FACULTY OF PHARMACEUTICAL SCIENCES

UNIVERSITÄT BERN

SIMULTANEOUS DETERMINATION OF PHOSPHATIDYLETHANOL AND PHOSPHATIDYLCHOLINE IN ANIMAL TISSUE BY LC-MS/MS

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A Master dissertation for the study programme Master in Drug Development

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SUMMARY

Phosphatidylethanol (PEth) represents a group of abnormal phospholipids, which are only formed in the presence of ethanol, from phosphatidylcholine (PC) through the action of phospholipase D (PLD). Because of its pharmacokinetic characteristics, direct formation after alcohol intake and an approximate half-life of four days, PEth is a promising direct alcohol marker. Several studies investigated PEth formation, in both human and animal tissues, and indicated that PEth formation is dependent on substrate availability. However, the endogenous amounts of PC were never analyzed.

To acquire more detailed insight in the PEth formation and in its substrate availability, a new LC-ESI-MS/MS method, with a core shell C8-column coupled to a Sciex 5500 QTrap triple quadrupole operated in SRM mode, was developed for the simultaneous determination of PEth and PC. To determine PEth and PC within the same method a polarity switch was made, whereby PC was measured in ESI positive mode and PEth in ESI negative mode. The method was validated according to the FDA guidance for human blood samples and applied for the determination of *in vitro* PEth formation in blood-, brain-, liver-, and kidney tissue from a pig, a cattle and a goat, freshly obtained from a local butcher. Tissue samples were spiked with ethanol concentrations ranging from 1-7 g/kg and incubated up to five hours at 37°C. Every 30 minutes an aliquot was taken and added to 1 mL of acetonitrile to stop the PEth formation (protein precipitation). The PEth formation was analyzed by means of Michaelis Menten kinetics and corrected for the actual amount of ethanol present in the tissue samples, which was determined by HS-GC-FID.

The PEth formation in animal blood remained below the LLOQ of 0.0075 µg/mL, which is in agreement with literature. For the PEth formation in the other tissues large variations in PEth formation among the different tissues and also among the different animals were observed. These variations could only partially be explained by the endogenous amounts of PC, present in the tissues, which indicates that also enzyme characteristics contribute to the observed differences. Whether differences in PLD regulation, expression, or the presence of different isotypes of PLD are responsible for variations in PEth formation remains unclear.

SAMENVATTING

Phosphatidylethanol (PEth) verwijst naar een groep abnormale fosfolipiden, die enkel in de aanwezigheid van ethanol gevormd worden, vanuit fosfatidylcholine door de inwerking van het enzym fosfolipase D (PLD). Zijn farmacokinetische eigenschappen, directe vorming na ethanol inname en een halfwaardetijd van ongeveer vier dagen, maken van PEth een veelbelovende directe alcohol merker. Verscheidene studies hebben de vorming van PEth, in zowel menselijk als dierlijk weefsel, onderzocht en toonden aan dat PEth synthese afhankelijk is van de PC beschikbaarheid. Desondanks werd de endogene hoeveelheid PC nooit mee bepaald.

Om een beter inzicht te krijgen in de PEth vorming en PC beschikbaarheid, werd een nieuwe LC-ESI-MS/MS methode, met een core shell C8-kolom gekoppeld aan een Sciex 5500 QTrap triple quadrupole in SRM modus, ontwikkeld om simultaan PEth en PC te bepalen. Hiervoor werd een polarity switch gemaakt, waarbij PC in ESI positieve modus en PEth in ESI negatieve modus gemeten werd. De methode werd gevalideerd voor menselijke bloedstalen volgens de FDA richtlijnen en toegepast om de *in vitro* PEth vorming in bloed-, hersen-, lever- en nierweefsel van een varken, een geit en een rund te bepalen. De weefsels konden verkregen worden bij een plaatselijke slager. De weefsel stalen werden belast met ethanol concentraties gaande van 1 tot 7 g/kg en werden 5 uur lang bij 37°C geïncubeerd. Ieder half uur werd een staal genomen en in 1 mL acetonitrile gebracht om de PEth vorming te stoppen. De PEth synthese werd geanalyseerd aan de hand van Michaelis Menten kinetiek en gecorrigeerd voor de precieze hoeveelheid ethanol aanwezig in het staal, bepaald met HS-GC-FID.

De PEth concentraties in dierlijk bloed gevormd, bleven onder de LLOQ van 0.0075 µg/mL. Dit is in overeenkomst met de literatuur. Grote variaties in PEth vorming tussen de verschillende weefsels en verschillende dieren werden waargenomen. De endogene hoeveelheden PC konden deze verschillen slechts gedeeltelijk verklaren, wat erop wijst dat ook enzym eigenschappen aan de basis van deze geobserveerde verschillen liggen. Of het gaat over verschillen in de regulatie, expressie of verschillende isotypes van PLD blijft onduidelijk.

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TABLE OF CONTENTS

LIST OF USED ABBREVIATIONS

ADH: Alcohol dehydrogenase

ALT: Alanine Aminotransferase

AST: Aspartate Aminotransferase

AUDIT: Alcohol Use Disorders Identification Test

BAC: Blood alcohol concentration

CDT: carbohydrate-deficient transferrin

CE: Collision energy

cps: counts per second

CV: Coefficient of variation

CXP: Collision cell exit potential

CYP: Cytochrome P450

DP: Declustering potential

ESI Electrospray ionization

EtG: Ethyl glucuronide

EtS: Ethyl sulfate

FAEEs: Fatty acid ethyl esters

FDA: U.S. Food and Drug Administration

GGT: Gamma-glutamyl transpeptidase

HS-GC-FID: Headspace gas chromatography coupled to a flame ionization detector

IS: Internal standard

LOD: Limit of detection

LLOQ: Lower limit of quantification

LC-ESI-MS/MS: Liquid chromatography- electrospray- tandem mass spectroscopy

MCV: Mean corpuscular volume

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

PC: Phosphatidylcholine

PC 16:0/18:1: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

PC 16:0/18:2: 1-palmitoyl-2-lineoyl-sn-glycero-3-phosphocholine

PEth: Phosphatidylethanol

PEth 16:0/18:1: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol

PEth 16:0/18:2: 1-palmitoyl-2-lineoyl -*sn*-glycero-3-phosphoethanol

PLD: Phospholipase D

RP: Reversed Phase

RBC: Red blood cell

RT: Retention time

SRM: Selected reaction monitoring

TIC: Total ion current

XIC: Extracted ion chromatogram

1. INTRODUCTION

1.1. ALCOHOL CONSUMPTION AND RELATED HARM

Ethanol, a colorless liquid produced by the fermentation of sugars, is a psychoactive drug which is present in different kinds of beverages such as beer and wine. The use of alcohol is related to all kinds of activities and occasions and considered as an integral part of the Western culture. However, alcohol consumption is not harmless.

Depending on the amount consumed, the effects of alcohol can range from an increase in self-confidence to coma. More than 200 diseases or injury conditions, both physical and mental, are associated with excessive drinking. Some common examples include alcohol use disorder (AUD), liver cirrhosis, and depression.(1, 2) Alcohol has toxic effects on the brain, liver, pancreas and heart and can increase the risk of developing certain cancers such as breast, liver, mouth, neck, and colorectal cancer.(3) These toxic effects are linked to the formation of acetaldehyde and reactive oxygen species after alcohol consumption, which can damage DNA and proteins.(4) It is estimated that alcohol is the cause of 3.3 million deaths worldwide, each year, corresponding to 5.9 % of all deaths.(1)

Whether or not alcohol-related harm is encountered, depends on environmental factors and drinking-related aspects such as the volume of alcohol consumed and drinking patterns. For most of the diseases and injuries affected by alcohol there is a dose-response relationship, i.e. the larger the consumption, the higher the risk. Besides the volume, the pattern of drinking plays a role as well. Drinking alcohol over dinner seems to be less harmful than drinking at other times.

Environmental factors incorporate age, gender, familial risk factors, socioeconomic status, culture, and context. Women reach higher blood alcohol concentrations than men after drinking the same amount of alcohol because they have less body water. Elderly people are more vulnerable for the toxic effects of alcohol due to the fact that they have less body fluid and that their liver and kidney

activity decreases. Adolescents on the other hand are more sensitive to the negative effects of alcohol on the brain since their brain is still developing.(1, 5)

In addition to the negative effects of alcohol on health, the harmful use of alcohol is frequently associated with socioeconomic consequences and burden. Heavy drinkers encounter unemployment, family problems and barriers to the access of health care. However, harms from drinking are not limited to the drinkers, their close circle is also affected, as alcoholics and their families are often marginalized and socially excluded. Traffic accidents, property damage, violence and significant social and economic costs illustrate the burden of alcohol abuse on society.(1, 6)

1.1.1. Trends in alcohol consumption

With an average consumption of 12.5 liters of pure ethanol per capita per year, Europe is the continent with the highest alcohol consumption in the world. The patterns and trends in alcohol consumption differ between different country groupings; Southern and Northern Europe, Central Eastern and Eastern Europe, and Central Western and Western Europe.(7) The average alcohol consumption in Belgium in 2014 was 12.6 L of pure ethanol per capita. Mainly beer and wine were consumed.(8)

Over the past 20 years (1992-2012), alcohol consumption in the industrialized world declined by 2.5%. However, a steady rise is seen for heavy episodic drinking.(9) Heavy episodic drinking or binge drinking is defined as the consumption of 4 or more drinks for women and 5 or more drinks for men within 2 hours.(10) Binge drinking is mainly seen among university students.

1.2. ALCOHOL METABOLISM

1.2.1. Absorption

The blood alcohol concentration reached after alcohol consumption depends on different factors. First of all, the absorption into the blood circulation. A small part of the consumed alcohol is absorbed from the stomach, but the absorption mainly (80-

90%) takes place in the duodenum and jejunum through passive diffusion. Therefore, the rate of absorption is dependent on the alcohol concentration. The higher the alcohol concentration, the higher the resulting concentration gradient and consequently, the more rapid the absorption takes place.

Furthermore the gastric emptying and alcohol oxidation in the stomach influence the rate and amount of absorption. Food retards the gastric emptying which results in both a decreased absorption rate and systematic bioavailability of ethanol.(13, 14) Part of the orally ingested alcohol is oxidized to acetaldehyde by ADH isoforms, such as σADH and class I and class III ADH, present in the stomach, before reaching the systemic circulation. The efficiency of this first pass metabolism determines the bioavailability of alcohol as well.(5, 15)

1.2.2. Distribution

Once ethanol has entered the systematic circulation, it distributes itself rapidly throughout the body tissues, proportional to the relative water content of each tissue. Its low molecular weight and polarity allows passing through biological membranes and thus the ethanol diffusion between blood and tissue depends on the rate of blood flow, the mass of the tissue and the water content.(16, 17) In general, women have a smaller volume of distribution than men of the same weight because they have less body water.(18)

1.2.3. Metabolism

1.2.3.1. Oxidative metabolism

Alcohol is metabolized by several pathways. The major pathway (nearly 95%) is oxidation in the liver. As illustrated in figure 1.1, there are three enzyme systems responsible for this: alcohol dehydrogenase (ADH) in the cytosol and to a lesser extent the microsomal ethanol oxidation system (MEOS) and catalase enzymes present in peroxisomes. All three catalyze the conversion of ethanol into

acetaldehyde. Acetaldehyde is then quickly oxidized to acetate by aldehyde dehydrogenase (ALDH) in the mitochondria.(5,19)

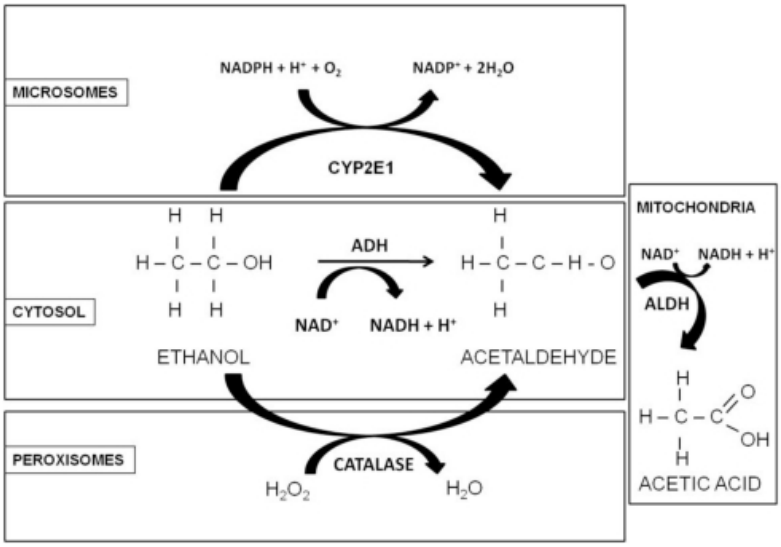

Figure 1.1: Oxidative alcohol metabolism is catalysed by three major enzyme systems; Alcohol dehydrogenase (ADH), the microsomal ethanol oxidation system (MEOS) and catalase enzymes.(19)

The primary enzyme responsible for ethanol oxidation is ADH. This enzyme has a high affinity for alcohol and thus a low Km (Michaelis constant). Therefore saturation of ADH is easily reached. Once saturation is reached the elimination becomes independent of the ethanol concentration and hence the alcohol elimination can be considered as a zero-order process. Multiple variants of ADH and ALDH, which can differ in their expression and activity, are known. This explains the inter-individual differences in alcohol metabolism and has shown to have an influence on the susceptibility of developing alcohol related problems.(19, 20)

The microsomal ethanol oxidation system (MEOS) pathway involves the cytochrome P450 enzymes. Especially CYP2E1 plays a role in alcohol metabolism. This enzyme has a lower affinity for ethanol than ADH and is mainly important for ethanol oxidation after the consumption of large amounts. CYP2E1 is induced in chronic alcohol consumers and is also important for the ethanol oxidation in the brain, where the presence of ADH is limited.

