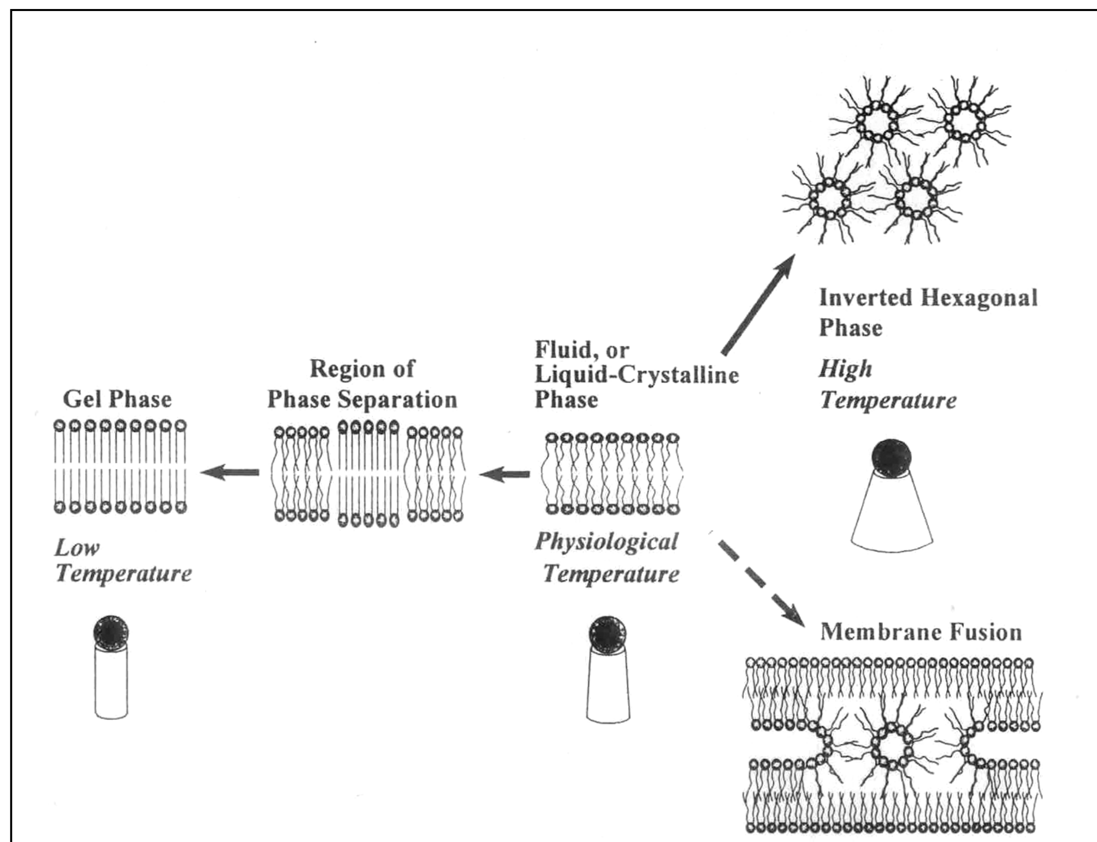

Figure 24: Solid arrows indicates the effect either a rise or drop in temperature on phase behavioural and molecular geometry of membrane phospholipids. The physiological temperature refers to the temperature at which an organism is either adapted or acclimated. The dashed arrows illustrate the presumed involvement of the inverted hexagonal phase in membrane fusion.

surrounding the lipid bilayer also influences these transitions primarily by affecting the size of the headgroup relative to the hydrophobic domain. $Ca^{2+}$and other divalent cations reduce the effective size of the negatively charged headgroups (Fig. 25) of cardiolipin or phosphatidyl acid allowing organization to H_{II} phase (cell directed transition to H_{II} phase is observed during membrane fusion, see Figure 26. Low pH has the similar effect on the headgroups of phosphatidylserine. Since Ca^{2+} is an important signalling ion, it is possible that the part of its effects may be transmitted through changes in physiological properties of membranes. Nusrat

et al found that Ca^{2+}regulates assembly of small micro domains which create a tight junction on membrane (Nusrat et al, 2000).

5.1.2.1 Homeoviscous adaptation versus homeophase adaptation

The inherit sensitivity of the phase behaviour and physical properties of membrane lipids to change in temperature restrict the thermal range over which a designed set of membrane constituents can function effectively. Poikilothermic organisms have to restructure their membranes to obtain appropriate physical properties tha matched to the prevailing thermal conditions, to function over a broad range of environmental temperatures. Remodelling of biological membranes is the most commonly observed cellular response to temperature changes is. Decrease of temperature usually leads to one or combine adjustments. First of them is an increased proportion of *cis* unsaturated fatty acids. There are evidences, required by Huang et al that both the position and the numbers of *cis* double bonds in *sn-2*acyl chain could exert noticeable influence on the gel to liquid crystalline phase transition behaviour of the lipid bilayer (Huang et al, 1997). When is the organism exposed to gentle cooling, the latent desaturase is activating and also the induction of desaturation gene transcription is observed (Trueman et al, 2000). The compositional adaptation for PC and PE is different. For PC largely occurs the adaptation by saturating fatty acid in the *sn*-2 position, whilst for PE is fatty acid changes involved at the *sn-1* position (Logue et al, 2000; Brooks et al, 2002). The other one is elevated proportion of phosphatidylethanolamines to phosphatidylcholines. Most common adaptive explanation for this event – membrane remodelling is a homeoviscous adaptation. This hypothesis declares that optimal membrane function is restricted to a limited range of membrane fluidities (Cossins et al, 1989). When the temperature arises acutely, membrane becomes hyperfluid and conversely, as temperature drops, fluidity falls below the optimal range and membrane activities are constrained. In addition, persisting exposure to temperatures above or below those required to maintain optimal fluidity initiates acclimatory (within the lifetime of an individual) or adaptational (over evolutionary time) alternations in lipid composition that largely offset the direct effect on temperature on membrane lipid fluidity. The rank sequence of membrane order correlates directly with body or habitat temperature, which indicates that evolutionary adaptation to cold environments produce membranes of significantly lower order. When compare at the respective cell or body temperatures, membrane order is roughly equivalent in all species, which illustrate the essence of homeoviscous adaptation. Study of membranes of *Bacillus subtilis* confirm that conformation order rather than rate of lipid motions is the feature of

membrane organization subject to regulation when temperature changes (Herman et al, 1994). The capacity of homeoviscous adaptation appears to be a basic cellular response displayed by microorganism and also by cells of vertebrate poikilotherms (Hazel, 1995).

There are numerous examples of membrane responses to altered growth temperature that are difficult to explain in terms of homeoviscous adaptation. This suggests that mechanisms other than the defence of lipid order may also contribute to the thermal compensation of membrane function. It is possible that moderate degree of homeoviscous adaptation could result in perfect compensation of function in some membranes, or the variable degree of homeoviscous adaptation could compensate function to different extents in different membranes. But the tendency of cold exposure disordered some membranes, while not influencing or ordering others, argues against the regulation of membrane order as a generally applicable paradigm of membrane adaptation (Hazel, 1995).

Thermal compensation of membrane function and capacity for homeoviscous adaptation are not tightly linked. Thermal compensation of membrane function can occur in the absence of homeoviscous adaptation and vice versa. Two aspects of temperature-induced membrane restructuring are particularly difficult to explain in terms of homeoviscous adaptation. First it is an accumulation of long-chain polyunsaturated fatty acids (PUFAs) at low temperature and a positive correlation between growth temperature and ratio of bilayer-stabilizing to bilayer-destabilizing lipids is the second. Reduced levels of lipid unsaturation promote survival at warm temperatures and the complete loss of PUFAs (desaturase mutants) reduces heat tolerance. The impact on membrane physical properties of all double bond is not similar. For example monoenoic fatty acids are superior to PUFAs with respect to the magnitude of the change they produce and also the lower metabolic cost of their production. Balance, between bilayer-stabilizing (PC) and bilayer-destabilizing (PE) lipids, is the second composition adjustment. Cold-adapted poikilotherms has elevated proportion of PE to PC, this is a commonly observed feature than fluidize a membrane. It is possible that the reason is about 20°C higher temperature for PE to transition to gel phase. Thermal modulation of headgroup composition may thus have a greater adaptive impact on membrane phase behaviour than on hydrocarbon order (Hazel, 1995).

The concept of homeoviscous adaptation is an adaptational extension of the fluid mosaic membrane model, which emphasizes the lacks of long-range order in membranes and the functional importance of appropriate lipid fluidity. This model has been very useful, but nowadays is not enough precise. Compensation of membrane function are continuously correlated with changes in acyl chain order, suggesting that features of membrane organization other than lipid order are subject to regulation when environmental conditions change. The existence of discrete membrane domains is one feature of membrane organization. The adaptive significance of temperature-induced alternations in membrane lipid composition may relate to conservation of dynamic membrane properties, including the maintenance of an appropriate balance between membrane microdomains and the ability to regulate intracellular membrane traffic. Biomembrane prefer dynamic phase behaviour of a membrane rather than to the fine tuning of lipid order. According these facts a new term has been proposed – homeophasic adaptation. According this model, it is the relationship between the ambient temperature (T_a) and the temperatures of gel/fluid and H_{II} phase transition that is conserved even when growth temperature changes. Temperature acclimation or adaptation, by

