POLAR LIPID SEPARATION AND IDENTIFICATION IN TURKEY MUSCLE TISSUE BY LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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Abstract – A method for the analysis of glycerophospholipids and sphingomyelins is described. The lipid fraction was extracted by a mix of chloroform and methanol. Polar lipids (PL's) were purified using zirconia coated silica SPE cartridges (HybridSPE®-PL). PL classes were separated by hydrophobic interaction chromatography (HILIC) and detected by an ion trap mass spectrometer. The structure of the PL's was established or confirmed by tandem mass spectrometry both in positive and negative mode. The application of the method in 15 turkey muscle meat samples (breast and tight) revealed the abundance of different PL classes as well as differences in the abundance of single PL´s between the individual samples. The method provides a tool to explore the variation and metabolism of PL's as a function of the investigated conditions.

Key Words – Glycerophospholipid, Hydrophilic interaction chromatography, Ion trap mass spectrometry, Sphingomyelin, SPE

INTRODUCTION

Polar lipids (PL's) are important functional components of meat and situated mostly in cell membranes. They consist of a polar phosphoryl head group with different fatty acid esters at the sn1 and/or sn2 position of a glycerol backbone. Sphingomyelins, being also phospholipids, have instead of a glycerol a sphingosine backbone and a phophocholine as polar head group [1]. Phospholipids have a significant role in the flavor development in meat products [2]. Prooxidant as well as antioxidant mechanisms of phospholipids are currently discussed [3]. Furthermore phospholipids containing nitrogen, such as phosphatidylethanolamine, are precursor of Strecker degradation products and Maillard reaction products [4] which are important for the acceptance of the product by the consumer [2].

Extraction procedures are necessary in the analysis of phospholipids to separate them from other constituents such as proteins or sugars – components that would interfere with also necessary chromatographic steps. There are several approaches to extract polar lipids. Most of them include solid-phase extraction procedures using several phases such as amino propyl- or silica. Recently zirconia coated SPE phases were developed, that bind PL's using a Lewis acid-base interaction [5].

The separation of the extracted PL classes can be achieved by reverse phase chromatography separating the compounds according to their hydrophobicity, or, according to the "effective carbon number" (number of carbon atoms minus number of double bonds in the acyl chains) [6]. Secondly a separation of PL classes can be obtained by means of normal phase chromatography by polar interactions of the stationary phase with the analytes [7]. However, normal phase HPLC can be difficult because strict pH conditions or non-volatile buffers are needed which are not compatible with electrospray ionization mass spectrometry (ESI-MS) [1]. To overcome these difficulties hydrophilic interaction chromatography (HILIC) is used achieving ESI – MS compatible separation conditions. Moreover, amphiphilic compounds as PL's can be separated very well using HILIC [8].

Aim of the current study was the development of a quick and reliable analytical method to separate and detect PL-classes as well as single PL's with as little as possible interferences from the complex

meat matrix. It is understood as a methodic preliminary work but also to provide systematic knowledge of the occurrence of phospholipids in meat of varying origin – which is, to our best knowledge – not available so far.

I. MATERIALS AND METHODS

Chemicals

Methanol (LC-MS grade) was purchased from Merck KGaA, Darmstadt. Ammonium acetate (LC-MS grade), ammonia solution in methanol (2.0M), HybridSPE®- Phospholipid cartridges, Phospholipid mixture for HPLC from Glycine max (soybean), sphingomyelin from chicken egg yolk and 3-sn-lysoethanolamines from egg yolk as well as Water (LC-MS grade) were purchased from Sigma-Aldrich (Taufkirchen). 1,2-Dipentadecanoyl-sn-glycero-3-phosphatidylcholin (PC (15:0, 15:0)) and Acetonitrile (LC-MS grade) were purchased from LGC Standards GmbH. Lipids were dissolved in methanol/chloroform (1:2). Their concentrations varied from 15µg/mL for lyso-phosphatidylcholines, 45µg/mL for phosphatidylinositols, 60µg/mL for phosphatidyl-ethanolamines, lyso-phosphatidylethanolamines and sphingomyelins and 75µg/mL for phosphatidylcholines.
Samples were purchased at local markets: turkey breast (n=7), tight (n=5) and drumstick (n=3).

Sample preparation

Samples were freed of all visible fat and sinews and subsequently homogenized thoroughly using a Grindomix (Retsch). After this, aliquots were freezed at -20°C until further analysis.

Lipid extraction

Lipids were extracted using a modified Folch method [9]. 3g of the homogenized sample were mixed with 5g sea sand, 10g anhydrous sodium sulphate, 25mL of a mixture of methanol and chloroform (2:1) and 1mL of a 100µg/mL PC (15:0, 15:0) solution at 3000rpm for 90s using a Bühler homogenizer. The resulting mixture is filtered and the solvent is evaporated at 337mbar and 40°C. For protein precipitation 1mL of 1% formic acid in acetonitrile is added and the mixture is transferred into an ultrasonic bath. After precipitation is complete 1mL of the mixture is used for solid phase extraction.

Solid phase extraction

Solid phase extraction of polar lipids was performed using an modified method according to Ferreiro-Vera [5]. 1mL of the extract was transferred to a HybridSPE®- Phospholipid cartridge and a slight vacuum was applied. Subsequently the cartridge was washed with 1mL of acetonitrile. The polar lipids were eluted using 1mL of ammonia solution in methanol (2.0M). The resulting mixture was dried under constant nitrogen flow and the solid residue dissolved in 1mL 90% acetonitrile, 10% water and 2,5mmol/L ammonia acetate. The solution was filtered and kept at -20°C until analysis.