ADH and MEOS both make use of a cofactor, NAD^+ and $NADP^+$ respectively, which becomes reduced by two electrons during the ethanol oxidation process. Hence the liver is in a reduced state and more vulnerable to damage during alcohol metabolism.(19)

The third enzyme, catalase, uses ethanol to reduce hydrogen peroxide (H_2O_2) to water and oxygen. This pathway is limited given that only small amounts of H_2O_2 are generated in the human body.(5)

The remaining 5-10 % of the ethanol is eliminated unchanged via urine (0.5-2%), the lungs (1.6-6%) and the skin (0.5%).(31)

1.2.3.2. Non-oxidative metabolism

Additionally, there are non-oxidative pathways responsible for a very small fraction (less than 1%) of ethanol metabolism. The metabolites generated by these pathways, such as ethyl glucuronide (EtG), ethyl sulfate (EtS), fatty acid ethyl esters (FAEEs), and phosphatidylethanol (PEth) are important alcohol biomarkers. They exhibit longer lifetimes and can be detected in several biological matrices.

Ethyl sulfate and ethyl glucuronide are formed by phase II conjugation reactions and are eliminated through the kidney. Ethyl glucuronide is formed through the glucuronidation of ethanol by UDP-glucuronosyltransferase. Ethyl sulfate is the ethyl ester of sulfuric acid, formed in the presence of ethanol by the enzyme sulfotransferase.(21)

Fatty acid ethyl esters (FAEEs) are formed through the esterification of ethanol with free fatty acids, catalyzed by specific esterases and non-specific enzymes. It has been demonstrated that they are toxic both *in vitro* and *in vivo*. They are formed in nearly all body tissues but mostly in the pancreas, liver, heart and brain, considering these are the organs with the highest concentrations of the enzymes related to FAEE formation. There are more than 20 different types of FAEEs from which some are used as alcohol biomarkers.(22-24)

Phosphatidylethanol (PEth) is formed by phospholipase D (PLD) in the presence of ethanol. PLD is a membrane associated enzyme present in many cell types and it is responsible for the hydrolysis of its principal substrate phosphatidylcholine into phosphatic acid (PA) and choline. PA is a lipid second messenger involved in several pathways influencing cellular metabolism, cell cycle progression, and cell growth.(25, 26)

In mammals, two isoforms of PLD have been identified, PLD1 and PLD2. They share 50% of their sequence and display similar domain structures. Due to this they have similar substance preferences.(27) PLD1 differs from PLD2 with respect to the localization, modes of activation, and functional roles. PLD1 is localized in the intracellular compartments and needs protein kinase C activation, while PLD2 is found in the submembranous vesicular structures and exhibits a high basal activity.(25, 28, 29). A large variation in the expression of PLD is seen among different animals and among different tissues of a single organism.(30).

In the presence of ethanol, a transphosphatidylation reaction resulting in PEth takes place at the expense of the normal hydrolysis. This is due to the fact that PLD has a higher binding affinity for primary alcohols, since they are stronger nucleophilic acceptors than water. This reaction occurs predominantly at high circulating ethanol concentrations due to the high K_m value of PLD for ethanol.(31)

PEth represents a group of glycerophospholipids, consisting of a glycerol backbone onto which a phosphoethanol head group and two long fatty acid chains are attached. The different homologues are named in the form PEth A:B/C:D. A represents the fatty acid chain in sn1 position and C the fatty acid chain in sn2 position. B and D indicate the number of double bonds. Up to now 48 homologues have been identified, of which the 16:0/18:1 and 16:0/18:2 homologues are most prominent, accounting on average for 37%–46% and 26%–28%, respectively of total PEth in blood. The occurrence of the different homologues may be influenced by nutrition. The elimination of PEth is not yet elucidated, but there is a clear disproportion between formation and degradation rates resulting in a half–life of approximately 4 days.(30)

1.3. ALCOHOL BIOMARKERS

Considering the serious health problems and socioeconomic consequences related to excessive alcohol consumption, it is necessary to detect hazardous drinking at an early stage to ensure successful clinical intervention. Questionnaires such as Alcohol Use Disorders Identification Test (AUDIT) and CAGE (Cut down, Annoyed, Guilty, and Eye-opener) provide an insight on the amounts of alcohol consumed. However, they rely on self-report and are therefore often questionable. For that reason, there is a need for objective measures. These are provided by alcohol related biomarkers.

The World Health Organization has in coordination with the United Nations and the International Labor Organization, defined a biomarker as "*any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease*."(32) An Ideal biomarker for alcohol consumption directly corresponds to the amount of alcohol consumed and can be measured accurately and reproducibly. It should be both sensitive and specific. However, this is hardly ever achieved and depending on the used cutoff value. Further are easy sampling and inexpensive quantification desirable. (33-36)

There are two types of alcohol biomarkers: direct and indirect biomarkers. Alcohol itself and its metabolites are referred to as direct biomarkers. Indirect biomarkers are substances indicating the toxic effects of ethanol on the body. They are valuable for monitoring chronic disorders or the diagnosis of organ damage caused by excessive drinking. Contrary to the direct alcohol biomarkers they do not contain the ethanol structure.(33)

1.3.1. Indirect biomarkers

Liver damage and cell death caused by alcohol abuse can be indicated by the presence of liver enzymes in the blood, such as aspartate aminotransferase (AST), alanine amino transferase (ALT) and gamma glutamyltransferase (GGT). Increased

levels of GGT are also seen in 30-50% of chronic heavy drinkers. If no liver disease is present, the levels normalize within 2-3 weeks upon abstinence.

Furthermore, heavy chronic drinking is commonly associated with an elevated erythrocyte macrocytic volume (MCV). MCV has limited clinical use as a relapse marker, since normalisation requires 2-4 months, after a person stops drinking. Elevated levels of carbohydrate deficient transferrin (CDT) indicate heavy drinking as well. Transferrin is a glycoprotein branched with sialic acid molecules, responsible for the iron transport in the blood. There are various forms of transferrin, which differ in the level of sialylation. After heavy alcohol intake the proportion of transferrin forms with zero, one or two sialic acid, which are referred to as CDT, increases.

However, all of these indirect biomarkers lack specificity since elevated levels can also be caused by non-alcoholic disease states. Besides that, the heterogeneity between individuals should be taken into account. Therefore, they are not very useful as stand-alone alcohol markers, but a combination improves the diagnostic value. (34-36)

1.3.2. Direct biomarkers

Ethanol and some of its metabolites can be rapidly detected in different matrices such as blood, urine, breath, and hair. The direct biomarkers are more specific since these metabolites are only formed in the presence of ethanol.

1.3.2.1. Ethanol

Ethanol itself can be detected in blood via the blood alcohol concentration (BAC), in breath (BrAC), and in urine. Measurements of ethanol in these matrices show whether or not a person has consumed alcohol. They are used in the context of traffic offences and traffic controls. During alcohol controls in traffic, the alcohol concentration is measured in exhaled air. Breath alcohol concentrations show a good correlation with the BAC and can be used to estimate the BAC.(37) The big

disadvantage of ethanol is its short detection window (<12h) due to rapid metabolism.(38, 39)

1.3.2.2. EtG and EtS

Ethyl glucuronide (EtG) and Ethyl sulfate (EtS) in urine are markers for recent alcohol intake.(40) After the consumption of small amounts of alcohol they are detectable by a few hours to 2-3 days and thus have a longer detection window than ethanol itself. However, there are a few factors that can give rise to unreliable test results. False positive results can be obtained after the use of daily products containing alcohol such as mouth wash. EtG false positive results can also be caused by post sampling production through E.Coli, which is the common cause of urinary tract infections. On the other hand, bacterial hydrolysis of EtG can lead to false negative results.(18, 19)

EtG in hair and nails can serve as a long term alcohol biomarker for abstinence monitoring and detection of excessive drinking.(41). Here, cosmetic hair treatments, such as bleaching and perming, can lead to false negative results.(26)

1.3.2.3. FAEE

FAEEs are present in nearly all body tissues and accumulate preferentially in adipose tissue and hair. They can serve as a post-mortem ethanol marker in blood, liver and adipose tissue. (42). The measurement of the sum amounts of ethyl myristate (14:0), palmitate (16:0), oleate (18:1), and stearate (18:0) in hair has proven to be a long term heavy drinking marker.(42) FAEEs are less sensitive to cosmetic hair treatments and washing than EtG, but ethanol containing hair care products may lead to false positive results. (23, 43, 44). Furthermore, FAEEs present in meconium indicate prenatal alcohol exposure (PAE). (23, 45)

1.3.2.4. PEth

Since PEth formation starts immediately after alcohol intake and the average elimination half-life is 4 days, PEth accumulates after repeated alcohol consumption. The concentration of PEth in whole blood can be correlated to the amount of alcohol consumed and is not influenced by age, gender, other ingested substances and non-alcohol associated diseases. These characteristics turn PEth into a promising biomarker to observe long-term drinking behavior and for the detection of alcohol abuse. Besides, it can potentially be used in abstinence monitoring, considering that PEth can be detected up to 12 days after a single drinking event.(25) The major advantage of PEth compared to previously established alcohol markers is its high diagnostic sensitivity and specificity.

An important drawback of PEth as a direct alcohol marker is the possible post sampling formation in blood samples, containing more than 0.1 g/kg ethanol, which can lead to false positive results. This *in vitro* PEth formation occurs both at room temperature and at -20°C, but is not observed for storage at +4°C up to three weeks. On top of that, it has been shown that PEth is unstable at -20°C.(66) Therefore storage at -80°C is recommended. At this temperature PEth is stable in whole blood for 30 days and post sampling formation is considered to be inhibited. Also dried blood spots (DBS) have been shown to be a convenient matrix to stabilize PEth.(46, 47)

1.4. INSTRUMENTATION

1.4.1. LC-MS/MS

In forensic toxicology liquid chromatography - tandem mass spectroscopy (LC-MS/MS) is a powerful analytical tool for the identification, characterization and quantification of substances in various matrices based on their polarity, molecular mass and fragmentation patterns. For the determination of PEth, LC-ESI-MS/MS has been described as a sensitive method to detect PEth with a limit of detection below 20 ng/mL.(48, 49)

Before compounds reach the mass spectrometer, they have been separated by liquid chromatography. Chromatographic separation is based on the difference in distribution between a mobile and stationary phase. In case of reversed phase (RP) liquid chromatography, the mobile phase is a polar liquid percolating through a non-polar stationary phase packed in a column. Depending on their affinity for the stationary and mobile phase, compounds elute at different time points (retention times). Compounds with a high affinity for the stationary phase interact with it and they are slowed down compared to compounds with a lower affinity. Therefore, compounds elute in order of increasing hydrophobicity. The retention time of hydrophobic analytes can be reduced by gradually increasing the amount of organic solvent in the mobile phase. This process is referred to as gradient elution.(51, 52)

In a mass spectrometer ions are created and separated based on their mass to charge ratio (*m/z*). The device consists of four components; an inlet, an ion source, a mass analyzer and a detector. In case of LC-MS an LC pump feeds the separated compounds into the mass spectrometer that subsequently get ionized under atmospheric pressure through electrospray ionization.(50)

Compounds in solution can be converted into single ionic molecules by the application of a charge and brought into the gaseous phase by electrospray ionization (ESI). As illustrated in figure 1.2, the electrospray ionization process involves three steps; nebulization of the solution into a fine spray of charged droplets, droplet disintegration and ion emission from the droplets.

The sample solution is fed through a capillary tube, with an electrical field being applied at the tip. The electrical field together with the surface tension lead to the deformation of the liquid into a conical shape, called the Taylor cone.(51) When the applied voltage reaches a threshold the cone shape inverts and a fine mist of droplets with the same polarity as the capillary voltage is created. By reversing the electric field, droplets with an opposite polarity can be obtained.