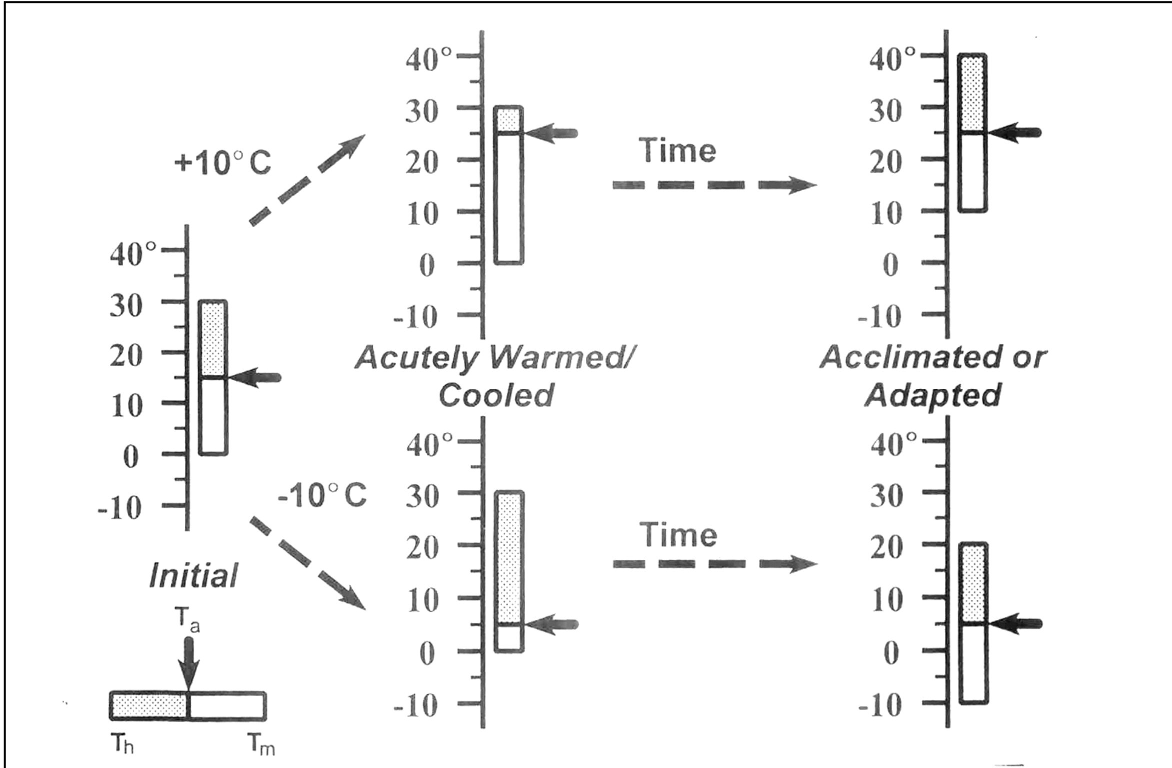

Figure 25: The dynamic phase behaviour model of thermal adaptation in biological membranes. An acute rise or drop temperature alter the relationship between the ambient or body temperature (T_a is the temperature which the membrane is functioning) and the transition to the gel (T_m) and H_{II} (T_h) phases (a rise in temperature decreases the interval between T_a and T_h, while increasing the interval between T_m and T_a, whereas a drop in temperature has the opposite effects). Acclimation or adaptation to an altered temperature restores the proximity of T_a to T_h and T_m.

altering the chemical composition of the membrane, modifies both T_m and T_h so that operational temperature (T_a) remains at a suitable interval above T_m and bellow T_h (Fig. 25). The dynamic phase behaviour model also explains some aspects of membrane restructuring not consistent with homeoviscous adaptation. The positive correlation between growth temperature and the PE/PC ratio in cell membranes can be viewed as a homeostatic mechanism to restore the appropriate interval between T_a and T_h (Hazel, 1995).

5.1.2.2 Lipid microdomains and rafts

Wide variety of lipids observed in biological membranes extends beyond the concept of a simple barrier function. One of the consequences of the chemical heterogeneity of lipids is the possibility of non-random mixing in the bilayer. All biological membranes are not uniform in respect to chemical composition and distribution of lipids and proteins.

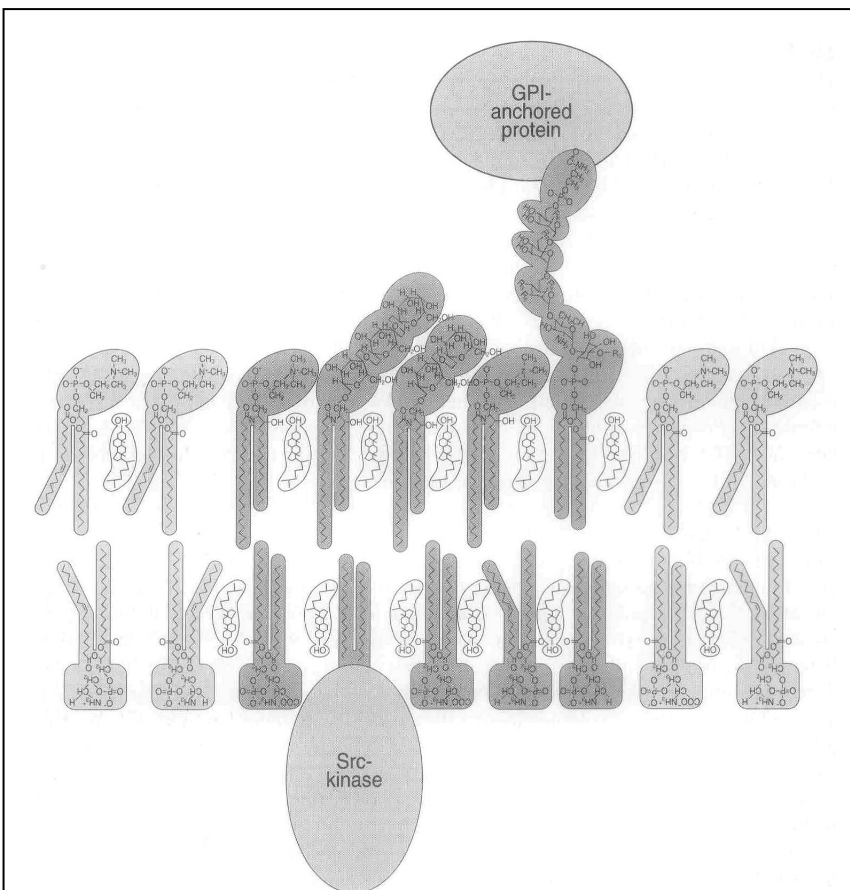

Figure 26: Model of a lipid raft. A glycosylphosphatidylcholinositol-linked protein is attached to the exterior monolayer of the membrane and a Src-kinase to the interior monolayer of the membrane by their respective covalently attached lipids. The mechanism for clustering and coupling Src-kinase with the glycosylphosphatidylinositol linked protein is hypothetical. Clustered (dark grey) around the glycosylphosphatidylinositol are ordered (straight alkyl chains) glycosphingolipids, sphingomyelin, and PC with intercalated cholesterol. The phospholipids with unsaturated chains indicate the more disordered liquid crystalline state of the surrounding bilayer (Dowhan and Bogdanov 2002).

Experimental evidence has been provided that phase separation does occur in biological membranes, resulting in the formation of microscopic domains with various size and life times. Domains also differ in composition of peptides and lipids (Figure 26). These domains serve to further compartmentise cellular processes and function in physiologically important cellular events, such as signal transduction or exocytosis (Williams, 1998). They can also strongly affect membrane structure by concentrating interacting species in particular regions or by excluding diffusion molecule from other region. Lipid domains exist in variety of forms and their formation can be caused by different mechanisms like lipid phase separation, protein barriers, and electrostatic interactions between membrane-associated components. There is also opinion presented by Azzam et al that microdomains may participate in the cellular mechanism of low temperature acclimation in hibernating animals. When the temperature is lowered in some membranes *in vitro*, the gelation of high-melting-point lipids induces lipid segregation and lateral displacement and exclusion of proteins. Exactly same protein-free domains were observed by Azzam et al in hibernating animals (Azzam et al, 2000). Some proteins or peptides bind to phosphatidic acid and are able to induce the formation of domains or affect phase separation. Heterogeneity in the lateral distribution of lipids implies that some lipid molecules may be transiently segregated into domains, while others staying out of these domains as disorganized lipids (Bandorowitz-Pikula, 2000).

Special type of domains rich in cholesterol, glycosphingolipids (gangliosides), sphingomyelin and proteins are called the rafts. Lipid rafts are defined as the membrane fraction resistant to a solubilisation in the cold by detergent. The proteins co clustered in lipid rafts are soluble globular proteins tethered to raft lipids via covalent linkage to fatty acid, cholesterol, or phosphatidylinositol. The latter glycosylphosphatidylinositol-linked proteins are attached directly to the amino group ethanolamine phosphate which in turn is linked to a trisaccharide and then to the inositol of PI. The sphingolipids and glycosylphosphatidylinositol-linked proteins occupy the outer surface monolayer of the plasma membrane bilayer, and the acyl chains of these lipids are generally more saturated and longer than those of the plasma membrane phospholipids. Lipid rafts and glycosylphosphatidylinositol-linked proteins are self-associating because of their dissimilarity with surrounding fluid phospholipids. All complex is stabilized by the hydrogen-bonding. Finally the planar shape of cholesterol favours its intercalation parallel to the ordered acyl chains of the raft lipids with its single hydroxyl group facing the surface (Dowhan and Bogdanov 2002). Cholesterol plays a crucial role in determining the physical properties of the rafts (Bandorowitz-Pikula, 2000).

Lipid rafts appear to be a mechanism to compartmentalize various processes on the cell surface by bringing together various receptor-mediated and signal transduction processes.

5.1.3 Common response of organism through membrane remodelling of thermal changes of environment

Consequence of poikilothermy is a remodelling of membrane organization when cell or body temperature changes. Harwood describe and divide these changes in several group. His study was focused on plant membrane behaviour during cold acclimation but most of these changes have been noticed in many invertebrates and microorganisms. Molecular species remodelling means a redistribution of acyl moieties within a particular lipid class and, since it involves no biosynthesis de novo, probably represent an emergency response (Harwood et al, 1994):

1. *Cis* / *trans* isomeration. It is well known that the melting point of *trans*-unsaturated fatty acid is much higher than that of equivalent *cis*/unsaturated molecules. This, in theory, changing of isomerism of a given fatty acid could have a dramatic effect on membrane fluidity. This phenomena has been observed for the psychronic bacterium *Vibrio* species (Harwood et al, 1994)
2. Changes in acyl chain length and fluidity. Because longer-chain fatty acids have higher melting temperatures than their shorter equivalents, regulation of fatty acids chain length provides another potential strategy for the control of membrane fluidity. The mechanism seems to be case for most plants (Harwood et al, 1994).
3. Increased unsaturation seems to be a common feature at lower temperatures. Given the huge decrease in melting temperature that is effected by the introduction of a single *cis* - double bond in to a saturated chain, the above change is easy understand. Furthermore, because many desaturases are membrane-bond enzymes (Trueman et al, 2000) that use complex lipid substrates, the increase of unsaturation can take place in situ, and membrane fluidity may also serve to control desaturase activity (Harwood et al, 1994).
4. Changes in lipid classes' proportion are observed during temperature acclimation. This is a slower method of adaptation to change in environmental temperature. Altering the proportion of individual lipid classes could affect membrane fluidity (Harwood et al, 1994).