High performance liquid chromatography

A HPLC System of the HP 1100 series (Agilent Technologies) was used. As stationary phase a Kinetex HILIC 2,6µm, 100Å, 150x3mm (phenomenex, Aschaffenburg) was applied. The separation method was adapted according to Losito et al. [8]. Eluent A was 90% acetonitrile, 10% water and 2,5mmol/L ammonia acetate, eluent B 90% methanol, 10% water and 2,5mmol/L ammonia acetate. Gradient elution with a flow of 0,3mL/min started isocratic at 9% B for 6 minutes followed by a linear increase of eluent B up to 36% in 11 minutes and a second isocratic part from minute 11 to minute 25 at 36% B. Subsequently a cleaning step at 100% B for 13 minutes and re-equilibration step at 9% B for 20 minutes followed. Column temperature was set at 30°C and injection volume at 2µL.

Mass spectrometry

Peak detection was done using an HP 1100 Series LC/MSD Trap SL (Agilent Technologies) equipped with an ESI ion source. Nebulizer was set at 40psi, dry gas at 8L/min, dry temperature at 350°C, capillary voltage was set at 3500V. All measurements were done in positive as well as in negative ionization mode over a scan range 100 –

1200m/z using an automatic data dependent MS/MS with an average MS/MS fragmentation amplitude of 1.7V and active exclusion after 2 spectra or 0,3 minutes.

II. RESULTS AND DISCUSSION

Baseline separation was achieved of six phospholipid classes within a runtime of 25 minutes. Fig 1 shows the base peak chromatogram within positive and negative mode, respectively. Elution order is as follows: phosphatidylinositols (PI) at about 2.5 minutes, phosphatidylethanolamines (PE) at about 5 minutes, lyso-phosphatidylethanolamines (LPE) at about 10 minutes, phosphatidylcholines (PC) at about 15 minutes, sphingomyelins (SM) at about 18 minutes and lyso-phosphatidylcholines (LPC) at about 22 minutes.

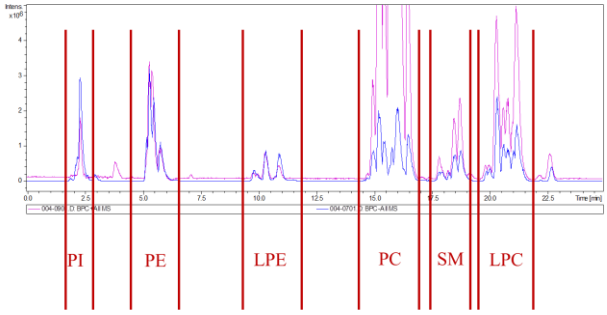

Fig. 1: Base peak chromatogram of HILIC separation of 6 PL classes (PI, PE, LPE, PC, SM and LPC).

Confirmation of lipid classes was done with the help of standard mixtures as well as with the help of characteristic ions: a neutral loss at 141 Da in positive mode for PE, a product ion $[M+H]^+$ at 184 Da for LPE, a neutral loss of 59 Da for PC in positive mode, LPC and SM in positive mode and a product ion $[M-H]^-$ at 241 Da for PI in negative mode.

Besides the baseline separation of the PL classes a partial separation of single components within the classes was observed. The elution order of these single compounds should depend on the effective carbon number (ECN) as described above. This is in agreement with other authors [1, 7].

Basing on the data, achieved with the data dependent automatic MS/MS 65 single PL`s were identified. Identification was done with the help of LipidBlast, an in-silico tandem mass spectrometry database for lipid identification [10]. The assignment of sn1 and sn2 fatty acids was confirmed using either signal intensities of the carboxylate ions ($R1\text{-}COO^-$ and $R2\text{-}COO^-$), assuming that the signal intensity of the ion deriving from the sn2 shows a higher intensity or signal intensities of ions deriving from a neutral loss of the acyl chains [7]. We are aware, that both methods have their own pitfalls, namely due to the dependence of signal intensities on chain length and degree of unsaturation. Table 1 shows only some examples of identified PL's that were identified exclusively in one of the cuts investigated.

Table: Examples for PL'sidentified in the cuts investigated

PL	sn1/sn2	breast	tight	drumstick	Ionisation mode
PI	18:0/18:2			x	Neg.
PE	20:4/18:0			x	Neg.
PE	20:5/18:0	x			Neg.
LPE	22:6/ 0:0		x		Pos.
PC	18:1/18:2		x		Pos.
PC	20:4/16:0	x			Pos.
LPC	18:3/ 0:0			x	Pos.
LPC	20:2/ 0:0	x			Pos.

In the case of sphingomyelins no unambiguous assignment of the acyl chains to sn1 and sn2 position was possible so far.

Recovery experiments with single PL classes showed, that all classes, except PI, can be found in the spiked samples as expected. In the case of PI we assume, that there is a difficulty in recovering the compounds from the SPE phase. Further investigation is under way.

CONCLUSION

In conclusion the HILIC method presented offers a fast and efficient method to separate 6 main PL

classes. The presented SPE method is effective reliable for 5 of the 6 classes. 65 single PL's were identified. The method provides a tool to explore the variation and metabolism of PL's as a function of the investigated conditions.

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