High temperature and a stream of nitrogen drying gas are responsible for the evaporation of the solvent and thus a reduction of the particle size and an increase in charge density. This process is referred to as ion evaporation. According to Coulomb’s law, electrostatic repulsion appears. When this exceeds the surface tension, the droplet disintegrates. The process repeats itself until a critical point is

reached at which it is energetically and kinetically possible for ions to be emitted from the droplet surface into the gaseous phase.(52, 53)

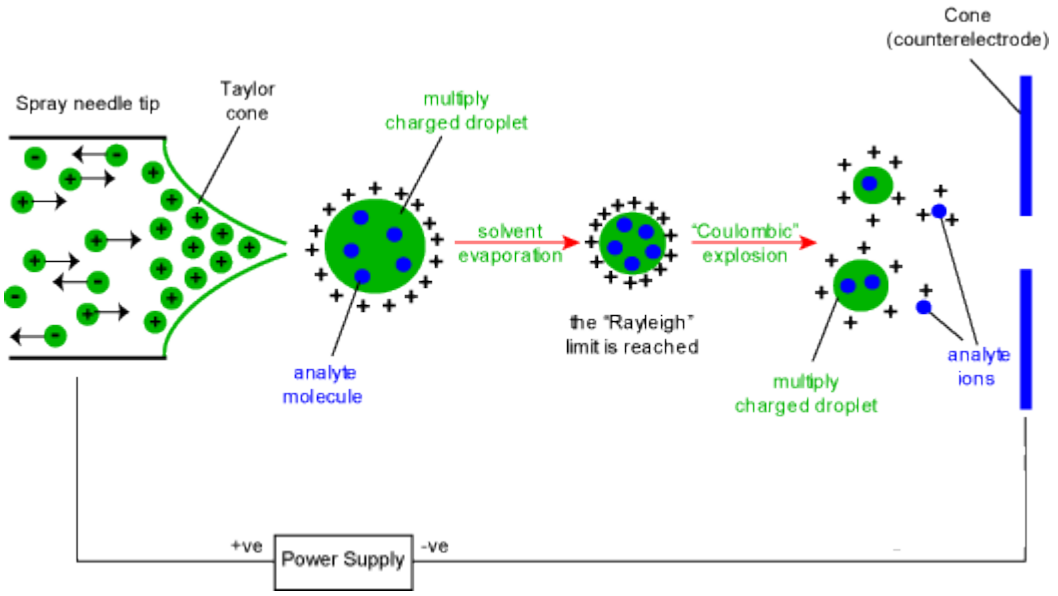

Figure 1.2: ESI involves three steps; nebulization of the solution into a fine spray of charged droplets, droplet disintegration, and ion emission from the droplets.(54)

After ionization, the desired PEth homologues were selected with a triple quadrupole setup according to their *m/z*. The first (Q1) and third (Q3) quadrupole consist of four parallel metal rods on which an electric and magnetic field is applied. They serve as mass filters, by providing stable trajectories for selected *m/z*, and they are connected through a second quadrupole (Q2), also referred to as a collision cell. Here, fragmentation occurs through interaction with a collision gas. This process is referred to as collision induced fragmentation (CID). For quantification, selected reaction monitoring (SRM) is used because of its high specificity and sensitivity. In SRM mode both the first and the third quadrupole filter specific precursor and fragment ions.(50, 52, 55, 56)

The selected ions are subsequently detected by an electron multiplier. The principle of an electron multiplier is based on a process called secondary electron emission. When an ion hits a dynode, secondary electrons from ions in the surface layer are released. These secondary electrons then hit a secondary dynode generating more secondary electrons. The process is repeated, resulting in a cascade of electrons.(57, 58)

2. OBJECTIVES

Phosphatidylethanol (PEth) in whole blood is a direct alcohol marker for alcohol abuse and shows potential for abstinence monitoring. Furthermore, it was demonstrated that the PEth concentration in human autopsy material could give insight in the alcohol use at the time of death. However, inter-individual variations are seen in PEth formation. The cause of these inter-individual variations has not been fully elucidated yet and therefore further investigations are necessary.

In order to obtain a more detailed insight in the PEth formation in different organ tissues, a Liquid chromatography-electrospray- tandem mass spectroscopy (LC-ESI-MS/MS) method was developed to simultaneously determine PEth and its precursor phosphatidylcholine (PC), as previous studies on organ tissue presumed that variations in PEth formation could be explained by PC availability.

With the newly developed method, the *in vitro* PEth formation in blood, brain, liver, and kidney tissue, from pig, cattle and goat, was investigated. The tissues were spiked with ethanol concentrations ranging from 1-7 g/kg and incubated at 37°C up to five hours. Every 30 minutes an aliquot was taken and added to acetonitrile to stop the PEth formation (protein precipitation). PEth and PC were extracted, separated by LC with a C8 core-shell column, and determined by a Sciex 5500 QTrap instrument, operated in selected reaction monitoring (SRM) mode. By switching ESI polarity, PC and PEth could be determined within the same run. PC was measured in ESI positive mode, while PEth was measured in ESI negative mode. The PEth formation was evaluated by means of Michaelis-Menten kinetics.

The accuracy and precision of the method were validated according to the FDA guidance for human blood samples. Matrix effects, extraction efficiency and process efficiency were determined for every tissue according to the method described by Matuszewski *et al*.

The possibility to investigate *in vitro* PEth formation, by spiking tissue with ethanol, was demonstrated by the work of Schröck *et al*. They investigated phospholipase D (PLD) activity in human blood after *in vitro* incubation with ethanol. Since the animal tissues were obtained from a local butcher, no animals had to be sacrificed for this study.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

Tetra-N-butylammoniumsalts of PEth 16:0/18:1 and PEth 16:0/18:2 were purchased from Sigma Aldrich (Paloma, USA). PC 16:0/18:1, PC 16:0/18:2 and PC 16:0/18:1-d_{31} were obtained from Avanti Polar Lipids (Alabaster, USA). Deuterated internal standards for the two PEth homologues, PEth 16:0/18:1-d_5 and PEth 16:0/18:2-d_5, were synthesized in the laboratory as described in section 3.2.2. Chloroform, diethyl ether, ethanol, ammonium acetate, calcium chloride and sodium chloride were acquired from Merck (Darmstadt, Germany). Acetonitrile, p.a., was provided by Acros Organics (Geel, Belgium). 2-propanol, High-performance liquid chromatography (HPLC) grade, was purchased from Fisher Scientific (Loughborough, UK). Formic acid, ethanol-d_6 and phospholipase D were obtained from Sigma Aldrich (Buchs, Switzerland) and methanol was provided by Biosolve Chimie SARL (Dieuze, France). Water was deionized in-house with a Milli-Q water system from Millipore (Billerica, USA).

3.1.2. Materials and instrumentation

For the homogenization of the animal tissues gentleMACS™ M tubes and a gentleMACS dissociator (Bergisch Gladbach, Germany) were used. The samples were incubated in a drying and heating chamber Classic.Line series FD model 23 (Binder, Tuttlingen, Germany). Samples were vortexed and shaken with a Vortex-Genie® from scientific industries, Inc. (New York, USA) and a Vibrax VXR basic from IKA® (Staufen, Germany), respectively. For centrifugation a MIKRO 220R centrifuge from Hettich (Tuttlingen, Germany) was used. Evaporation of the samples occurred in a Sample Concentrator from Techne (Staffordshire, UK) under a stream of nitrogen at 50°C. An AT200 Mettler Toledo balance (Bern, Switzerland) was used for weighing.

Different types of pipettes were used: the pipetman® 200M (systematic error ±1.00 µL, random error ≤0.26 µL) and 1000M (systematic error ±0.15 µL, random error ≤0.06 µL), the Microman® 10M (systematic error ±0.15 µL, random error ≤0.06 µL), 25M (systematic error ±0.30 µL, random error ≤0.10 µL) and 50M (systematic error ±0.70 µL, random error ≤0.30 µL) and the Repetman® from Gilson, Inc. (Middleton, USA).

The LC–ESI-MS/MS system was composed of an UltiMate™ 3000 ultra-high performance liquid chromatography (UHPLC) focused system with an UltiMate™ 3000 RS autosampler and a heated column compartment from Dionex (Olten, Switzerland) connected to a QTrap® 5500 triple-quadrupole mass spectrometer from Sciex (Rotkreuz, Switzerland) controlled by Analyst 1.6.2 Software.

3.1.3. Animal tissue

The different animal tissues were freshly obtained from a local butcher (Schlieren, Switzerland) and processed on the same day. Liver, brain, and kidney tissue from a 100 kg female domestic pig, a 170 kg steer and a 10 kg goat were provided. Additionally cattle blood from the 170 kg steer and pooled blood from 9 pigs was obtained. Blood from the goat was not available. The blood was collected in Lithium heparin S-monovettes (Sarstedt, Nümbrecht, Germany) to prevent it from clotting. PEth negative human blood was collected in Lithium heparin S-monovettes.

3.2. METHODS

3.2.1. LC-ESI-MS/MS method

A LC-ESI-MS/MS method was developed in order to simultaneously quantify the PC homologues, PC 16:0/18:1 and PC 16:0/18:2, and the PEth homologues, PEth 16:0/18:1 and PEth 16:0/18:2, in animal tissue. The deuterated PC 16:0/18:1-d_{31} served thereby as internal standard for PC and PEth 16:0/18:1-d_5 and PEth 16:0/18:2-d_5 as internal standards for the corresponding PEth homologues. The PC

and PEth homologues were separated by liquid chromatography on a Kinetex® 2.6 µm C8 100 Å, RP column 100 x 2.1 mm (Brechbühler, Schlieren, Switzerland). In the Kinetex® columns core-shell silica is used as solid support. This particle morphology decreases the three types of band broadening, described by the Van Deemter theory, leading to better separation efficiency.(59) The column was heated to 60°C and the samples were injected at a flow rate of 0.3 mL/min. Acetonitrile/water (70:30, v/v) with 0.1% formic acid was used as mobile phase A and 2-propanol served as mobile phase B in a 10 minutes gradient elution as outlined in table 3.1.

Table 3.1: LC-gradient

Time (min)	%B
0.0	40.0
1.0	40.0
7.0	80.0
7.1	99.0
8.5	99.0
8.6	40.0
10.0	40.0

This gradient resulted in the following retention times: 5.72 min for PC 16:0/18:1, 5.46 min for PC 16:0/18:2, 5.63 min for PC 16:0/18:1-d_{31}, 6.36 min for PEth 16:0/18:1, 6.34 min for PEth 16:0/18:1-d_5, 6.10 min PEth 16:0/18:2, and 6.08 min for PEth 16:0/18:2-d_5. Seen these retention times, the compounds eluting in the first four minutes were directed to waste by using the six valve system from the LC-system in order to minimize potential contamination of the mass spectrometer.

PEth and PC were analyzed within the same MS method by switching the ESI polarity. A switch from positive to negative mode was made at 5.85 min. First, PC was measured in ESI positive SRM mode with an ionspray voltage of 2000 V and a source temperature of 550°C, collision gas at medium, curtain gas at 30, ion source gas 1 at 15, ion source gas 2 at 20 and a cycle time of 0.200 s (arbitrary units). After the polarity switch, PEth was measured in ESI negative SRM mode with an ionspray voltage of -4250 V and a source temperature of 500°C, collision gas at medium, curtain gas at 30.0, ion source gas 1 at 15, ion source gas 2 at 20, and a cycle time of 0.250 s (arbitrary units). The two transitions, SRM1 and SRM2, measured for each

PEth- and PC homologue are together with their corresponding mass spectrometric parameters displayed in table 3.2 and 3.3, respectively. The SRM 1 transitions served as qualifiers and SRM 2 transitions as quantifiers.

Table 3.2: SRM transitions for the PEth homologues and IS.

Transitions	Q1 Mass (Da)	Q2 Mass (Da)	Dwell time (msec)	DP (volts)	CE (volts)	CXP (Volts)	retention time (min)
PEth 16:0/18:1 SRM1	701.30	255.20	20.00	-20.00	-40.00	-14.00	6.36
PEth 16:0/18:1 SRM2	701.30	281.30	20.00	-32.00	-40.00	-14.00	
PEth 16:0/18:2 SRM1	699.50	279.40	20.00	-5.00	-40.00	-14.00	6.10
PEth 16:0/18:2 SRM2	699.50	255.30	20.00	-5.00	-40.00	-14.00	
PEth 16:0/18:1- d_5 SRM1	706.30	281.10	20.00	-20.00	-40.00	-14.00	6.34
PEth 16:0/18:1- d_5 SRM2	706.30	255.30	20.00	-32.00	-40.00	-14.00	
PEth 16:0/18:2- d_5 SRM1	704.50	279.50	20.00	-5.00	-40.00	-14.00	6.08
PEth 16:0/18:2- d_5 SRM2	704.50	255.30	20.00	-5.00	-40.00	-14.00	

Table 3.3: SRM transitions for the PC homologues and IS.

Transitions	Q1 Mass (Da)	Q2 Mass (Da)	Time (msec)	DP (volts)	CE (volts)	CXP (volts)	retention time (min)
PC 16:0/18:1 SRM1	760.60	124.80	20.00	14.00	111.00	7.00	5.72
PC 16:0/18:1 SRM2	760.60	184.10	20.00	11.00	37.00	11.00	
PC 16:0/18:2 SRM1	758.30	125.30	20.00	10.00	94.00	9.00	5.46
PC 16:0/18:2 SRM2	758.30	184.10	20.00	10.00	40.00	9.00	
PC 16:0-d_{31}-18:1 SRM1	791.70	791.70	20.00	7.00	15.00	40.00	5.63
PC 16:0-d_{31}-18:1 SRM2	791.70	184.10	20.00	5.00	45.00	42.00	

3.2.2. Preparation of new PEth internal standard solution

A deuterated PEth internal standard solution was synthesized from PC 16:0/18:1 and PC 16:0/18:2 by the addition of ethanol-d_6. The reaction was catalyzed by phospholipase D.(26) Afterwards the newly prepared internal standard solution was tested for possible PC residues with LC-MS/MS to check if the reaction was completed. Remaining PC residues in the internal standard solution would interfere with the simultaneous PC determination in animal tissue since the PC homologues used to prepare the PEth IS are identical to the ones determined in animal tissue.

First, two internal standard stock solutions, PEth 16:0/18:1-d_5 and PEth 16:0/18:2-d_5, were prepared. 10 mg PC 16:0/18:1 and 10 mg PC 16:0/18:2 were dissolved separately in 1.5 mL diethyl ether. Subsequently 200 µL ethanol-d_6 and 2.25 mL buffer solution (100 mM ammonium acetate and 100 mM calcium chloride in water) were added to each solution. By adding 200 µL PLD solution (1 mg/mL in deionized water) the reaction was started. The vials were closed and wrapped in parafilm to prevent the diethyl ether from evaporating. The mixtures were shaken for six hours at room temperature and stored in the fridge overnight, where phase separation took place. Then the organic phase was transferred to a 6 mL glass vial. The remaining aqueous phase was extracted with 1.5 mL diethyl ether three times. All the organic phases were collected and evaporated under a stream of nitrogen at 50°C. The white residue was dissolved in a 5 mL chloroform/methanol (5:8) mixture and filtered. The solution was again evaporated. The residue was dissolved in 1 mL chloroform and stored in 2 mL crimp-top vials in the freezer at -20°C.

For the final PEth internal standard solution 5 µL PEth 16:0/18:2-d_5 and 6.6 µL PEth 16:0/18:1-d_5 stock solution were evaporated and reconstituted in 15 mL 2-propanol.