5. The final type of change that may occur as a result of a low growth temperature alternation is another long-term response where the ratio of lipid to protein in membranes increases (Harwood et al, 1994).

The similar membrane change features were observed during cold acclimation of several insect or higher poikilotherm organisms like fish. Increasing level of fatty acid desaturation, increasing length of fatty acid chains and also change in ratio of phosphatidylcholines to phosphatidylethanolamines are very common and significant changes. For example the investigation were done in tissues homogenates of the larvae of *Chymomisa costata* and aestivating insect prepupa (Kostal et al, 1998, 2000, 2003), adult of heteropteran *Pyrrhocoris apterus* (Slachta et al. 2002), (Hodkova et al, 1999, 2002). It also appears that marine crustacean *Gammarus sp.* follows the same strategy to control the membrane fluidity (Lahdes, 2000). Also the embryo of see urchin *Litochynus pictus* changes the ratio of PC to PE (Tremper et al 1999).

5.1.4. Insect lipid study No.2.: Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature desiccation and diapause progression 2006

Abstract

Adults of the insect Pyrrhocoris apterus acquire chill tolerance through the process of autumnal acclimatization. Field and laboratory experiments were conducted to separate the triggering effects of low temperatures, desiccation and diapause progression on the physiological characteristics related to chill tolerance with emphasis on the restructuring of glycerophospholipid (GPL) composition. Changes in relative proportions of major molecular species of glycerophosphoethanolamines (GPEtns) and glycerophosphocholines (GPChols) in thoracic muscle and fat body tissues were followed using HPLC coupled to electrospray ionisation mass spectrometry. The increase in relative proportion of 1-palmitoyl-2-linoleyl-sn-GPEtn at the expense of 1,2-dilinoleyl-sn-GPChol was the most prominent feature of the complex change observed in both tissues during autumnal acclimatization in the field. The relative proportion of total GPEtns increased, while the proportion of total GPChols decreased. The relative proportion of unsaturated fatty acyls slightly decreased. A similar restructuring response was seen during acclimatization in the field and cold acclimation in the laboratory. By contrast, the GPL changes related to desiccation and diapause progression were relatively small, differed qualitatively from the cold-acclimation response, and were accompanied with no increase of chill tolerance. Other features of autumnal acclimatization, i.e. depression of supercooling capacity and accumulation of polyhydric alcohols, were also triggered solely by low temperatures.

5.1.5. Insect lipid study No.3.: Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster* 2008

Abstract

Adaptative responses of ectothermic organisms to thermal variation typically involve the reorganization of membrane glycerophospholipids (GPLs) to maintain membrane function. We investigated how acclimation at 15, 20 and 25 1C during preimaginal development influences the thermal tolerance and the composition of membrane GPLs in adult Drosophila melanogaster. Long-term cold survival was significantly improved by low acclimation temperature. After 60 h at 0 1C, more than 80% of the 15 1C-acclimated flies survived while none of the 25 1C-acclimated flies survived. Cold shock tolerance (1 h at

subzero temperatures) was also slightly better in the cold acclimated flies. LT50 shifted down by ca 1.5 1C in 151C-acclimated flies in comparison to those acclimated at 25 1C. In contrast, heat tolerance was not influenced by acclimation temperature. Low temperature acclimation was associated with the increase in proportion of ethanolamine (from 52.7% to 58.5% in 25 1C-acclimated versus 15 1C-acclimated flies, respectively) at the expense of choline in GPLs. Relatively small, but statistically significant changes in lipid molecular composition were observed with decreasing acclimation temperature. In particular, the proportions of glycerophosphoethanolamines with linoleic acid (18:2) at the sn-2 position increased. No overall change in the degree of fatty acid unsaturation was observed. Thus, cold tolerance but not heat tolerance was influenced by preimaginal acclimation temperature and correlated with the changes in GPL composition in membranes of adult *D. melanogaster*.

5.2 LIPID MOBILIZATION AFTER THE ADIPOKINETIC HORMONE ACTION

5.2.1 Response to bioenergetic demands

Flight is one of the most energy demanding insect behaviour. Metabolism of insect flight muscle involves an integration of biochemical and physiological processes controlled by hormonal and neural mechanisms. The demand for substrates providing energy requires activation and control of mobilization and transport of fuel reserves. Regarding the utilization of substrates during flight of the insects can be divided into several groups. (1) Group exclusively generates energy from carbohydrates; (2) group using lipid oxidation; (3) group using both strategies and finally, (4) group in which proline act as a direct and in some species most prominent substrate (Beenakkers et al, 1984). Utilization of energetic resources is mostly controlled by small neuropeptides from AKH/RPCH family (for details see below).

We used the African migratory locust *Locusta migratoria* as a model species for our studies. Locusts use predominantly lipids as energy source. Hovewer, lipid reserves in the flight muscle themselves are very limited, so reserves from the fat body have to be mobilized, transported through haemolymh in to the flight muscles (Beenakkers et al, 1984; Oudejas et al, 1991).

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5.2.2 Regulation of lipid mobilization by AKH

The adipokinetic peptides are octa-, nona- or decapeptides with N-terminus blocked by a pyroglutamate residue and the C-terminus by an amide (Gade et al, 1997). The AKHs are usually synthesized and stored by corpus cardiacum (CC), a neurosecretory gland connected with the insect brain. Major function of these hormones is the control of insect metabolism. However, these peptides are pleotropic with a number action related to their metabolic role (Kodrík, 2008).. Generally, they behave as typically stress hormones stimulating catabolic reactions (mobilise lipids, carbohydrates and/or certain amino acids), making energy more available, while inhibiting synthetic reactions. The main target tissue for AKH is fat body. Flight is the first stimulus for releasing of AKH from CC to haemolymph. AKH is than a trigger a cascade of processes leading to production of energy (Van der Horst et al, 2001).

The peptide characteristics prevent AKHs to penetrate a cell membrane , their signal transduction is mediated via specific membrane receptor. Although a wealth of information has been available about the action of AKHs at the cellular level, information about the

putative AKH receptors has been obtained only in recent years. An early biochemical characterization of an AKH receptor protein from the fat body of the tobacco hawkmoth *Manduca sexta*, but subsequently molecular biological methods have been used to characterize AKH receptors from several insect species. AKH receptors have been cloned from the fruit fly *Drosophila melanogaster* , the silkworm *Bombyx mori*, the American cockroach *Periplaneta americana* and the African malaria mosquito *Anopheles gambiae*. Receptor specificity was proved in the ligand binding studies with recombinant receptor produced in Chinese hamster ovary cells or Xenopus oocytes. These receptors are typical G protein-coupled proteins with seven membrane spanning domains, and they are structurally related to receptors of the vertebrate gonadotropin-releasing hormone (Kodrík 2008)

5.2.3 Lipid mobilization from fat body

The model of mobilization of lipids is based on research with the locust *Locusta migratoria* and the moth *Manduca sexta* (Gade et al, 2003). This study deals with the locust model organism.

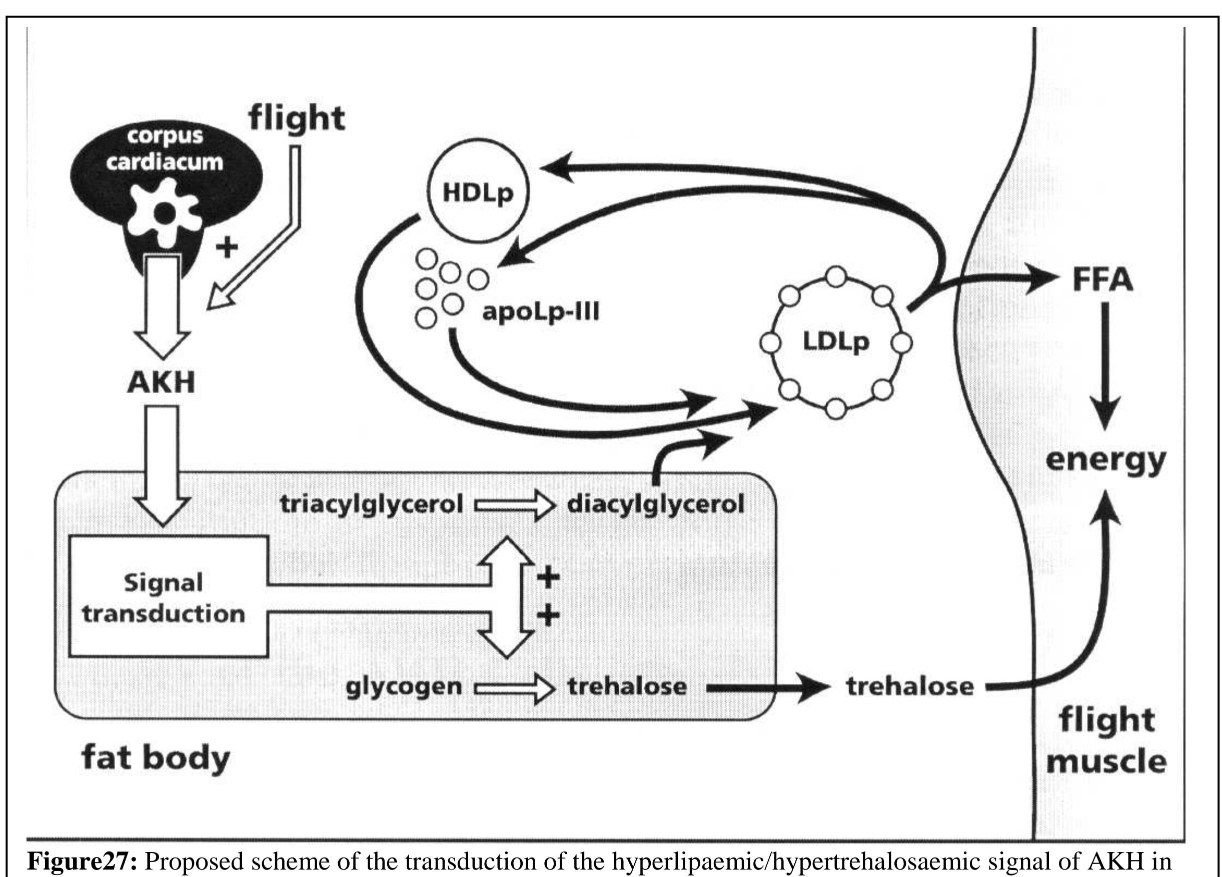

Figure27: Proposed scheme of the transduction of the hyperlipaemic/hypertrehalosaemic signal of AKH in the fat body of insects and general scheme of lipid mobilization (Van der Horst et al. 2001).