3.2.3. Preparation of calibrators and quality controls

3.2.3.1. PEth calibrators and quality controls

New PEth calibrator working solutions (C1-C7) with a concentration range from 0.25-50µg/mL PEth tetra-N-butylammoniumsalt were prepared in chloroform. For this, 100 µL PEth 16:0/18:1 tetra-N-butylammoniumsalt stock solution (1 mg/mL) and 100 µL PEth 16:0/18:2 tetra-N-butylammoniumsalt stock solution (1 mg/mL) were pipetted in a glass vial and evaporated under a stream of nitrogen at 50°C. The residue was reconstituted in 1 mL chloroform. The obtained stock solution, named V_0, had a concentration of 100 µg/mL. The calibrators C1-C7 were prepared from the V_0 solution by dilution with chloroform as illustrated in table 3.4. Further, four quality control solutions (LLOQ-QC3) were prepared similarly to control the accuracy. These were also prepared by dilution from a V_0 solution with a concentration of 100 µg/mL PEth tetra-N-butylammoniumsalt, as illustrated in table 3.5.

Table 3.4: dilution series for the PEth calibrator solutions

PEth calibrator working solutions	C7	C6	C5	C4	C3	C2	C1
chloroform (µL)	500	400	400	400	600	800	500
from solution	V_0	C7	C6	C5	C4	C3	C2
addition (µL)	500	400	400	400	400	200	500
Concentration [µg/mL]	50	25	12.5	6.25	2.5	0.5	0.25

Table 3.5: dilution series for the PEth quality control solutions

Quality control working solutions	QC3	QC2	QC1	LLOQ
chloroform (µL)	375	960	600	500
from solution	V_0	QC3	QC2	QC1
addition (µL)	225	40	300	500
Concentration [µg/mL]	37.5	1.5	20	10

3.2.3.2. PC calibrators and quality controls

Seven PC calibrator working solutions with a concentration range from 12.5-2500 µg/mL were made in chloroform by dilution. The concentration range of these calibrators was based on the amount of PC found in human blood. As illustrated in table 3.6, the dilution started from a V_0 solution with a concentration of 5000 µg/mL. The V_0 solution was prepared from a PC 16:0/18:1 stock solution (25 mg/mL) and a PC 16:0/18:2 stock solution (25 mg/mL). 200 µL of both stock solutions was pipetted in a vial and evaporated. The residue was reconstituted in 1 mL chloroform. Also 4 PC quality control working solutions were prepared by dilution from a V_0 solution with a concentration of 5000 µg/mL as illustrated in table 3.7.

Table 3.6: dilution series for the PC calibrator solutions

PC calibrator working solutions	C7	C6	C5	C4	C3	C2	C1
chloroform (µL)	500	400	400	400	600	800	500
from solution	V_0	C7	C6	C5	C4	C3	C2
addition (µL)	500	400	400	400	400	200	500
Concentration [µg/mL]	2500	1250	625	312.5	156.25	62.5	12.5

Table 3.7: dilution series for the PC quality control solutions

PC quality control working solutions	QC3	QC2	QC1	LLOQ
chloroform (µL)	375	960	600	500
from solution	V_0	QC3	QC2	QC1
addition (µL)	225	40	300	500
Concentration [µg/mL]	1875	75	25	12.5

3.2.4. PEth determination in animal tissue

The *in vitro* formation of PEth was tested in four different tissues: blood, liver, kidney and brain, from three different animals: a pig, a cattle and a goat. From each tissue eight different samples were taken. Seven of these were spiked with 1, 2, 3, 4,

5, 6 and 7 g/kg ethanol, respectively. The first sample was kept as a blank sample to make a calibration series with, and to evaluate the initial amount of PC. It was assumed that saturation of PLD would be reached at 7 g/kg ethanol, based on the work of Schröck *et al*.: “Standardization of an enzyme activity test for phospholipase D (PLD) in blood and testing of two selective PLD inhibitors”.

3.2.4.1. Sampling

From the brain (cerebral cortex), the liver and the kidney 8 different samples of approximately 3.5 gram were taken and transferred to a gentleMAC® M tube. For each gram of tissue, 2.5 mL 150 mM sodium chloride buffer was added. Then the samples were homogenized with the gentleMAC dissociator at the 45 second gradient and incubated at 37°C for one hour.

Blood from nine pigs was obtained from the butcher. This blood was pooled first and then eight samples of 8 mL were taken. Since not enough bovine blood was provided, 8 samples of 2.5 mL instead of 8 mL were taken. Goat blood was not available. No sodium chloride buffer was added to the blood samples. After sampling, the blood samples were together with the other tissue samples incubated at 37°C for one hour.

3.2.4.2. Spiking with ethanol

After one hour of incubation the samples were spiked with pure ethanol to obtain concentrations from 1 to 7 g/kg ethanol. The required volume of ethanol for this was calculated based on the total weight of the homogenized sample (tissue and buffer). But first a zero aliquot of each tissue sample was taken by transferring 175 µL of the brain, the kidney, and the liver samples and 250 µL of blood samples to 1mL of acetonitrile. This sample served as a negative control. Immediately after the addition of ethanol aliquots of 750 µL from each sample were taken for the ethanol quantification.

3.2.4.3. Evaluation of PEth formation rate

After spiking, the samples were again incubated at 37°C for 5 hours and every 30 minutes an aliquot of each sample was taken and transferred to a 2 mL Micro tube (Sarstedt, Nümbrecht, Germany), containing 1 mL acetonitrile. The acetonitrile was used for protein precipitation and thus for the inactivation of PLD. In doing so, the formation of PEth is stopped. Before sampling, the sample tubes were vortexed for 10 seconds. 175 µL aliquots from the brain-, liver- and kidney- samples were taken. This volume corresponds to 50 mg tissue. From the pig blood 250 µL aliquots were taken and from the bovine blood 125 µL aliquots. After the addition of the tissue the Eppendorf tubes were closed and vortexed.

3.2.4.4. Sample preparation for LC-ESI-MS/MS measurements

PEth and PC were extracted from the tissues by adding 300 µL 2-propanol. During this step also 10 µL of the PEth IS was added to compensate for variabilities during sample preparation and ionization differences. The samples were shaken for 10 minutes, centrifuged at 16,000 g at 8 °C for 10 min and the supernatant was transferred to a glass vial. Then the solvent was evaporated under a stream of nitrogen at 50°C. The residue was reconstituted in 400 µL mobile phase acetonitrile/2-propanol/water (42/40/18, v/v). 2µL of the reconstituted solution was injected into the LC-ESI-MS/MS system. Except for brain tissue samples, 15 µL was injected because the obtained signal with a 2 µL injection was too low.

To quantify the amount of PEth formed in a tissue, a calibration series was made from the calibrator sample of that tissue, after 5 hours of incubation. For this 13 aliquots for C1-C7, LLOQ-QC3, BW- and BW+, of the calibrator sample were transferred to 1 mL of acetonitrile. BW- was a blank tissue sample without internal standard and BW + a blank tissue sample with internal standard, used to check for contamination. By adding 10 µL PEth calibrator- or quality control working solution, a PEth concentration range from 0.0375-7.5000 µg/mL was obtained for the calibrators and a concentration range of 0.0375-5.6250 µg/mL for the quality controls, after

correction for the actual amount of PEth in the reference solution. The LLOQ was set at 0.0075 µg/mL, the LOD at 0.0035 µg/mL in solvent. Further, 10 µL PEth IS and 300 µL 2-propanol were added. The samples were shaken for 10 minutes, centrifuged at 16,000 g, at 8 °C for 10 min and the supernatant was transferred to glass vials. Then the solvent was evaporated under a stream of nitrogen at 50°C. The residue was reconstituted in 400 µL mobile phase. Additionally, a calibration series and quality controls were prepared in solvent for comparison. For the calibrators in animal tissue 2µL was injected into the LC-ESI-MS/MS system, while for the calibrators in solvent 0.4 µL was injected.

3.2.5. PC determination in animal tissue

To determine the initial amount of PC in the different samples, four aliquots: 175, 87.5, 43.75 and 22 µL from the brain, liver and kidney calibrator samples and 250, 125, 62.5 and 31.25 µL from the blood calibrator samples, were taken and added to 1 mL acetonitrile. In this way a dilution series of PC was obtained to make sure that one of the dilutions would fit the calibration range. The calibration series for PC (0.5-100 µg/mL) was made in solvent as well as three quality controls (1, 3, 75 µg/mL). The LLOQ was set at 1 µg/mL, the LOD at 0.2 µg/mL. For the brain tissue additional aliquots of 10 and 5 µL had to be taken. The same sample preparation for LC-ESI-MS/MS as previously described, was performed. For the PC samples 0.4 µL was injected into the LC-ESI-MS/MS system. The PC concentrations found in the dilutions within the calibration range were calculated regressively to assess the amount of PC within the original tissue.

3.2.6. Ethanol quantification

The exact ethanol concentration, present in each tissue sample after spiking, was determined by headspace gas chromatography coupled to a flame ionization detector (HS-GC-FID). This was performed by the IRM lab technicians. According to the Swiss regulation for ethanol determination in blood, this was done in four-fold;

two samples were tested on two different GC-FID systems with a different GC-column.(60) For this purpose, the samples, mentioned in section 3.2.4.2, were 1:1 diluted with water by weighing, to keep the alcohol concentrations within the calibration range.

3.2.7. Calculations

The peak areas from the determined homologues were divided by the peak areas from their corresponding deuterated internal standards. For PEth quantification, calibration curves were generated in each matrix by spiking with the PEth calibrators. Since the calibrators were prepared with a tetra-N-butylammomiumsalt of PETh, the obtained concentrations were corrected for the actual amount of PEth present in the used stock solutions, which was 74.4% for PEth 16:0/18:1 and 74.2% for PEth 16:0/18.2. For the SRM1 and SRM2 transition of both PEth homologues, formation rates were calculated and the mean formation rate of both transitions was taken to draft formation rate vs time curves. The V_{max} and K_m constants were calculated by means of Lineweaver-Burk.

3.2.8. Method validation

3.2.8.1. Accuracy and precision

Accuracy and precision were determined according to the FDA guidelines.(61) To determine intra-run accuracy and precision, four PEth quality controls (0.0075, 0.015, 0.045, and 1.125 µg/mL) were prepared six times in human blood as well as two calibration series (0.0075-1.5 µg/mL). This procedure was repeated on three days to determine the inter-run precision and accuracy. To determine the inter- and intra- run precision and accuracy for the PC measurements, two calibration series and six series of the three quality controls (1, 3, and 75 µg/mL) were made in solvent. For the blood samples an amount of 2 µL was injected, for PC and calibration samples in solvent, 0.4 µL was injected into the LC-ESI-MS/MS system.

Accuracy, expressed as the deviation of the mean measured concentration from the true concentration, is achieved if the mean is within 15% of the actual value. At the LLOQ a maximum deviation of 20% is allowed. The coefficient of variation, which serves as a measure for precision, should not exceed 15% for each quality control concentration level. Except for the LLOQ, where it may not exceed 20%.(61)

3.2.8.2. Extraction efficiency, matrix effects and process efficiency

The matrix effect, extraction efficiency and process efficiency were evaluated by comparing the peak areas, obtained with three different sets of every tissue. The sample preparation of every set is illustrated in table 3.8. For every set PEth IS was added during the reconstitution step in order to obtain comparable IS peak areas. Every set was made in triplicate for two different concentrations of PEth solutions, 40 ng/mL and 500 ng/mL. These concentrations cover the PEth concentration range that was formed in the different animal tissues.

Table 3.8: Sample preparation to determine the extraction efficiency, the matrix effects and the process efficiency.

Sample	preparation
Set 1	The tissues were spiked with PEth before extraction.
Set 2	Blank tissue samples were extracted and spiked with PEth after extraction.
Set 3	PEth solution is directly injected in the mobile phase.

The matrix effect is calculated by dividing the peak areas from set 2 by the peak areas from set 3. Extraction efficiency is determined by the quotient of the peak areas from set 1 and set 2. The process efficiency, which reflects the combined effect of extraction and the sample matrix, is calculated by dividing the peak areas from set 1 by the peak areas from set 3. The peak areas were calculated based on a 1/x weighted calibration curve in solvent and divided by the peak area from the internal standard.(62, 63)

4. RESULTS

4.1. LC-ESI-MS/MS METHOD

As illustrated in figure 4.1 both PC and PEth homologues can be separated by the new LC-ESI-MS/MS method. The polarity switch from ESI positive mode to ESI negative mode, indicated by the red bar, at 5.85 minutes occurs when all PC is eluted. The figure also shows the large concentration difference between the amount of PEth formed and the amount of PC present in the animal tissues. The extracted ion currents for PC are shown in figure 4.2 and for PEth in figure 4.3.

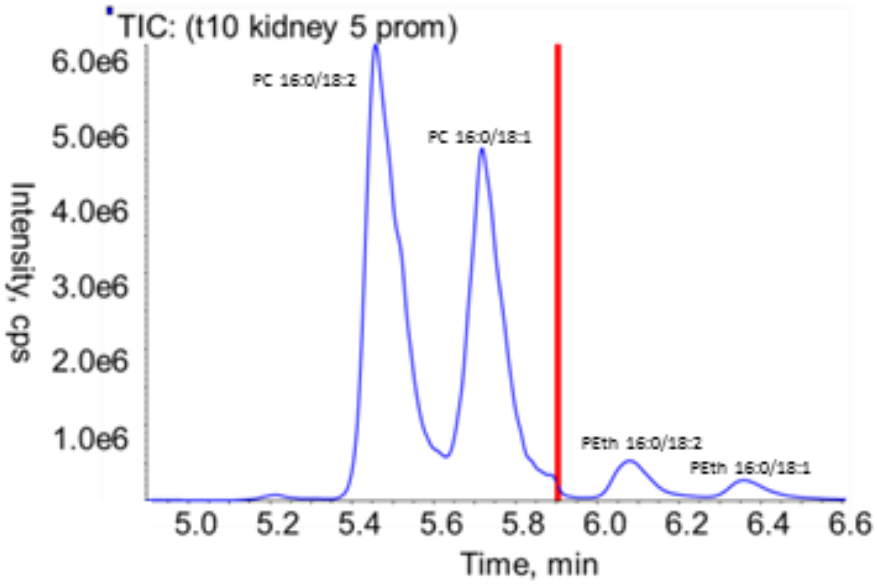

Figure 4.1: Total ion current chromatogram from a kidney sample spiked with 5 g/kg ethanol. PC 16:0/18:1 and PC 16:0/18:2 elute at 5.72 min and 5.46 min, respectively. At 5.85 minutes, after all PC is eluted, a polarity switch, indicated by the red bar, is made. Subsequently PEth 16:0/18:1 and PEth 16:0/18:2 elute at 6.36 min and 6.10 min, respectively.