The AKH action initialized by its binding to a receptor (Fig. 28) causes G-protein conformation. Change leads to activation of adenylate cyclase. The process increases cAMP

level and is followed by the activation of A-kinase activity of fat body cells, which results to activation of the TG-lipase. Activated lipase hydrolysis TG, stored in the fat droplets, into DG, and the DG is moved to the membrane by a cytosolic carrier for delivery to the haemolymph (Beenakkers, et al., 1984, Arrese et al, 2001). Receptor binding of AKHs also causes an entry of Ca^{2+} via channels in to the fat body of *L.migratoria*. The role of intracellular Ca^{2+} in adipokinetic signalling is not well investigated so far, and knowledge is fragmentary (Gade et al, 2003).

5.2.4 Lipid transport by lipophorin

Haemolymph is an aqueous environment therefore some type of DGs shuttle is important for transport. This role belongs to lipophorins – a special group of transport lipoproteins (Fig. 28). Their main function is moving lipids from side of absorption or storage to side of utilization.

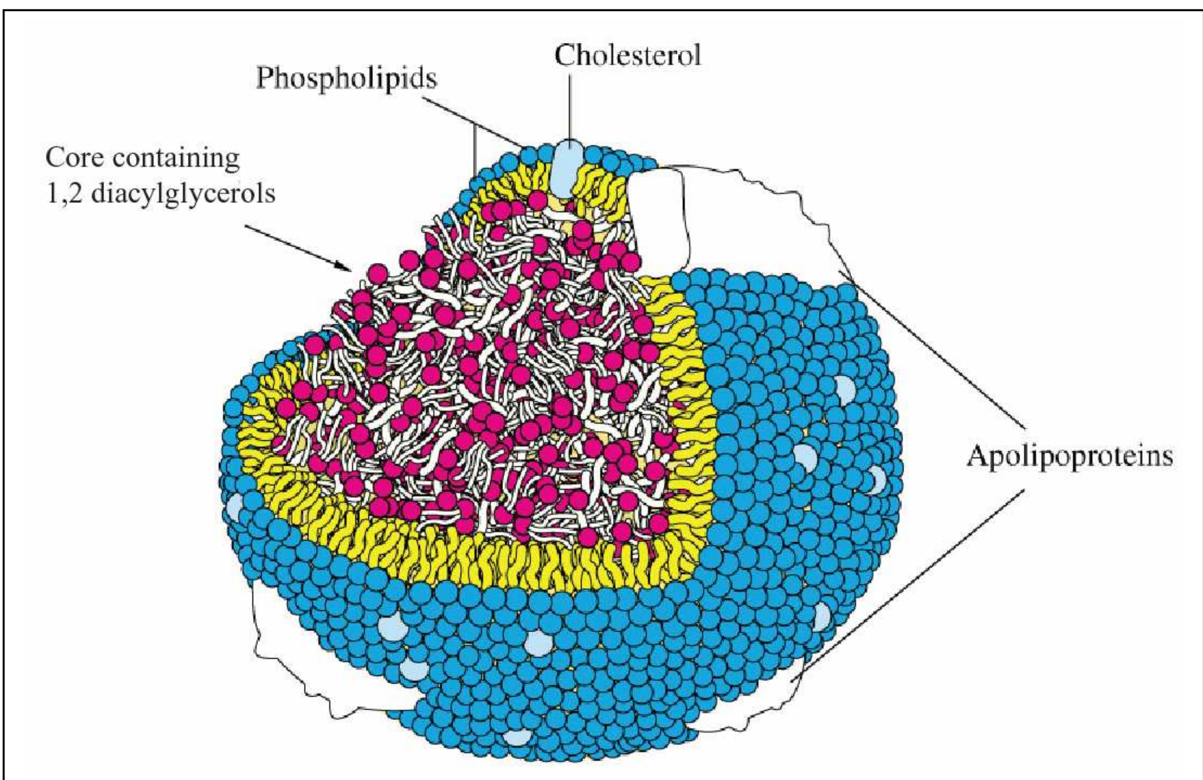

Figure 28: Illustration describes a model of common lipoprotein. Phospholipid capsule coupled with apolipoprotins covers transported diacylglycerol mostly wit FA attached in *sn-1* and *sn-2* position.

Insect haemolymph generally contains abundant amount of a single multifunctional lipoprotein particle, high density lipophorin (HDLp). A characteristic feature of insect HDLp is its ability to function as a reusable shuttle for variety of lipids by the selective loading and unloading of lipid content at target tissues. This provides energetical advantage because loading and unloading does not require lipophorin internalization by either lipid donor or

recipient. Thus increases capacity for lipid transport without additional lipophorin synthesis (Van der Horst et al, 2001).

HDLp generally contains two non-exchangeable apoliporoteins – apolipohorin I, apolipophorin II and is present circulating in insect haemolymph. In response to the AKH stimulated mobilization of fat body TG stores, DGs are loaded on to pre-existing HDLp particles, ultimately transforming them in to light-density lipophorins (LDLp). During loading processes facilitated by lipid transfer particle (LTP) several copies of amphipatic exchangeable apolipoprotein – apolipophorin III (apoLp-III) associate with the particle (Arrese et al, 2001; Canavoso et al. 2001). At the flight muscles, the DGs are depleted from the lipoprotein through the action of a membrane-boud lipophorin lipase in the extracellular space. The effusion of the lipid content from the particle induces dissociation of apoLp-III and both protein constituents (HDLp and apoLp-III) are recovered in the haemolymph and may return another cycle of DG transport. Consequently, apoLp III in the haemolymph alternates between a lipid-free and lipophorin-bound state (Van der Horst et al, 2001).

Many aspects of lipid metabolism in insects remain unclear. Good example is a presence of more than one AKH in one insect species – a phenomenon which has not been satisfactory elucidated yet. A certain degree of AKH specialization was recorded already a long time ago. For example the Locmi-AKH-I is the most potent in a lipid mobilization assay (Goldsworthy, 1994). Further, the Locmi-AKH-II is somewhat stronger than AKH-I in activation of glycogen phosphorylase (Oudejans et al., 1992; Van der Horst et al., 2001). Also the inhibition of total RNA synthesis in *L. migratoria* FB is decreasing in the order Locmi-AKH-III > AKH-II > AKH-I (Kodrík and Goldsworthy, 1995). To contribute to the elucidation of the problem we tried in the following study to find if there is any specialization of particular *L. migratoria* AKHs in mobilization of particular lipids.

5.2.5 Insect lipid study No.4: Locust adipokinetic hormones mobilize diacylglycerols selectively (submitted)

Abstract:

Insects use diacylglycerols (DG) as an essential transport form of lipids. The DG molecular species and their fatty acid (FA) composition were investigated by electrospray mass spektrometry (ESI-MS) and by gas chromatography with flame ionisation detection (GC-FID) in haemolymph of *Locusta migratoria* after application of adipokinetic hormones Locmi-AKH-I, -II and -III. The analyse showed (1) a heterogeneous distribution of individual DGs on nmol/ml level in haemolymph after the hormone application. The results revealed that mobilization of the DGs is molecular species-specific with the highest proportion of 34:1 DG (16:0/18:1 - molecular weight 595.0 Da) for all Locmi-AKHs bearing palmitic acid (C16:0) and oleic acid (C18:1) residues, and forming in summary about 20% of the total mobilized DG content. (2) Additional analysis of fat body triacylglycerols revealed that all Locmi-AKHs mobilize the DGs selectively with the preference of those possessing the C18 and C16 FAs.The fat body FAs with carbon chain longer than 18 did not participate on the mobilization. (3) A derived representation of FAs (using the ESI MS analysis) as well as their direct GC FID determination indicated a certain degree of Locmi-AKH selectivity toward mobilized DGs and hence the FAs. The Locmi-AKH-I significantly prefers mobilization of DGs containing unsaturated FAs (linoleic acid), while Locmi-AKH-II and -III prefer mobilization of saturated FAs (stearic and myristic).

6.0 CONCLUSION

Complex analytical method for biological lipid extract determination was proposed (partly presented in Chapter 4.6).This method covers extraction and separation techniques coupled with mass spectrometry, providing structural information about particular lipid classes and even species. Published insect physiology applications of this method proved suitability for routine usage. Robustness and reproducibility were confirmed due animal samples variability (species/tissue) and both PLs and NLs detailed determination in single run analyses.

First application deals with determination of triggering factors of chill tolerance of *Pyrrhocoris apterus* occurred due process of autumnal acclimatization with emphasis on the restructuring of PLs. Three triggering effect were proposed and investigated – low temperature, desiccation and diapauses progression. The increase in relative proportion of 1-palmitoyl-2-linoleyl-sn-GPE at the expense of 1,2-dilinoleyl-*sn*-PC was the most prominent feature of the complex change observed in both tissues due the autumnal acclimatization in the field. The relative proportion of total PEs increased, while the proportion of total PCs decreased. The relative proportion of unsaturated fatty acyls slightly decreased. A similar restructuring response was seen during acclimatization in the field and cold acclimation in the laboratory. By contrast, the GPL changes related to desiccation and diapause progression were relatively small, differed qualitatively from the cold-acclimation response.

We also investigated how acclimation at 15, 20 and 25 °C during preimaginal development influences the thermal tolerance and the composition of membrane PLs in adult *Drosophila melanogaster*. Acclimation was confirmed by survival test when After 60 h at 0°C, more than 80% of the 15 °C-acclimated flies survived while none of the 25 °C-acclimated flies survived. Experiments resulted to similar trends like in previous case and proved that low temperature acclimation was associated with increase of PE at the expense of PC and small but statistically significant up regulation of PE with linoleic acid but no overall change in the degree of fatty acid unsaturation.

Last paper demonstrates, for the first time, that mobilization of DGs and/or FAs from the FB of *Locusta migratoria* after injection of AKHs is not uniform. The individual DG species are mobilized selectively, preferably with the DGs containing the C18 and C16 FAs, and especially the C18 unsaturated oleic acid. Additionally, it seems there is also at least partial specificity of individual locust AKHs to certain DGs or FAs therewith Locmi-AKH-I prefers mobilization of unsaturated FAs (mostly linoleic acid) while AKH-II and AKH-III prefer saturated FAs (mostly stearic and myristic acids).

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8.0 **ABBREVIATIONS**

AKH	adipokinetic hormone
AKH/RPCH	adipokinetic hormone/ red pigment concentring hormone
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
cAMP	cyclic adenosine monophosphate
CC	corpora cardiaca
CID	collision induced decomposition
CL	cardiolipin
DG	diacylglycerol
ELSD	evaporative light scattering detector
ESI	electro spray ionization
FA	fatty acid
FID	flame ionization detector
GC	gas chromatography
H_{II}	hexagonal phase
HPLC	high performance liquid chromatography
Chol	cholesterol
LC/MS	liquid chromatography/ mass spectrometry
LyPL	lysophospholipid
Lyso PC	lysophosphatidylcholine
Lyso PE	lysophosphatidylethanolamine
L_{α}	liquid crystalline phase
L_{β}	gel phase
MG	monoacylglycerol
MS	mass spectrometry
NL	neutral lipid
PC	phosphatidylcholine
PCA	principal component analysis
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidyl acid
PI	phosphatidylinositole
PL	polar lipid / phospholipid
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
SM	sphingomyelins
SPE	solid phase extraction
SL	sphingolipid
T_a	ambient temperature
TG	triacylglycerol
T_h	transition temperature to hexagonal phase
TIC	total ion current
TLC	thin layer chromatography
T_m	transition temperature to gel phase
UV	ultra violet

Attachment 1 – Table of separation techniques used for lipid determination

9.0 Attachments

Source	Separation			Column	Detector	Mobile phases	Lipid species and time consumption
Patto et al, 1990		HPLC	N	LiChrospher Si-100		n-hexane/ 2-propanol/methanol	
Kim et al, 1994		HPLC	R	C 18	MS	methanol/ n-hexane/NH_4COOH	
Neff and Byrdwell 1995		HPLC	R	Absorbosphere UHS C18 250x4,6mm ,10um Absorbosphere UHS C18 250x4,6mm, 5um	MS/ELSD	n-hexane/propionitrile	TG/ 120min
Byrdwell et al 1996		HPLC	R	Absorbosphere UHS C18 250x4,6mm, 5um Absorbosphere UHS C18 250x4,6mm ,10um	MS	propionitrile/ dichloromethane/ acetonitrile.	TG/70 min
Caboni et al, 1996		HPLC	R		ELSD	methanol/ chloroform/ 30%NH_4OH	
Marcato et al, 1996	TLC/SPE	HPLC	R	C8	ELSD	0,1% acetic acid/ acetonitrile/ methylene chloride	
Murphy et al. 1996				Selectosil silica 250x4,6 mm, 5-6um	UV/ELSD	n-hexane/ 2-propanol/0,1% acetic acid	Pl+NL/ 50min
McHowat et al, 1996		HPLC	R	C18	UV	methanol/ water/ acetonitrile	PL /110min
Laakso and Manninen 1997		SFC	N		MS		TG / 50min
Silversand et al, 1997	TLC	HPLC	N	LiChrospher 100 Diol	ELSD	chloroform/ methanol/ acetic acid / water/ TMP	
Fang et al, 1998		HPLC	R	Alltech Jordi gel 150x4,6, 5um	MS	NH_4OH 2%< water/ acetonitrile/ methanol	
Homan et al, 1998		HPLC		Spherisorb SW silica 100x4,6mm	ELSD	isooctane/ tetrahydrofuran/ acetone/ dichlomethane/ 2-propanol/ water	Only classes of PL/20 min
Hvattum et al, 1998		HPLC	N	LiChrospher 100 Diol	MS	chloroform/ methanol	
Landi et al, 1998		HPLC	R	Lichrosorb Si-60 250x4,6mm, 10um	ELSD	chloroform/methanol/water n-hexane/ 2- propanol/water acetonitrile/methanol/water	PL
Lin et al, 1998		HPLC	R	C8	UV	methanol/ NH_4OH	PL standards/ 50min
Brouwers et al, 1999		HPLC	R	LiChrospher followed by RP 18 250x4mm	MS/ ELSD	methanol/ acetonitrile	
Lin et al, 1999		HPLC	R	C18	UV/ELSD	methanol/ acetic acid / 2-propanol/ water	

Attachment 1 – Table of separation techniques used for lipid determination

Sas et al, 1999		HPLC	N	YMC-PAK Diol 250x4,6 mm, 5um	ELSD	n-hexane: methanol:aceton: acetic acid	
Carrier et al, 2000		HPLC	R	AAgilent Hypersil 100x2,1mm, 5um	MS		
Lesnefsky et al, 2000		HPLC	N	Alltech Hypersil Silica 250x4,6mm, 5□m	UV	n-hexane/ 2-propanol/ ethanol/ acetic acid	
Lesnefsky et al, 2000		HPLC	R	Alltech Hypersil 150x2,1mm, 5um	MS		
Morril et al 2000		HPLC	N	300x3,9mm	UV	n-hexane/ 2-propanol/0,1% acetic acid	Only classes of Lipids/15 min
Mu and Hoy 2000		HPLC		Supelcosil LC-C18 250x4,6mm, 5um	MS	n-hexane/ 2-propanol	
Qiu et al, 2000	TLC	HPLC	R	C18 Beckman Hypersil 250x4,6mm, 5um	MS	NH_4OH/n-hexane/ methanol	
Ostrawska et al, 2000		HPLC	R	C18	UV	methanol/water/ acetonitrile	PL/150min
Uran et al, 2001		HPLC	N	LiChrospher 100 Diol	MS	chloroform/ methanol	
Larsen et al, 2002		HPLC	R	C18	ELSD/MS	NH_4CH_3COO	
Fagan et al, 2004		HPLC	N	PVA-guard column	ELSD	dichlormethane /trimetylpentane	
Adachi et al, 2004	SPE	HPLC	R	C8 Luna 150x1mm, 5um	Q-TOF	methanol/ ethanol/ NH_4COO< water	
Malone and Evans 2004		HPLC	RP	C 18 BetaBasic 100x1,0mm, 3um	MS	methanol/ 2-propanol	Partially separated TG/35min
Neron et al, 2004	SPE/TLC	HPLC		LiChrospher 100 Diol 100x4mm, 5um	ELSD	n-hexane/2-propanol/water	
Ohler et al, 2004					ELSD/NMR		
Persson et al, 2007	SPE	HPLC			ELSD	methanol/triethylamine/0,5% formic acid	Only classes of PL/ 30 min NL/ 60 min
Lee et al, 2007		HPLC	R/N	N – Chrompack Si60 300x3mm 5um R – Ultrasphare ODS 250x4,6mm 5um	ELSD/MS		Only classes of NL/ 35 min
Buytovitch et al, 2007		HPLC	N	Lichrosorb Si-60 250x4,6mm, 10um	APCI/MS	n-hexane/2-propanol	70 minutes
Mawatari et al, 2007		HPLC		LiChrospher 100 Diol 100x4mm, 5□m	ELSD/UV	n-hexane/2-propanol/water	Only classes of PL/ 30 minut
Sinanoglou et al,		HPLC	R	RP Novac pack	GC/MS	acetone/acetonitrile	NL/ 60 min

2008				C18 300x3,9mm, 4um			
Cvačka et al, 2008	TLC	HPLC		RP Novac pack C18 300x3,9mm, 4um coupled with 150x3,9mm 4um	APCI MS	acetonitrile/2-propanol	TG /130 min.
Pang et al, 2008		HPLC	N	Diol 250x3mm, 5um	MS	n-hexane/2-propanol/ water /formic acid	Only classes of PL/ 50min
Lee et al, 2008			R	Hypersil BDSCPS 250x4,6mm 5um	ELSD/MS	n-hexane/methyl-t-butyl ether/acetic acid	Only classes of NL/ 30 min
Van der Klift et al, 2008		HPLC		Nucleosil 100 5-SA (Ag) coupled Zorbax elipse XDB-C18 30x4,6mm, 1,8um	UV/ELSD/MS	methanol/methyl-t-butyl ether	TG/ 260min
Graeve et al 2009		HPLC		Chromolith Performance – Si100x4,6mm 2um	ELSD	isooctane/ethylacetate/2-propanol/ water	Only lipid classes / 35 min