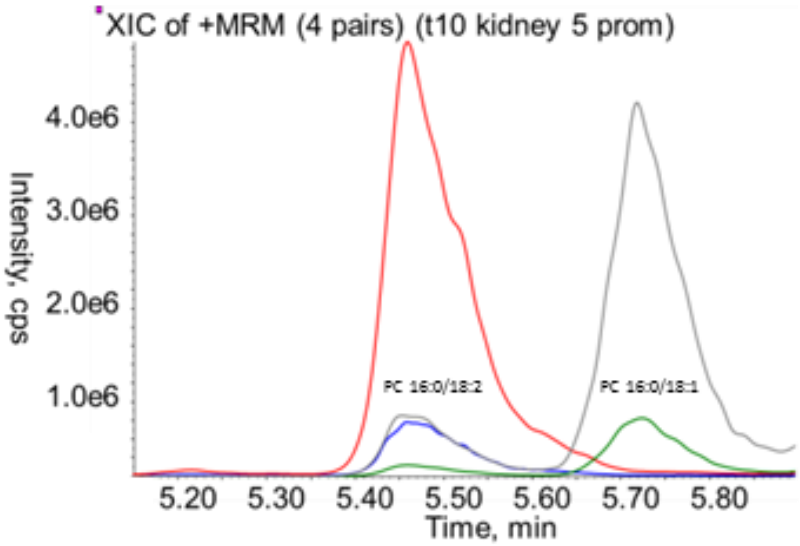

Figure 4.2: The extracted ion currents (XIC) for PC 16:0/18:2 (758.3→125.3 blue, 758.3→184.1 red) and PC 16:0/18:1 (760.6→124.8 green, 760.6→184.1 grey).

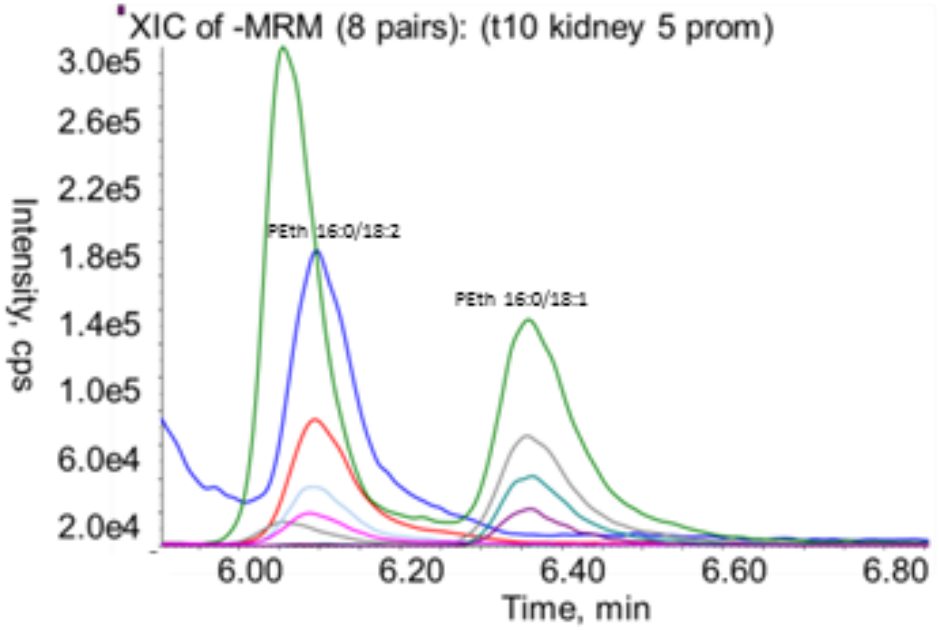

Figure 4.3: The extracted ion currents for PEth 16:0/18:2 (699.5→279.4 blue, 699.5→ 255.3 red) with its deuterated internal standard PEth 16:0/18:2-d_5 (704.5→279.5 light blue, 704.5→255.3 pink) and PEth 16:0/18:1 (701.3→281.3 green, 701.3→255.2 grey) with its deuterated standard PEth 16:0/18:1-d_5 (706.3→281.1 mint, 706.3→255.3 purple).

4.2. PEth INTERNAL STANDARD

No PC residues were detected in the synthesized PEth internal standards, PEth 16:0/18:1-d_5 and PEth 16:0/18:2-d_5. Hence, PEth and PC could be determined simultaneously, without interference.

4.3. CALIBRATION CURVES

From the calibration series made in every animal tissue and solvent, a calibration curve was drafted with a linear calibration model using 1/x weighting. The calibration curves in animal tissues were compared to a calibration curve in solvent to evaluate the influence of the different matrices. The obtained concentrations for the calibrators in solvent and animal tissue were within an acceptable 15% range and did not exceed the accepted 15% deviation from the expected concentration. Also the LLOQ's were within the accepted 20% deviation. However, the calibration curves in goat brain and kidney deviated more than 15% from the calibration curve in solvent. Especially in goat brain large deviations, up to 50%, were seen. Nevertheless a correlation coefficient R>0.99 was achieved for every calibration curve, both for the ones in solvent as the ones in animal tissue.

4.4. INITIAL AMOUNTS OF PC IN ANIMAL TISSUE

The endogenous amounts of the two PC homologues present in blood, brain, liver and kidney are depicted in table 4.1. Also the total amount of both homologues and their ratio is shown in the table. For all the tested animals, the highest amounts of PC were found in brain tissue. In brain a large distinction was observed between PC 16:0/18:1 and PC 16:0/18:2. The ratio PC 16:0/18:2 to 16:0/18:1 was 4.1% (pig), 4.5% (cattle) and 5.2% (goat). In the liver and kidney tissues, there was also more PC 16:0/18:1 present, but the difference was smaller, resulting in a larger ratio of PC 16:0/18:2 to PC 16:0/18:1, 48.2-85.5% (kidney) and 45.3-77.5% (liver). The largest

variability in PC concentrations between the different animals is seen in the liver tissue. The lowest amounts of PC were found in blood. In pig blood the amounts of PC 16:0/18:1 (0.009%) and PC 16:0/18:2 (0.008%) are similar. In bovine blood, contrary to all other tissues, more PC 16:0/18:2 is present, which resulted in a 355% ratio.

Table 4.1: The amounts of PC in blood, brain, liver, and kidney tissue from pig, cattle, and goat, expressed as a percentage of the total tissue weight. The percentages of the PC homologues, PC 16:0/18:1 and PC 16:0/18:2, and their ratio.

Animal	**Tissue**	**% PC**			
		PC 16:0/18:1	***PC 16:0/18:2***	**Total**	**Ratio (PC 16:0/18:2 / PC16:0/18:1)**
PIG					
	Brain	12.5%	0.5%	13.0%	4.1%
	Kidney	3.4%	2.9%	6.3%	85.5%
	Liver	4.9%	3.8%	8.6%	77.5%
	Blood	0.009%	0.008%	0.017%	88.9%
VEAL					
	Brain	11.0%	0.5%	11.5%	4.5%
	Kidney	3.6%	2.3%	5.9%	63.8%
	Liver	3.6%	1.4%	5.0%	40.4%
	Blood	0.011%	0.039%	0.050%	355%
GOAT					
	Brain	12.3%	0.6%	12.9%	5.2%
	Kidney	3.5%	1.7%	5.2%	48.2%
	Liver	4.1%	1.9%	6.0%	45.3%

4.5. PEth FORMATION IN ANIMAL TISSUE

A difference was observed between the PEth concentrations, formed in animal tissue, obtained with SRM1 and SRM2. However, this difference was not seen in the samples spiked with PEth. To normalize these differences the mean concentration obtained with both transitions was taken to calculate the formation rates. Further, large differences in PEth formation between different tissues and between different animals were seen.

4.5.1. Liver

The Michaelis-Menten constants calculated by means of Lineweaver-Burk are depicted in table 4.2. The corresponding Michaelis-Menten curves are shown in figure 4.4.

4.5.2. Kidney

The results of PEth formation in kidney tissue are shown in table 4.3 and figure 4.5.

4.5.3. Brain

The Michaelis Menten constants, calculated by means of Lineweaver-Burk are shown in table 4.4. However, it is uncertain if the constants for goat brain and for the PEth 16:0/18:1 formation in bovine brain represent the true values, because as illustrated in figure 4.6 the formation rates in bovine and goat brain fluctuate heavily from 3-7 g/kg. Therefore, the back-up samples of these tissues were measured, but these gave the same results. Fluctuations were also seen in the Lineweaver-Burk plots of these tissues as a result of which the correlation coefficient was smaller than 0.9. The Lineweaver-Burk plots can be found in the appendix.

4.5.4. Blood

The PEth concentrations formed in bovine and pig blood remained below the LLOQ of 0.0075 µg/mL. So these could not further be analyzed by means of Michaelis Menten.

Table 4.2: Michaelis Menten (MM) constants for PEth formation in liver tissue.

Animal	MM-constant	*PETH 16:0/18:1*	*PETH 16:0/18:2*
PIG			
	K_m (g/kg)	5.63	4.53
	V_{max} (µmol*kg^{-1}*h^{-1})	0.172	0.146
CATTLE			
	K_m (g/kg)	3.80	2.0
	V_{max} (µmol*kg^{-1}*h^{-1})	0.320	0.094
GOAT			
	K_m (g/kg)	2.96	2.11
	V_{max} (µmol*kg^{-1}*h^{-1})	0.264	0.103

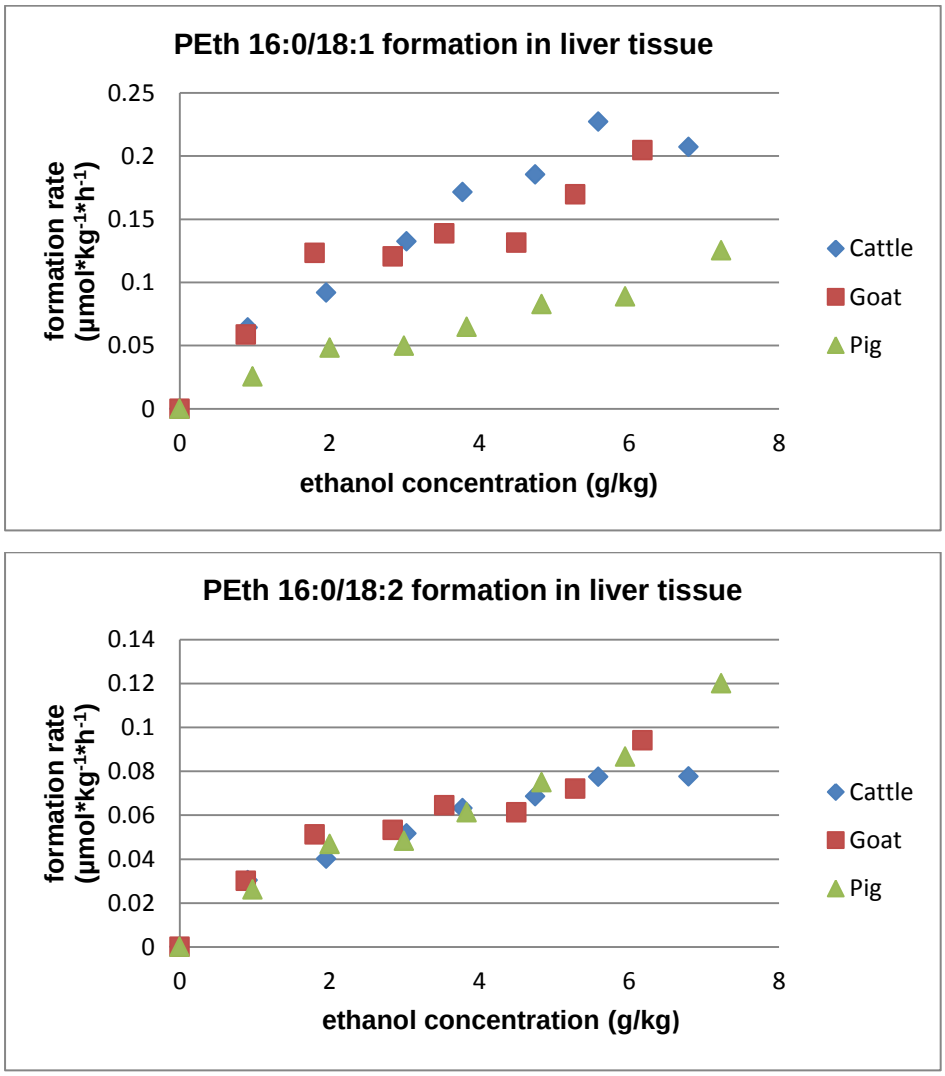

Figure 4.4: Observed PEth formation rates for each ethanol concentration in liver tissue.

Table 4.3: Michaelis Menten (MM) constants for PEth formation in kidney tissue.

Animal	MM-constant	*PETH 16:0/18:1*	*PETH 16:0/18:2*
PIG			
	K_m (g/kg)	4.37	5.60
	V_{max} (µmol*kg^{-1}*h^{-1})	0.318	0.364
CATTLE			
	K_m (g/kg)	5.86	5.61
	V_{max} (µmol*kg^{-1}*h^{-1})	1.271	0.966
GOAT			
	K_m (g/kg)	6.0	6.9
	V_{max} (µmol*kg^{-1}*h^{-1})	1.178	0.917

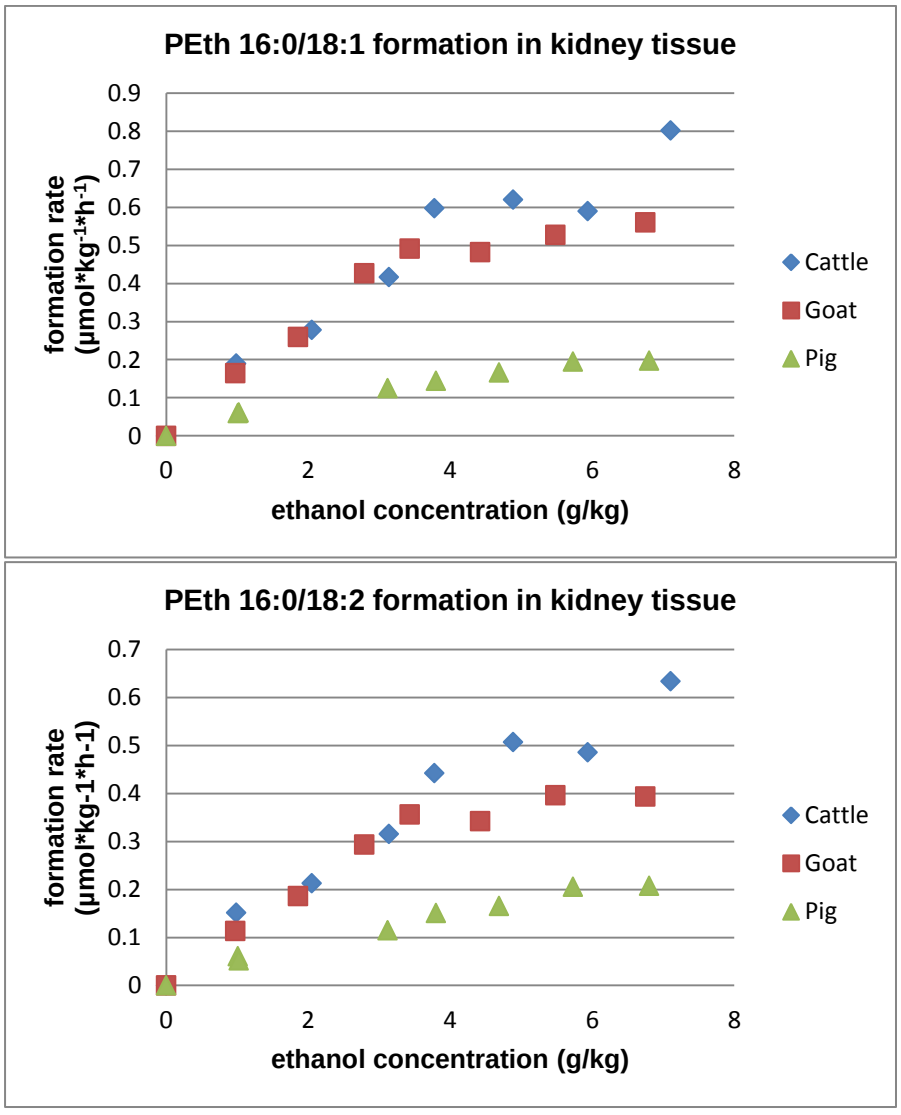

Figure 4.5: Observed PEth formation rates for each ethanol concentration in kidney tissue.

Table 4.4: Michaelis Menten (MM) constants for PEth formation in brain tissue.

animal	MM-constant	*PETH 16:0/18:1*	*PETH 16:0/18:2*
PIG			
	Km (g/kg)	5.16	2.05
	Vmax (µmol*kg-1*h-1)	1.375	0.041
CATTLE			
	Km (g/kg)	1.53	0.93
	Vmax (µmol*kg-1*h-1)	0.612	0.024
Goat			
	Km (g/kg)	6.20	6.73
	Vmax (µmol*kg-1*h-1)	2.788	0.143

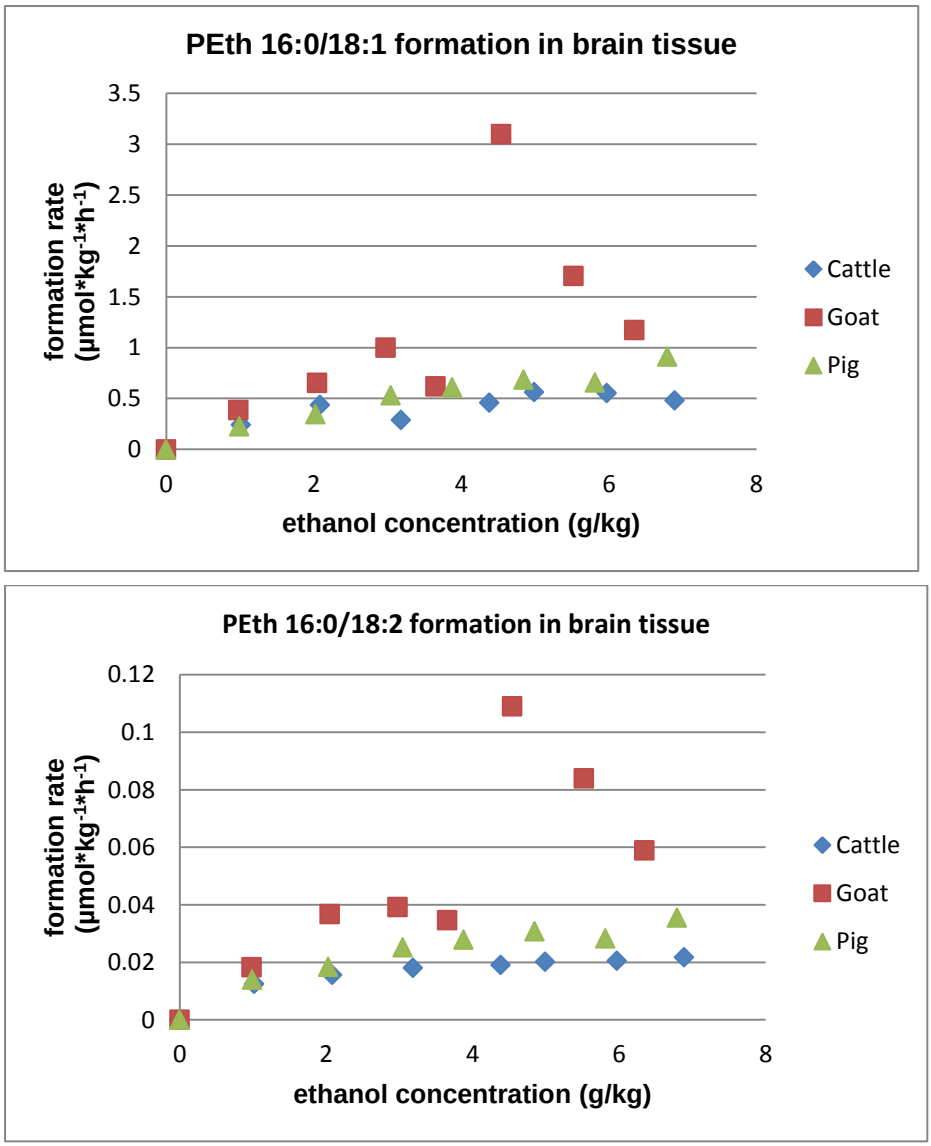

Figure 4.6: Observed PEth formation rates for each ethanol concentration in brain tissue.

4.6. METHOD VALIDATION

4.6.1. Accuracy and precision

The results for intra- and inter-run accuracy for the PC measurements, by measuring six series of the PC quality controls in solvent on three different days, are shown in table 4.5. The intra- and inter-run precision for PC is represented in table 4.6. The intra- and inter-run accuracy and precision for PEth in human blood are depicted in table 4.7 and 4.8, respectively. For both PEth and PC the mean result of the six series was within the allowed 15% deviation from the expected concentration. Also the %CV remained below 15% for PC and PEth.

Table 4.5: Intra- and inter-run accuracy for the PC analytes, determined with six series of every PC quality control in solvent on three different days.

Analyte	concentration (µg/mL)	Intra-run accuracy series 1 (%, n=6)	Intra-run accuracy series 2 (%, n=6)	Intra-run accuracy series 3 (%, n=6)	Inter-run accuracy (%, n=18)
PC 16:0/18:1 SRM 1	1	95.4	98.5	97.9	97.3
	3	102.0	107.5	103.7	104.4
	75	103.3	103.3	95.3	100.6
PC 16:0/18:1 SRM 2	1	93.1	96.2	97.4	95.6
	3	102.3	103.9	105.1	103.7
	75	104.9	106.6	99.0	103.5
PC 16:0/18:2 SRM 1	1	98.8	100.8	111.0	103.5
	3	101.2	98.0	98.6	99.2
	75	109.9	109.3	102.5	107.2
PC 16:0/18:2 SRM 2	1	95.8	97.9	109.9	101.2
	3	99.3	100.4	98.3	99.3
	75	110.5	108.8	106.4	108.6

Table 4.6: Intra- and inter-run precision for the PC analytes, determined with six series of every PC quality control in solvent on three different days.

Analyte	concentration (µg/mL)	Intra-run precision series 1 (%, n=6)	Intra-run precision series 2 (%, n=6)	Intra-run precision series 3 (%, n=6)	Inter-run precision (%, n=18)
PC 16:0/18:1 SRM 1	1	4.0	3.5	3.2	37
	3	3.8	2.8	1.4	3.5
	75	2.3	2.7	3.6	4.7
PC 16:0/18:1 SRM 2	1	3.8	4.0	3.9	4.1
	3	3.0	4.2	2.1	3.2
	75	1.8	3.0	4.5	4.4
PC 16:0/18:2 SRM 1	1	2.1	3.9	3.0	6.0
	3	7.2	6.2	4.6	5.9
	75	6.2	2.1	2.7	5.1
PC 16:0/18:2 SRM 2	1	3.9	3.2	3.0	7.0
	3	7.2	5.1	4.8	5.5
	75	6.4	2.1	2.7	4.8

Table 4.7: Intra- and inter-run accuracy for the PEth analytes, determined with six series of every PEth quality control in human blood on three different days.

Analyte	Concentration (µg/mL)	Intra-run accuracy series 1 (%, n=6)	Intra-run accuracy series 2 (%, n=6)	Intra-run accuracy series 3 (%, n=6)	Inter-run accuracy (%, n=18)
PEth16:0/18:1 SRM 1	0.01	101.4	100.5	106.8	102.9
	0.02	100.1	104.9	101.8	102.3
	0.06	95.1	99.1	96.7	97.0
	1.50	96.4	97.9	94.8	96.4
PEth16:0/18:1 SRM 2	0.01	107.2	102.8	98.5	102.8
	0.02	97.4	97.0	102.0	98.8
	0.06	93.9	98.8	95.0	95.9
	1.50	97.2	97.7	92.9	96.0
PEth16:0/18:2 SRM 1	0.01	110.3	108.2	111.0	109.9
	0.02	95.4	105.8	99.9	100.4
	0.06	92.9	98.1	95.6	95.5
	1.50	95.5	97.5	91.2	94.8
PEth16:0/18:2 SRM 2	0.01	102.7	102.7	109.8	105.1
	0.02	92.8	98.4	99.2	96.8
	0.06	96.5	96.7	94.1	95.8
	1.50	95.5	97.5	94.8	97.5

Table 4.8: Intra- and inter-run precision for the PEth analytes, determined with six series of every PEth quality control in human blood on three different days.

Analyte	Concentration (µg/mL)	Intra-run precision series 1 (%, n=6)	Intra-run precision series 2 (%, n=6)	Intra-run precision series 3 (%, n=6)	Inter-run precision (%, n=18)
PEth16:0/18:1 SRM 1	0.01	4.0	6.3	7.5	6.4
	0.02	38	3.5	5.4	4.5
	0.06	4.2	3.8	5.5	4.6
	1.50	8.4	3.5	7.2	6.4
PEth16:0/18:1 SRM 2	0.01	5.0	4.0	3.1	5.3
	0.02	3.6	2.6	4.8	4.3
	0.06	4.0	5.0	6.4	5.4
	1.50	8.7	3.0	8.9	7.3
PEth16:0/18:2 SRM 1	0.01	5.6	6.8	7.6	6.4
	0.02	2.5	6.3	6.9	6.9
	0.06	5.7	4.7	5.4	5.4
	1.50	5.8	5.1	4.4	5.6
PEth16:0/18:2 SRM 2	0.01	8.6	3.0	4.7	6.4
	0.02	3.6	3.7	8.0	6.0
	0.06	5.7	1.4	5.2	4.2
	1.50	6.3	3.8	4.4	5.2

4.6.2. Extraction efficiency, matrix effects and process efficiency

The average extraction efficiency, matrix effects, and process efficiency, which were measured in triplicate for a PEth concentration of 0.04 µg/mL are depicted in table 4.9. Table 4.10 shows the average extraction efficiency, matrix effects and process efficiency determined of the triplicates with the 0.50 µg/mL PEth solution. The coefficients of variation are indicated between brackets in the tables.

Table 4.9: Extraction efficiency, matrix effects and process efficiency of PEth determined with a 0.04 µg/mL solution.