Attachment 2 – Table of ionizations techniques and used detectors for lipid determination

literature	ionization	mode	detector	target lipids PL	NL	
Jensen and Gross 1988	CI/FAB/					
Duffin and Henion 1991	ESI	+	triple-quadrupole		MG, DG, TG	fragmentation are shown
Matsubara and Hayashi	FAB	+/-		PC, PE, PS, CER		fragmentation are shown
Kerwin et al 1994	ESI	+/-	triple-quadrupole	PC, PE, PS, PI, SM		
Kim et al 1994	ESI	+	triple-quadrupole	PC, PE, PS, PI		fragmentation are shown
Neff and Byrdwell 1995	APCI	+			TG	fragmentation are shown
Byrdwell et al 1996	APCI	+			TG	fragmentation are shown
Han and Gross 1996	ESI	+	triple-quadrupole	Lyso PC, Lyso PE		fragmentation are shown
Karlsson et al 1996	ESI/APCI	+/-	triple-quadrupole	PC, PE, PS, PI, SM		
Brugger et al 1997	nano ESI	+/-	triple-quadrupole	PC, PE, PS, PI, SM		fragmentation are shown
Laakso and Manninen 1997	APCI	+	trap		TG	fragmentation are shown
Cheng and Gross 1998	ESI/APCI	+	trap		TG	fragmentation are shown
Browers et al 1999	ESI	+/-	quadrupole	PE		fragmentation are shown
Fridriksson et al 1999	ESI	+/-	Fourier transform ion cyclotron resonance	PC, PE, PS, PI, PG,PA		
Khaselev and Murphy 1999	ESI	+/-		PE		fragmentation are shown
Hsu and Turk 2000	ESI	+	triple-quadrupole	PE		fragmentation are shown
Carrier et al 2000	APCI	+	triple-quadrupole	PC,PE, Lyso-PC		
Mu et al 2000	APCI		trap		DG, TG	fragmentation are shown
Mu et al 2000	APCI	+	trap		TG	fragmentation are shown
Rutters et al 2000	ESI	+	trap	PC, PE, PS, PI, PG,PA		
Taguchi et al 2000	ESI		triple-quadrupole	PC,PE,PS		fragmentation are shown
Lytle et al 2000	ESI	-	triple-quadrupole	PE, PC, PG		fragmentation are shown
DeLong et al 2001	ESI	+	triple-quadrupole	PC (PC-O), PE, PS		fragmentation are shown
Koivusalo et al 2001	ESI	+/-	triple-quadrupole/ trap	PC, PE, PS, SM		fragmentation are shown
Ekroos et al 2003	ESI	+	quadrupole - time of flight/ trap			fragmentation are shown
Han and Gross 2003	ESI	+		PC, PE, PG, PI, PS,SM	TG	
Hsu et al 2003	ESI	+	triple-quadrupole	PC, LysoPC, PC-o-alkyl		fragmentation are shown
Forrester et al 2004	ESI			PC, PE, PS, PI, SM,		
Malone and Evans 2004	ESI	+	ion trap		TG	fragmentation are shown
Byrdwell 2005	APCI	+	ion trap		TG	fragmentations and regioizomers

Attachment 2 – Table of ionizations techniques and used detectors for lipid determination

Han and Gross 2005	ESI	+/-				
McAnoy et al 2005	ESI	+	ion trap		TG	fragmentation are shown
Taguchi et al 2005	ESI	+/-	triple-quadrupole	PC, PE, PS, PI, PG,SM		
Cai et al 2006	APCI/APPI/ESI	+/-				
Murphy et al 2006	ESI	+/-	triple-quadrupole/ trap	PC, PE, PS, PI, SM	MG, DG, TG	fragmentation are shown
Schwundke et al 2006	ESI	+/-	quadrupole - time of flight	PC, PE	TG	
Thomas et al 2006	ESI (O2)	-	ion trap	PA		double bound within fatty acid determination
Zhang and Reid 2006	nano ESI	-	ion trap	PC		
Cvacka et al 2008	APCI	+	ion trap		TG	
Pang et al 2008	ESI	-	triple-quadrupole/ trap	PC, PE, PS, PI, PG, SM		
Cui and Thomas 2009	ESI	+	triple-quadrupole		MG, DG, TG	fragmentation/ more ionization study
Hsu and Turk 2009	ESI/MALDI	+/-	triple-quadrupole/ trap	PC, PE, PS, PA, PG		fragmentation and its mechanisms are shown.

Attachment 3 – Table of ions caused by loose of particular fatty acid in particular TG

m/z	no. of carbons : no. of double bonds	12:0	14:0	14:1	15:0	16:0	16:1	16:2	17:0	17:1	18:0	18:1	18:2	18:3	19:0	19:1	20:4
[M+HN4]+	mass of fatty acid + NH4	217	245	243	259	273	271	269	287	285	301	299	297	295	315	313	321
816	C 48:4	599	571	573	557	543	545	547	529	531	515	517	519	521	501	503	495
818	C 48:3	601	573	575	559	545	547	549	531	533	517	519	521	523	503	505	497
820	C 48:2	603	575	577	561	547	549	551	533	535	519	521	523	525	505	507	499
822	C 48:1	605	577	579	563	549	551	553	535	537	521	523	525	527	507	509	501
824	C 48:0	607	579	581	565	551	553	555	537	539	523	525	527	529	509	511	503
826	C 49:6	609	581	583	567	553	555	557	539	541	525	527	529	531	511	513	505
828	C 49:5	611	583	585	569	555	557	559	541	543	527	529	531	533	513	515	507
830	C 49:4	613	585	587	571	557	559	561	543	545	529	531	533	535	515	517	509
832	C 49:3	615	587	589	573	559	561	563	545	547	531	533	535	537	517	519	511
834	C 49:2	617	589	591	575	561	563	565	547	549	533	535	537	539	519	521	513
836	C 49:1	619	591	593	577	563	565	567	549	551	535	537	539	541	521	523	515
838	C 49:0	621	593	595	579	565	567	569	551	553	537	539	541	543	523	525	517
840	C 50:6	623	595	597	581	567	569	571	553	555	539	541	543	545	525	527	519
842	C 50:5	625	597	599	583	569	571	573	555	557	541	543	545	547	527	529	521
844	C 50:4	627	599	601	585	571	573	575	557	559	543	545	547	549	529	531	523
846	C 50:3	629	601	603	587	573	575	577	559	561	545	547	549	551	531	533	525
848	C 50:2	631	603	605	589	575	577	579	561	563	547	549	551	553	533	535	527
850	C 50:1	633	605	607	591	577	579	581	563	565	549	551	553	555	535	537	529
852	C 50:0	635	607	609	593	579	581	583	565	567	551	553	555	557	537	539	531
854	C 51:6	637	609	611	595	581	583	585	567	569	553	555	557	559	539	541	533
856	C 51:5	639	611	613	597	583	585	587	569	571	555	557	559	561	541	543	535
858	C 51:4	641	613	615	599	585	587	589	571	573	557	559	561	563	543	545	537
860	C 51:3	643	615	617	601	587	589	591	573	575	559	561	563	565	545	547	539
862	C 51:2	645	617	619	603	589	591	593	575	577	561	563	565	567	547	549	541
864	C 51:1	647	619	621	605	591	593	595	577	579	563	565	567	569	549	551	543
866	C 51:0	649	621	623	607	593	595	597	579	581	565	567	569	571	551	553	545
868	C 52:6	651	623	625	609	595	597	599	581	583	567	569	571	573	553	555	547
870	C 52:5	653	625	627	611	597	599	601	583	585	569	571	573	575	555	557	549
872	C 52:4	655	627	629	613	599	601	603	585	587	571	573	575	577	557	559	551
874	C 52:3	657	629	631	615	601	603	605	587	589	573	575	577	579	559	561	553
876	C 52:2	659	631	633	617	603	605	607	589	591	575	577	579	581	561	563	555

Attachment 3 – Table of ions caused by loose of particular fatty acid in particular TG

878	C 52:1	661	633	635	619	605	607	609	591	593	577	579	581	583	563	565	557
880	C 52:0	663	635	637	621	607	609	611	593	595	579	581	583	585	565	567	559
882	C 53:6	665	637	639	623	609	611	613	595	597	581	583	585	587	567	569	561
884	C 53:5	667	639	641	625	611	613	615	597	599	583	585	587	589	569	571	563
886	C 53:4	669	641	643	627	613	615	617	599	601	585	587	589	591	571	573	565
888	C 53:3	671	643	645	629	615	617	619	601	603	587	589	591	593	573	575	567
890	C 53:2	673	645	647	631	617	619	621	603	605	589	591	593	595	575	577	569
892	C 53:1	675	647	649	633	619	621	623	605	607	591	593	595	597	577	579	571
894	C 53:0	677	649	651	635	621	623	625	607	609	593	595	597	599	579	581	573
896	C 54:6	679	651	653	637	623	625	627	609	611	595	597	599	601	581	583	575
898	C 54:5	681	653	655	639	625	627	629	611	613	597	599	601	603	583	585	577
900	C 54:4	683	655	657	641	627	629	631	613	615	599	601	603	605	585	587	579
902	C 54:3	685	657	659	643	629	631	633	615	617	601	603	605	607	587	589	581
904	C 54:2	687	659	661	645	631	633	635	617	619	603	605	607	609	589	591	583
906	C 54:1	689	661	663	647	633	635	637	619	621	605	607	609	611	591	593	585
908	C 54:0	691	663	665	649	635	637	639	621	623	607	609	611	613	593	595	587
910	C 55:6	693	665	667	651	637	639	641	623	625	609	611	613	615	595	597	589
912	C 55:5	695	667	669	653	639	641	643	625	627	611	613	615	617	597	599	591

Attachment 4 – Table of ions caused by loose of particular fatty acid in particular DG

DG	Residuum of fatty acids															
m/z	**12:0**	**14:0**	**14:1**	**15:0**	**16:0**	**16:1**	**16:2**	**17:0**	**17:1**	**18:0**	**18:1**	**18:2**	**18:3**	**19:0**	**19:1**	**20:0**	**20:4**
[M+HN4]+	217	245	243	259	273	271	269	287	285	301	299	297	295	315	313	329	321
582	365	337	339	323	309	311	313	295	297	281	283	285	287	267	269	253	261
584	367	339	341	325	311	313	315	297	299	283	285	287	289	269	271	255	263
586	369	341	343	327	313	315	317	299	301	285	287	289	291	271	273	257	265
596	379	351	353	337	323	325	327	309	311	295	297	299	301	281	283	267	275
598	381	353	355	339	325	327	329	311	313	297	299	301	303	283	285	269	277
606	389	361	363	347	333	335	337	319	321	305	307	309	311	291	293	277	285
608	391	363	365	349	335	337	339	321	323	307	309	311	313	293	295	279	287
610	393	365	367	351	337	339	341	323	325	309	311	313	315	295	297	281	289
612	395	367	369	353	339	341	343	325	327	311	313	315	317	297	299	283	291
614	397	369	371	355	341	343	345	327	329	313	315	317	319	299	301	285	293
622	405	377	379	363	349	351	353	335	337	321	323	325	327	307	309	293	301
624	407	379	381	365	351	353	355	337	339	323	325	327	329	309	311	295	303
626	409	381	383	367	353	355	357	339	341	325	327	329	331	311	313	297	305
628	411	383	385	369	355	357	359	341	343	327	329	331	333	313	315	299	307
630	413	385	387	371	357	359	361	343	345	329	331	333	335	315	317	301	309
632	415	387	389	373	359	361	363	345	347	331	333	335	337	317	319	303	311
634	417	389	391	375	361	363	365	347	349	333	335	337	339	319	321	305	313
636	419	391	393	377	363	365	367	349	351	335	337	339	341	321	323	307	315
638	421	393	395	379	365	367	369	351	353	337	339	341	343	323	325	309	317
640	423	395	397	381	367	369	371	353	355	339	341	343	345	325	327	311	319
642	425	397	399	383	369	371	373	355	357	341	343	345	347	327	329	313	321
650	433	405	407	391	377	379	381	363	365	349	351	353	355	335	337	321	329
652	435	407	409	393	379	381	383	365	367	351	353	355	357	337	339	323	331
664	447	419	421	405	391	393	395	377	379	363	365	367	369	349	351	335	343
666	449	421	423	407	393	395	397	379	381	365	367	369	371	351	353	337	345
668	451	423	425	409	395	397	399	381	383	367	369	371	373	353	355	339	347
690	473	445	447	431	417	419	421	403	405	389	391	393	395	375	377	361	369

Attachment 5 – Table of ions caused by loose of particular fatty acid in particular PE

PE [M+1] FullScan m/z		**PE [M-141]** MS/MS m/z	**Residuum of fatty acids *sn*1**															
			12:0	14:0	14:1	15:0	16:0	16:1	16:2	17:0	17:1	18:0	18:1	18:2	18:3	19:0	19:1	20:4
			200	228	226	242	256	254	252	270	268	284	282	280	278	298	296	304
660	C30:2	519	319	291	293	277	263	265	267	249	251	235	237	239	241	221	223	215
662	C30:1	521	321	293	295	279	265	267	269	251	253	237	239	241	243	223	225	217
664	C30:0	523	323	295	297	281	267	269	271	253	255	239	241	243	245	225	227	219
688	C32:2	547	347	319	321	305	291	293	295	277	279	263	265	267	269	249	251	243
690	C32:1	549	349	321	323	307	293	295	297	279	281	265	267	269	271	251	253	245
704	C33:1	563	363	335	337	321	307	309	311	293	295	279	281	283	285	265	267	259
714	C34:3	573	373	345	347	331	317	319	321	303	305	289	291	293	295	275	277	269
716	C34:2	575	375	347	349	333	319	321	323	305	307	291	293	295	297	277	279	271
718	C34:1	577	377	349	351	335	321	323	325	307	309	293	295	297	299	279	281	273
720	C34:0	579	379	351	353	337	323	325	327	309	311	295	297	299	301	281	283	275
730	C35:2	589	389	361	363	347	333	335	337	319	321	305	307	309	311	291	293	285
732	C35:1	591	391	363	365	349	335	337	339	321	323	307	309	311	313	293	295	287
736	C36:6	595	395	367	369	353	339	341	343	325	327	311	313	315	317	297	299	291
738	C36:5	597	397	369	371	355	341	343	345	327	329	313	315	317	319	299	301	293
740	C36:4	599	399	371	373	357	343	345	347	329	331	315	317	319	321	301	303	295
742	C36:3	601	401	373	375	359	345	347	349	331	333	317	319	321	323	303	305	297
744	C36:2	603	403	375	377	361	347	349	351	333	335	319	321	323	325	305	307	299
746	C36:1	605	405	377	379	363	349	351	353	335	337	321	323	325	327	307	309	301
757	C37:3	616	416	388	390	374	360	362	364	346	348	332	334	336	338	318	320	312
759	C37:2	618	418	390	392	376	362	364	366	348	350	334	336	338	340	320	322	314
761	C37:1	620	420	392	394	378	364	366	368	350	352	336	338	340	342	322	324	316
771	C38:3	630	430	402	404	388	374	376	378	360	362	346	348	350	352	332	334	326
773	C38:2	632	432	404	406	390	376	378	380	362	364	348	350	352	354	334	336	328
775	C38:1	634	434	406	408	392	378	380	382	364	366	350	352	354	356	336	338	330
777	C38:0	636	436	408	410	394	380	382	384	366	368	352	354	356	358	338	340	332
779	C39:6	638	438	410	412	396	382	384	386	368	370	354	356	358	360	340	342	334
781	C39:5	640	440	412	414	398	384	386	388	370	372	356	358	360	362	342	344	336
783	C39:4	642	442	414	416	400	386	388	390	372	374	358	360	362	364	344	346	338
785	C39:3	644	444	416	418	402	388	390	392	374	376	360	362	364	366	346	348	340
786	C39:2	645	445	417	419	403	389	391	393	375	377	361	363	365	367	347	349	341

Attachment 5 – Table of ions caused by loose of particular fatty acid in particular PE

788	C39:1	647	447	419	421	405	391	393	395	377	379	363	365	367	369	349	351	343
790	C39:0	649	449	421	423	407	393	395	397	379	381	365	367	369	371	351	353	345
792	C40:6	651	451	423	425	409	395	397	399	381	383	367	369	371	373	353	355	347
810	C41:4	669	469	441	443	427	413	415	417	399	401	385	387	389	391	371	373	365

	Acylium residuum of fatty acids															
	12:0	14:0	14:1	15:0	16:0	16:1	16:2	17:0	17:1	18:0	18:1	18:2	18:3	19:0	19:1	20:4
	183	**211**	**209**	**225**	**239**	**237**	**235**	**253**	**251**	**267**	**265**	**263**	**261**	**281**	**279**	**287**

Attachment 6 – Table of ions caused by loose of particular fatty acid in particular PC

PC [M+1]-RCOOH m/z		Residuum of fatty acids																
		12:0	14:0	14:1	15:0	16:0	16:1	16:2	17:0	17:1	18:0	18:1	18:2	18:3	19:0	19:1	20:0	20:4
		200	228	226	242	256	254	252	270	268	284	282	280	278	298	296	312	304
664	C27:0	464	436	438	422	408	410	412	394	396	380	382	384	386	366	368	352	360
674	C28:2	474	446	448	432	418	420	422	404	406	390	392	394	396	376	378	362	370
676	C28:1	476	448	450	434	420	422	424	406	408	392	394	396	398	378	380	364	372
688	C29:2	488	460	462	446	432	434	436	418	420	404	406	408	410	390	392	376	384
690	C29:1	490	462	464	448	434	436	438	420	422	406	408	410	412	392	394	378	386
704	C30:1	504	476	478	462	448	450	452	434	436	420	422	424	426	406	408	392	400
714	C31:3	514	486	488	472	458	460	462	444	446	430	432	434	436	416	418	402	410
716	C31:2	516	488	490	474	460	462	464	446	448	432	434	436	438	418	420	404	412
718	C31:1	518	490	492	476	462	464	466	448	450	434	436	438	440	420	422	406	414
720	C31:0	520	492	494	478	464	466	468	450	452	436	438	440	442	422	424	408	416
728	C32:3	528	500	502	486	472	474	476	458	460	444	446	448	450	430	432	416	424
730	C32:2	530	502	504	488	474	476	478	460	462	446	448	450	452	432	434	418	426
732	C32:1	532	504	506	490	476	478	480	462	464	448	450	452	454	434	436	420	428
740	C33:4	540	512	514	498	484	486	488	470	472	456	458	460	462	442	444	428	436
742	C33:3	542	514	516	500	486	488	490	472	474	458	460	462	464	444	446	430	438
744	C33:2	544	516	518	502	488	490	492	474	476	460	462	464	466	446	448	432	440
746	C33:1	546	518	520	504	490	492	494	476	478	462	464	466	468	448	450	434	442
751	C34:6	551	523	525	509	495	497	499	481	483	467	469	471	473	453	455	439	447
753	C34:5	553	525	527	511	497	499	501	483	485	469	471	473	475	455	457	441	449
755	C34:4	555	527	529	513	499	501	503	485	487	471	473	475	477	457	459	443	451
757	C34:3	557	529	531	515	501	503	505	487	489	473	475	477	479	459	461	445	453
759	C34:2	559	531	533	517	503	505	507	489	491	475	477	479	481	461	463	447	455
761	C34:1	561	533	535	519	505	507	509	491	493	477	479	481	483	463	465	449	457
779	C36:6	579	551	553	537	523	525	527	509	511	495	497	499	501	481	483	467	475
781	C36:5	581	553	555	539	525	527	529	511	513	497	499	501	503	483	485	469	477
783	C36:4	583	555	557	541	527	529	531	513	515	499	501	503	505	485	487	471	479
785	C36:3	585	557	559	543	529	531	533	515	517	501	503	505	507	487	489	473	481
786	C36:2	586	558	560	544	530	532	534	516	518	502	504	506	508	488	490	474	482
788	C36:1	588	560	562	546	532	534	536	518	520	504	506	508	510	490	492	476	484
790	C36:0	590	562	564	548	534	536	538	520	522	506	508	510	512	492	494	478	486
792	C37:6	592	564	566	550	536	538	540	522	524	508	510	512	514	494	496	480	488

Attachment 6 – Table of ions caused by loose of particular fatty acid in particular PC