Tissue	Extraction efficiency		Matrix effects		Process efficiency	
	PEth 16:0/18:1	***PEth 16:0/18:2***	***PEth 16:0/18:1***	***PEth 16:0/18:2***	***PEth 16:/18:1***	***PEth 16:0/18:2***
PIG						
liver	86% (3%)	85% (4%)	106% (7%)	109% (6%)	91% (5%)	93% (2%)
kidney	95% (2%)	92% (8%)	113% (4%)	121% (10%)	108% (6%)	111% (6%)
brain	92% (21%)	83% (7%)	130% (6%)	114% (3%)	118% (16%)	94% (10%)
blood	74% (7%)	82% (14%)	101% (1%)	100% (8%)	75% (7%)	81% (5%)
CATTLE						
liver	76% (5%)	86% (4%)	111% (4%)	116% (13%)	84% (6%)	99% (10%)
kidney	83% (1%)	84% (2%)	103% (2%)	123% (3%)	86% (3%)	104% (3%)
brain	83% (5%)	91% (2%)	12% (6%)	108% (4%)	106% (5%)	98% (6%)
GOAT						
liver	78% (3%)	89% (8%)	109% (4%)	113% (5%)	84% (7%)	101% (5%)
kidney	86% (9%)	99% (6%)	132% (3%)	119% (3%)	115% (10%)	118% (2%)
brain	83% (14%)	98% (12%)	140% (9%)	110% (4%)	116% (4%)	108% (8%)
HUMAN						
blood	75% (10%)	78% (5%)	102% (8%)	102% (8%)	77% (3%)	80% (4%)

Table 4.10: Extraction efficiency, matrix effects and process efficiency of PEth determined with a 0.50 µg/mL solution.

Tissue	Extraction efficiency		Matrix effects		Process efficiency	
	PEth 16:0/18:1	***PEth 16:0/18:2***	***PEth 16:0/18:1***	***PEth 16:0/18:2***	***PEth 16:/18:1***	***PEth 16:0/18:2***
PIG						
liver	78% (8%)	84% (10%)	96% (1%)	93% (8%)	75% (8%)	78% (6%)
kidney	86% (8%)	93% (1%)	97% (1%)	97% (3%)	84% (8%)	90% (8%)
brain	72% (7%)	79% (2%)	87% (2%)	90% (6%)	63% (5%)	71% (7%)
blood	75% (0.3%)	76% (2%)	103% (9%)	104% (9%)	77% (9%)	80% (7%)
CATTLE						
liver	80% (4%)	85% (2%)	95% (3%)	98% (4%)	76% (2%)	84% (6%)
kidney	80% (8%)	87% (2%)	100% (3%)	102% (6%)	79% (6%)	89% (8%)
brain	67% (3%)	82% (6%)	96% (3%)	95% (8%)	64% (3%)	78% (2%)
GOAT						
liver	76% (6%)	83% (7%)	97% (5%)	96% (4%)	74% (4%)	80% (9%)
kidney	74% (5%)	83% (4%)	99% (5%)	99% (5%)	73% (4%)	82% (2%)
brain	71% (9%)	80% (6%)	92% (4%)	93% (6%)	65% (6%)	74% (2%)
HUMAN						
blood	80% (25%)	91% (26%)	74% (16%)	88% (19%)	74% (7%)	79% (6%)

5. DISCUSSION

With the newly developed LC-ESI-MS/MS method both PC- and PEth homologues could successfully be separated and detected. However, for the quantification of both phospholipid homologues it was required that the amounts, present in the samples, fitted the calibration curve. For the PC quantification this was a challenge, since the endogenous amounts, present in the investigated tissues, were unknown. A calibration series based on the amounts of PC in human blood was used, but as illustrated in table 4.1 the PC amounts in blood are far below the amounts present in the other animal tissues. Therefore a dilution series of the animal tissues was made to analyze the PC.

Another requirement for the simultaneous determination of PEth and PC is that the synthesized PEth internal standard does not contain PC residues, to avoid interference. No PC residues were found in both synthesized, deuterated PEth homologues. For an optimal compensation of the variabilities during sample preparation and the matrix effects, a second PC internal standard for PC 16:0/18:2 could be used.

Method validation, based on the FDA guidance, was performed by measuring six series of quality controls on three different days. The mean value, obtained from six measurements at every quality control concentration, was within 15% of the expected concentration and the coefficient of variation did not exceed 15%, for both PEth in human blood and PC in solvent. So the method fulfilled the FDA criteria for accuracy and precision.(61)

With the exception of the calibrators in goat brain and kidney, all calibrators were well within the range of the defined limits. Concerning linearity, for all calibration curves a correlation coefficient >0.99 was obtained. For the PC quantification it was not possible to make a calibration curve in animal tissue as recommended by the FDA guidance, since it is an endogenous compound ubiquitous in all tissues. Therefore a calibration curve in solvent was made, which did not account for possible matrix effects.

The fact that PC is an endogenous compound made it also impossible to determine the extraction efficiency, matrix effects and process efficiency by the method described by Matuszewski *et al*. The use of the deuterated internal standard, PC 16:0/18:1-$d_{31,}$ however, should be able to rectify the matrix effects, as it undergoes the same degree of ion enhancement or suppression.(64)

For the PEth determination, extraction efficiency, matrix effects and process efficiency were analyzed. Generally the extraction efficiency and process efficiency of the PEth 16:0/18:2 homologue was larger than the extraction efficiency and process efficiency of the PEth 16:0/18:1 homologue. The matrix effects on both PEth homologues were similar, but the concentration of the spiked PEth solution seemed to have an influence on the matrix effects. For the samples spiked with 0.04 µg/mL PEth solution ion enhancement was seen, while for the samples spiked with 0.5 µg/mL no matrix effect was observed, with the exception of the ion suppression seen for PEth 16:0/18:1 in human blood. This could be due to the fact that a 1/x weighted calibration curve was used for the calculation of the peak areas. Overall the process efficiency of PEth determination was the smallest in the blood samples and relatively consistent for a type of tissue between the different animals. The differences in extraction efficiency between the different tissues and animals were during the PEth measurements in animal tissue corrected for by the deuterated internal standard. The deuterated internal standard also corrected for the matrix effects together with the calibration curves, made in every tissue.(64)

For full method validation selectivity and stability should be tested as well. According to the FDA guidance, selectivity must be analyzed by determining the interference in blank samples of at least six sources. In this experiment the selectivity was ensured by testing a blank tissue sample from each tissue for the presence of PEth. No PEth was found in these blank samples. Stability was not tested, since PEth stability has already been investigated in numerous studies.(65, 66) Besides, the samples were tested within two days. Post sampling PEth formation, as observed by Aradottir *et al*.(65), was avoided by adding acetonitrile to precipitate the PLD enzyme.

After dilution of the blank tissue samples, the endogenous amounts of PC could be determined. For all the tested animals, most PC was found in brain tissue. In brain a large distinction was seen between PC 16:0/18:1 and PC 16:0/18:2. The ratio PC 16:0/18:2 to 16:0/18:1 was 4.1% (pig), 4.5% (cattle) and 5.2% (goat). In the liver- and kidney tissues, there was also more PC 16:0/18:1 present, but the difference is smaller, resulting in a larger ratio of PC16:0/18:2 to PC 16:0/18:1, 48.2-85.5% (kidney) and 45.3-77.5% (liver). The largest variability in PC concentrations between the different animals is seen in the liver tissue. The lowest amounts of PC for pig and goat were found in kidney tissue, when blood tissue is left out of account. The amounts of PC in both pig and bovine blood were at least ten times lower than the amounts of PC in the other tissues. Contrary to all other tissues, more PC 16:0/18:2 than PC 16:0/18:2 is present in bovine blood, which results in a 354% ratio. For pig blood the amounts of both homologues were similar.

An increase in PEth formation was seen, with increasing ethanol concentrations. However, different concentrations were obtained for the SRM1 and SRM2 transitions of both PEth homologues, formed in animal tissue, while this was not the case for the concentrations in solvent after spiking with the PEth reference solution. A possible explanation for the different PEth concentrations obtained with SRM1 and SRM2, in the animal tissues, can be found in the research done by the team of Wolf D Lehman from the University of Heidelberg. They found that two structural isomers of PC, PC 18:0/18:1 and PC 18:1/18:0, give a slightly different fragmentation pattern, due to the fact that fragmentation at the SN2 position is preferred and thus a higher signal is obtained for the fatty acid chain in this position.(67) Since no difference was seen in the samples spiked with ethanol, probably a mixture of PEth 16:0/18:1 and PEth 18:1/16:0 and of PEth 16:0/18:2 and PEth 18:2/16:0 is formed in animal tissue. By taking the mean concentration of both SRM transitions this difference should be normalized with respect to the reference solution, used to spike the calibration curve in solvent. We assumed that the reference PEth solution contained 100% PEth 16:0/18:1 or 16:0/18:2.

After analyzing the normalized concentrations by means of Michaelis Menten kinetics, the following observations were made: When comparing liver, kidney, and brain tissue, the lowest formation rates for PEth 16:0/18:1 were seen in liver tissue and the highest formation rates in brain. For PEth 16:0/18:2 the lowest formation

rates were seen in brain and the highest in kidney. This can partially be explained by the endogenous amounts of PC. The highest amount of PEth 16:0/18:1 as well as the lowest amounts of PEth 16:0/18:2 were seen in brain. However, the lowest amounts of PEth 16:0/18:1 were seen in kidney for pig and goat. Only in cattle the lowest amounts of PC 16:0/18:1 were found in liver tissue. For PEth 16:0/18:2 the highest amounts of its PC precursor were indeed seen in bovine kidney, but for pig and goat the highest amounts were seen in liver.

When comparing the PEth formation in one type of tissue between the tested animals, following observations were made. In liver tissue the formation rate of PEth 16:0/18:1 was the lowest in pig tissue, while the PEth 16:0/18:2 formation rate was similar for the three animals. The fact that for PEth 16:0/18:2 the same formation rate was seen in pig tissue, as in the other tissues, is possibly due to the amount of PC 16:0/18:2, which was twice as high in pig liver compared to cattle and goat. Also in kidney tissue lower formation rates were seen in pig tissue, both for PEth 16:0/18:1 as for PEth 16:0/18:2. In brain tissue the highest formation rates were found in goat. However the formation rate vs. time curves of goat tissue fluctuated heavily. Due to this the calculated Michaelis Menten constants do probably not fully represent the true kinetics. Also for the PEth 16:0/18:1 formation in bovine brain heavy fluctuations were seen in the formation rate vs. time curve. When the back-up samples for both goat and bovine brain were tested the same results were obtained. The problem is most likely inhomogeneous sampling. Therefore it is recommended for future experiments to work with large organs so homogeneity can be ensured. Another possible explanation can be found in the calibration curve. Some of the calibrators in brain tissue exceeded the allowed 15% deviation from the expected concentration, possibly indicating that these were not fully compensated for the matrix effects.

In all three the animals the difference between the formation rates of both PEth homologues in brain tissue was conspicuous. The ratio PEth 16:0/18:2 to PEth 16:0/18:1 in brain tissue was 3.9% (pig), 2.9% (cattle), and 6.0% for goat. These ratios roughly correspond to the ratio PC 16:0/18:2 to PC 16:0/18:1, 4.1% (pig), 4.5% (cattle), and 5.2% (goat), in brain tissue and indicate that PC 16:0/18:1 is the main substrate. The distinction between the PEth homologues was also seen in human brain by Thompson *et al*. They mentioned that the PEth 16:0/18:1 levels were at least a ten-fold higher than PEth 16:0/18:2 in all subjects.(68)

In general, large variations in PEth formation were seen among the tested animals and also among the tissues of a single animal. Variations in PEth concentrations were also seen in rat organs, after *in vivo* alcohol exposure, by Aradottir *et al*.(69) Some of these varying concentrations of PEth, such as the difference in formation rate between PEth 16:0/18:1 and PEth 16:0/18:2, could be explained by the endogenous amounts of PC.

However, the differences in formation rates observed between the three animal species could not solely be correlated to the endogenous amounts of PC, indicating that also enzyme linked characteristics are likely responsible for the observed differences. It has been demonstrated that there are different PLD isoforms, which are differently regulated and expressed in different organ tissues.(70, 71) Whether the differences in PEth formation, between the different tissues and animal species, are caused by varying expression, regulation, or characteristics of PLD is still unclear and needs further investigation.

The fact that the PEth formation in pig and bovine blood remained below the LLOQ supports the perception of Viel G. *et al*. that human RBCs seem to be peculiar in the in vitro formation of PEth when ethanol is present.(30) A possible explanation is that the PLD activity or abundance in animal RBCs is much lower than in human RBCs. The presence of PLD in animal RBCs cannot be excluded, since PLD activity in rabbit RBCs was demonstrated by Ochi *et al*.(65, 72)

6. CONCLUSION

With the newly developed LC-ESI-MS/MS method it is possible to determine the most abundant PEth homologues, PEth 16:0/18:1 and PEth 16:0/18:2, together with their PC precursors. The quantification of both phospholipid homologues within one run is however, only possible for samples with low PC concentrations, such as blood samples. The method was validated for PEth in human blood samples for PC in solvent and fulfilled the FDA criteria for accuracy and precision. A drawback for optimal accuracy is the fact that a calibration curve in solvent is necessary for PC determination, given that PC is an endogenous substance.

Considering PEth formation, the PEth concentration in animal blood remained below the LLOQ, which implies the incapability of animal RBC's to form PEth or a fast PEth degradation in animal RBC's. Further large variations were seen in the calculated V_{max} and K_m constants between the liver, kidney, and brain tissue from one animal species, as well as between the different animal species: pig, goat and cattle.

The endogenous amounts of PC accounted for some of the observed differences in PEth formation. However, the observed differences could not entirely be explained by the amounts of PC present in the tissues. This indicates that also PLD enzyme characteristics are responsible for the observed differences. Whether the differences are caused by different regulation, expression, or the presence of different isotypes of PLD is unclear and needs further investigation.

For further investigations in animal tissue, it is recommended to use animals with large organs in order to ensure homogeneity. Inhomogeneous sampling could give rise to large variabilities, as seen for the PEth formation in goat brain.