PC [M+1]- R2CH=O m/z		**Residuum of fatty acids from *sn-1*position**																
		12:0	14:0	14:1	15:0	16:0	16:1	16:2	17:0	17:1	18:0	18:1	18:2	18:3	19:0	19:1	20:0	20:4
		182	210	208	224	238	236	234	252	250	266	264	262	260	280	278	294	286
674	C28:2	492	464	466	450	436	438	440	422	424	408	410	412	414	394	396	380	388
676	C28:1	494	466	468	452	438	440	442	424	426	410	412	414	416	396	398	382	390
688	C29:2	506	478	480	464	450	452	454	436	438	422	424	426	428	408	410	394	402
690	C29:1	508	480	482	466	452	454	456	438	440	424	426	428	430	410	412	396	404
704	C30:1	522	494	496	480	466	468	470	452	454	438	440	442	444	424	426	410	418
714	C31:3	532	504	506	490	476	478	480	462	464	448	450	452	454	434	436	420	428
716	C31:2	534	506	508	492	478	480	482	464	466	450	452	454	456	436	438	422	430
718	C31:1	536	508	510	494	480	482	484	466	468	452	454	456	458	438	440	424	432
720	C31:0	538	510	512	496	482	484	486	468	470	454	456	458	460	440	442	426	434
728	C32:3	546	518	520	504	490	492	494	476	478	462	464	466	468	448	450	434	442
730	C32:2	548	520	522	506	492	494	496	478	480	464	466	468	470	450	452	436	444
732	C32:1	550	522	524	508	494	496	498	480	482	466	468	470	472	452	454	438	446
740	C33:4	558	530	532	516	502	504	506	488	490	474	476	478	480	460	462	446	454
742	C33:3	560	532	534	518	504	506	508	490	492	476	478	480	482	462	464	448	456
744	C33:2	562	534	536	520	506	508	510	492	494	478	480	482	484	464	466	450	458
746	C33:1	564	536	538	522	508	510	512	494	496	480	482	484	486	466	468	452	460
750	C34:6	568	540	542	526	512	514	516	498	500	484	486	488	490	470	472	456	464
752	C34:5	570	542	544	528	514	516	518	500	502	486	488	490	492	472	474	458	466
755	C34:4	573	545	547	531	517	519	521	503	505	489	491	493	495	475	477	461	469
757	C34:3	575	547	549	533	519	521	523	505	507	491	493	495	497	477	479	463	471
759	C34:2	577	549	551	535	521	523	525	507	509	493	495	497	499	479	481	465	473
761	C34:1	579	551	553	537	523	525	527	509	511	495	497	499	501	481	483	467	475
779	C36:6	597	569	571	555	541	543	545	527	529	513	515	517	519	499	501	485	493
781	C36:5	599	571	573	557	543	545	547	529	531	515	517	519	521	501	503	487	495
783	C36:4	601	573	575	559	545	547	549	531	533	517	519	521	523	503	505	489	497
785	C36:3	603	575	577	561	547	549	551	533	535	519	521	523	525	505	507	491	499
786	C36:2	604	576	578	562	548	550	552	534	536	520	522	524	526	506	508	492	500
788	C36:1	606	578	580	564	550	552	554	536	538	522	524	526	528	508	510	494	502
790	C36:0	608	580	582	566	552	554	556	538	540	524	526	528	530	510	512	496	504
792	C37:6	610	582	584	568	554	556	558	540	542	526	528	530	532	512	514	498	506

Attachment 7 – Unpublished PLs and NLs determination

Unpublished determinated PLs in *D.melanogaster* whole body sample		positive mode	negative mode		neutral loose
mark	PL ID	$[M+H]^+$	$[M-H]^-$	$[M+59]^-$	
476	Lyso PE 18:3	476.4	474.4	-	-18/-141
466	Lyso PC 14:1	466.4	464.4	-	-18
478	Lyso PE 18:2	478.4	476.4	-	-18/-141
454	Lyso PE 16:0	454.4	452.4	-	-18/-141
480	Lyso PE 18:1	480.4	478.4	-	-18/-141
494	Lyso PC 16:1	494.4	492.4	-	-18
520	Lyso PC 18:2	520.4	518.4	-	-18
496	Lyso PC 16:0	496.4	494.4	-	-18
522	Lyso PC 18:1	522.4	520.4	-	-18
468	Lyso PC 14:0	468.4	466.4	-	-18
452	Lyso PE 16:1	452.4	450.4	-	-18/-141
660	PE 14:1/16:1	660.5	658.5	-	-141
662	PE 16:1/14:0	662.5	660.5	-	-141
688	PE 16:1/16:1	688.5	686.5	-	-141
688_1	PE 14:0/18:2	688.5	686.5	-	-141
714	PE 16:1/18:2	714.5	712.5	-	-141
714_1	PE 16:0/18:3	714.5	712.5	-	-141
740	PE 18:2/18:2	740.5	738.5	-	-141
702	PC 14:1/16:1//16:1/14:1	702.4	-	760.4	
690	PE 16:0/16:1//16:1:16:0	690.5	688.5	-	-141
786_PS	PS 18:1/18:2	786.5	784.5	-	-185
716	PE 16:1/18:1//18:1/16:1	716.5	714.5	-	-141
716_1	PE 16:0/18:2	716.5	714.5	-	-141
742	PE 18:2/18:1//18:1/18:2	742.5	740.5	-	-141
704	PC 14:0/16:1	704.5	-	762.5	
788_PS	PS 18:1/18:1	788.6	786.6	-	-185
730	PC 16:1/16:1	730.6	-	788.6	
704_1	PC 16:0/14:1	704.5	-	762.5	
730_1	PC 14:0/18:2	730.6	-	788.6	
756	PC 16:1/18:2	756.6	-	814.6	
718	PE 16:0/18:1	718.5	716.5	-	-141
744	PE 18:1/18:1	744.5	742.5	-	-141
782	PC 18:2/18:2	782.6	-	840.6	
744_1	PE 18:0/18:2	744.5	742.5	-	-141
790_PS	PS 18:0/18:1	790.5	788.5	-	-185

732	PC 16:0/16:1//16:1/16:0// 14:0/18:1	732.6	-	790.6	
758	PC 16:1/18:1//18:1/16:1	758.6	-	816.6	
792_PS	PS 18:0/18:0	792.5	790.5	-	-185
758_1	PC 16:0/18:2//18:216:0	758.6	-	816.6	
746	PE 18:0/18:1	746.5	744.5	-	-141
784	PC 18:1/18:2//18:2/18:1	784.6	-	842.6	
760	PC 16:0/18:1//18:1/16:0	760.6	-	818.6	
786	PC 18:1/18:1	786.6	-	844.6	
786_1	PC 18:0/18:2	786.6	-	844.6	
788	PC 18:1/18:0	788.6	-	846.6	

Unpublished determinated TGs in *P.apterus* fat body sample					
mark	**[M+NH4]$^+$**	**C:DB**	**TG ID**		
828	828.6	C 49:5	13:1/18:2/18:2		
830	830.6	C 49:4	13:1/18:1/18:2		
842_1	842.6	C 50:5	14:0/16:0/20:5		
842_2	842.6	C 50:5	13:1/18:2/19:2		
846	846.6	C 50:3	14:0/18:1/18:2		
848	848.6	C 50:2	16:0/16:0/18:2		
850	850.6	C 50:1	16:0/16:0/18:1		
860	860.6	C 51:3	16:0/17:1/18:2		
870_1	870.6	C 52:5	16:1/18:2/18:2		
870_2	870.6	C 52:5	16:2/18:1/18:2		
872_1	872.6	C 52:4	16:/18:1/18:2		
872_2	872.6	C 52:4	16:0/18:2/18:2		
874	874.6	C 52:3	16:0/18:1/18:2		
876_1	876.6	C 52:2	16:0/18:1/18:1		
876_2	876.6	C 52:2	16:0/18:0/18:2		
878.6	878.6	C 52:1	16:0/18:0/18:1		
882_1	882.6	C 53:6	17:2/18:2/18:2		
882_2	882.6	C 53:6	17:3/18:1/18:2		
884	884.6	C 53:5	17:1/18:2/18:2	17:2/18:1/18:2	2:1
886_1	886.6	C 53:4	17:1/18:1/18:2		
886_2	886.6	C 53:4	16:0/18:2/19:2		
888	888.6	C 53:3	17:1/18:0/18:2	16:0/18:1/19:2	1:1
890_1	890.7	C 53:2	16:0/18:1/19:1		
890_2	890.7	C 53:2	15:0/18:2/20:0		
894_1	894.7	C 54:7	18:1/18:2/18:4		
894_2	894.7	C 54:7	18:2/18:2/18:3		
896	896.7	C 54:6	18:2/18:2/18:2	18:1/18:2/18:3	
898_1	898.7	C 54:5	18:1/18:2/18:2		
898_2	898.7	C 54:5	18:2/18:2/18:1		
900_1	900.7	C 54:4	18:1/18:1/18:2		
900_n	900.7	C 54:4	18:0/18:1/18:3		
900_2	900.7	C 54:4	18:0/18:2/18:2		
902_1	902.7	C 54:3	18:1/18:1/18:1		
902_2	902.7	C 54:3	18:0/18:1/18:2		
904_1	904.7	C 54:2	18:0/18:1/18:1		
904_2	904.7	C 54:2	18:0/18:0/18:2		

910_1	910.7	C 55:6	18:2/18:2/19:2
912_1	912.7	C 55:5	18:2/18:2/19:1
912_2	912.7	C 55:5	18:1/18:2/19:2
914	914.7	C 55:4	18:1/18:2/19:1
914_n	914.7	C 55:4	18:0/18:3/19:1
916_1	916.7	C 55:3	18:0/18:2/19:1
916_2	916.7	C 55:3	18:1/18:2/19:0
926_1	926.7	C 56:5	18:2/18:2/20:1
928	928.7	C 56:4	18:1/18:2/20:1
930	930.7	C 56:3	18:1/18:2/20:0
942	942.7	C 57:4	18:2/19:1/20:1
944	944.7	C 57:3	18:2/19:0/20:1
946	946.7	C 57:2	18:2/19:0/20:0
956	956.7	C 58:6	18:2/18:2/22:0

Unpublished determinated DGs in *P.apterus* haemolymph sample					
mark	**[M+NH4]$^{+}$**	**C:DB**	**DG ID**		
582	582.3	C 32:2	16:1/16:1		
584	584.3	C 32:1	16:0/16:1		
586	586.3	C 32:0	16:0/16:0		
608	608.4	C 34:3	16:1/18:2		
610	610.4	C 34:2	16:0/18:2		
612	612.4	C 34:1	16:0/18:1		
614	614.4	C 34:0	16:0/18:0		
620	620.4	C 35:4	17:2/18:2		
622	622.4	C 35:3	17:1/18:2	17:2/18:1	1:1
622_1	622.4	C 35:3	17:0/18:3		
624	624.4	C 35:2	17:1/18:1		
624_1	624.4	C 35:2	17:0/18:2		
626	626.4	C 35:1	17:0/18:1		
630	630.4	C 36:6	18:3/18:3		
632	632.4	C 36:5	18:2/18:3		
634	634.4	C 36:4	18:2/18:2		
634_1	634.4	C 36:4	18:1/18:3		
636	636.4	C 36:3	18:1/18:2		
636_1	366.4	C 36:3	18:0/18:3		
638	638.4	C 36:2	18:1/18:1		
638_1	638.4	C 36:2	18:0/18:2		
640	640.4	C 36:1	18:0/18:1		
642	642.4	C 36:0	18:0/18:0		