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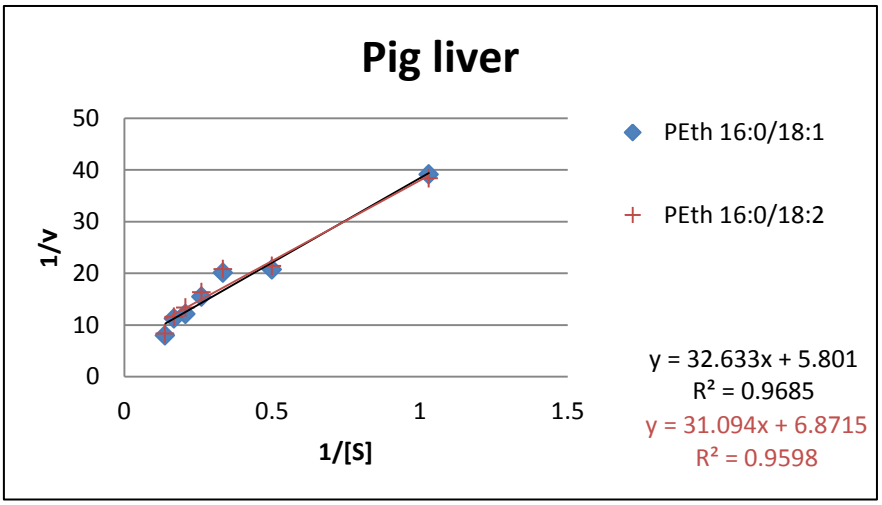
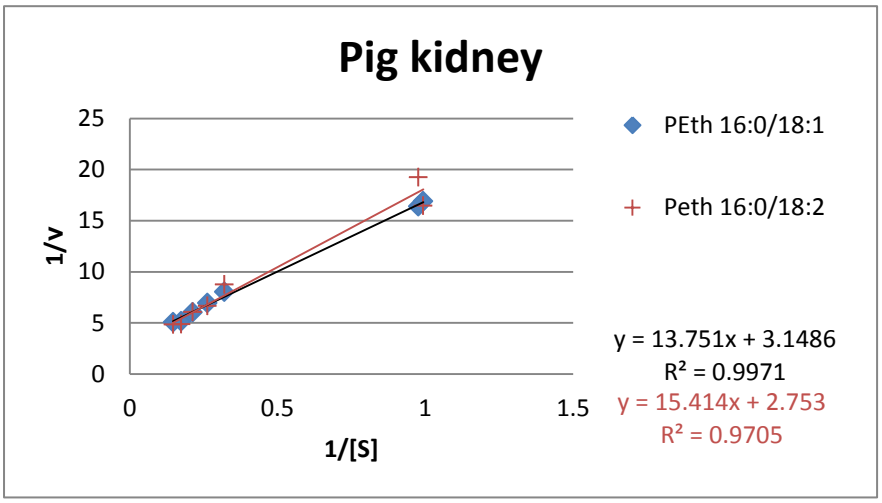
8. APPENDIX

a)



b)



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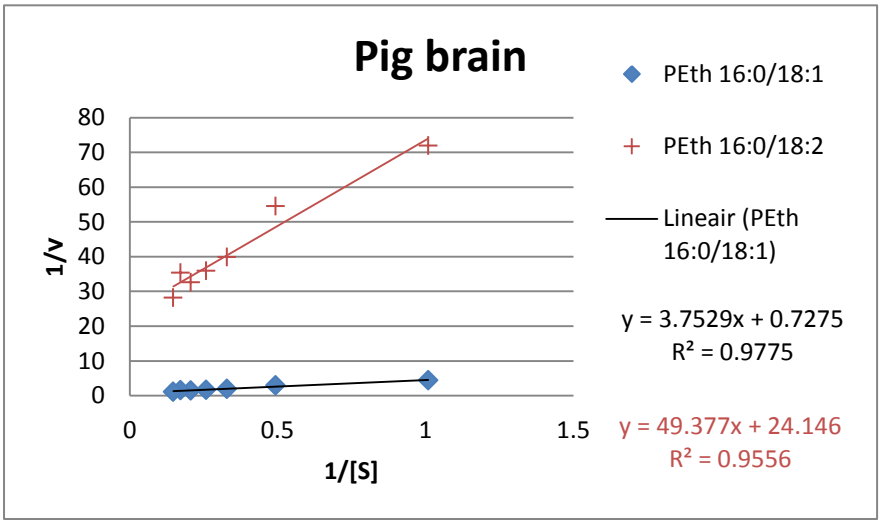
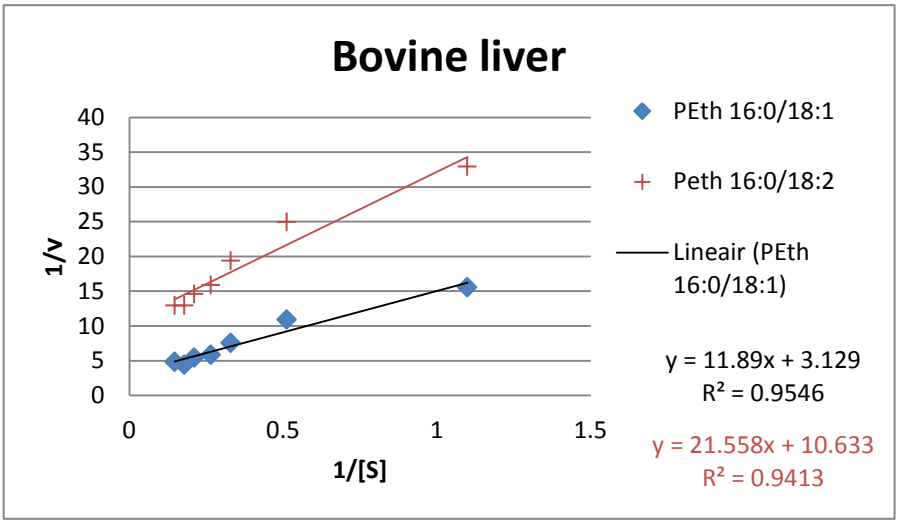


Figure 8.1: Lineweaver-burk plots for the PEth formation in pig liver (a), kidney (b) and brain (c).

a)



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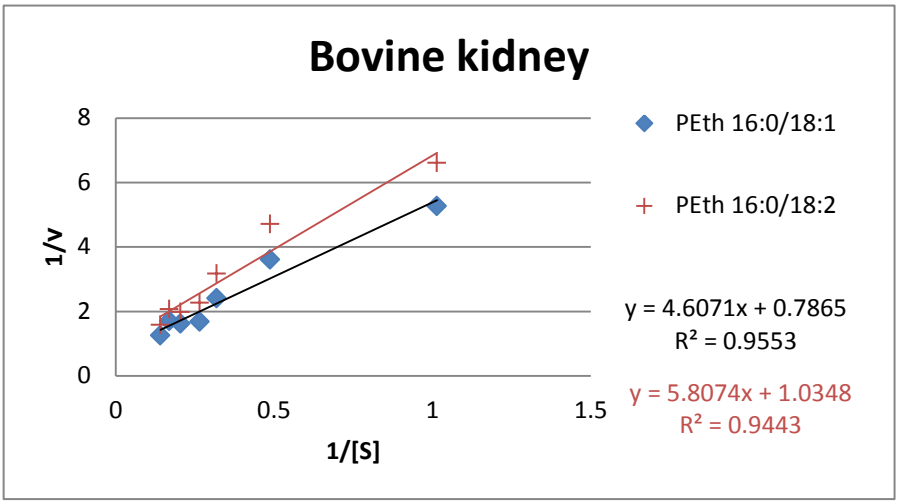

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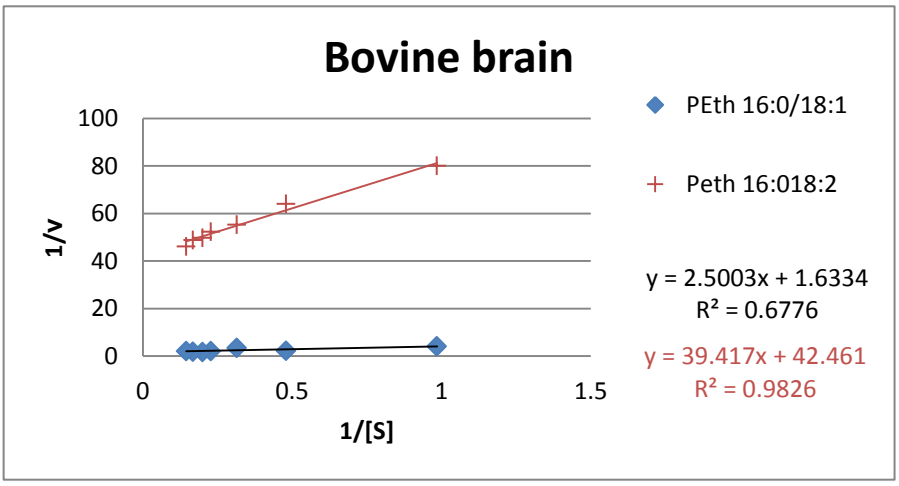
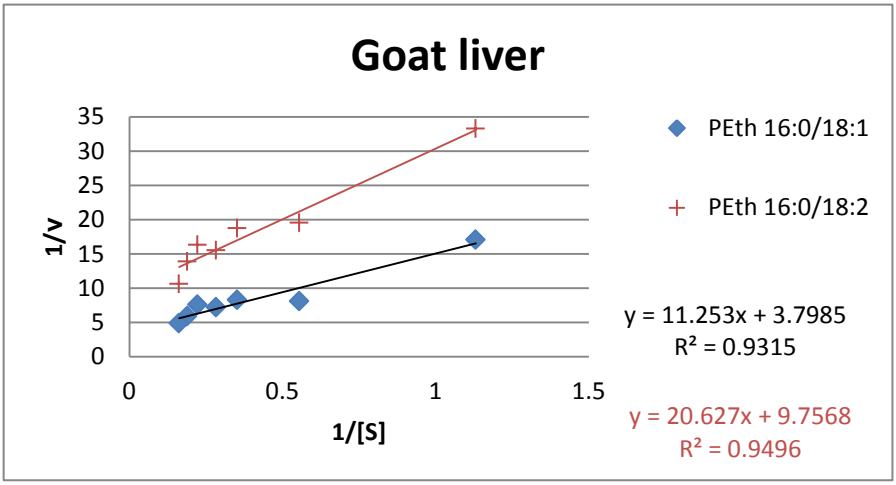


Figure 8.2: Lineweaver-Burk plots for the PEth formation in bovine liver (a), brain (b), and kidney (c).

a)



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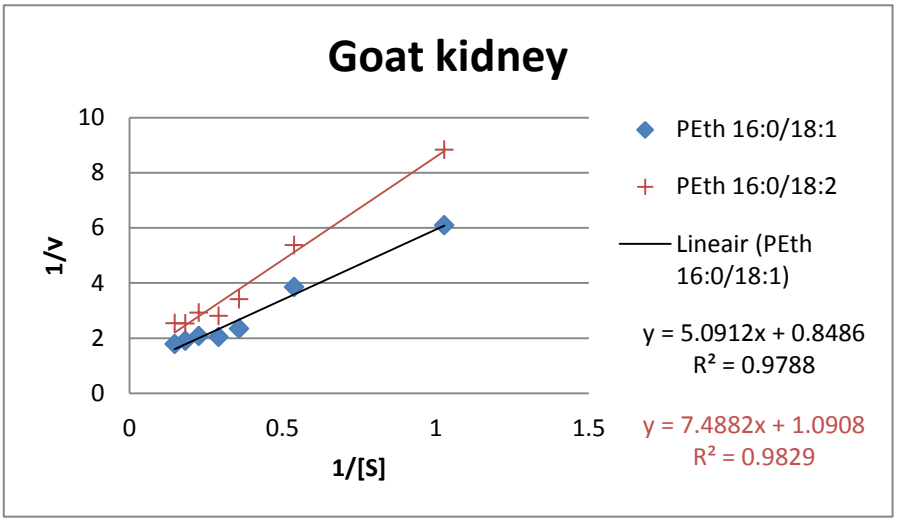

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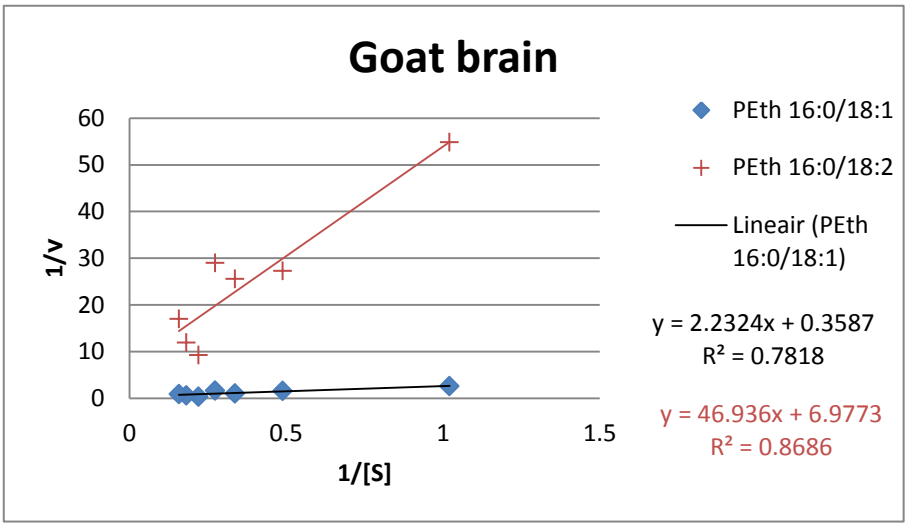

Figure 8.3: Lineweaver-Burk plots for the PEth formation in goat liver (a), kidney (b) and brain (c).