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Hubert Curien

Départment des Sciences Analytiques
Equipe de Chimie Analytique des Molécules Bioactives
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THÈSE

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Li Zhou

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Mise au point de modèles d'oxydation et de techniques analytiques spécifiques des phospholipides et de leurs produits de dégradation dans les aliments fonctionnels

THÈSE dirigée par :

Eric Marchioni	**Professeur, Université de Strasbourg**
Françoise Bindler	**Docteur, Université de Strasbourg**

RAPPORTEURS :

Henri Portugal	**Professeur, Université de Marseille**
Karen Gaudin	**Professeur, Université de Bordeaux**

MEMBRES DU JURY :

Jean-Philippe Loeffler	**Docteur, Université de Strasbourg**
Peter Horvatovich	**Docteur, Université de Groningen**

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LI ZHOU

Email: zhouli.strasbourg@hotmail.com

Education

2009-2012 **Doctoral study in analytical chemistry**—IPHC-DSA (CAMBA) University of Strasbourg, France

2006-2009 **Master degree in food science**—Southwest University, China. Research direction: Food quality and safety

2002-2006 **Undergraduate degree in Food science and technology**-Southwest University, China. Research direction: Aquatic products quality and safety

Work Experience

2009-2012 Laboratory of analytical chemistry of bioactive compounds
IPHC-UMR 7178, CAMBA, faculty of pharmacy, Strasbourg University, France
Subject : Development of analytical techniques for phospholipids and their degradation products in functional foods
Extraction of phospholipid by pressurized liquid extraction
Separation of phospholipids by solid phase extraction
Identification of molecular species of phospholipid by LC-ESI-MS^2 and volatile oxidized compounds by HS-SPME-GC-MS
Development of chromatographic analysis methods (HPLC, GC-MS, LC-MS)

2007-2009 Chongqing key Laboratory of Produce Processing and Storage, Chongqing, China
Subject: Quality changes of edible oil during frying and research on rapid detection technology of polar compounds (Natural Science Foundation of Chongqing, CSTC, 2007BB0237)
Solid phase extraction of polar compound in fried oil
Development of analytical methods (fluorescence spectrometry, electrical conductivity)
Acute toxicity test, Mutagenicity test

2005-2006 Laboratory of Food Science, Southwest University, China
Subject: Atomic absorption spectrometry determination of lead and cadmium in live fish from Jialing river
Digestion of samples by sulfuric acid and perchlorine acid
Atomic absorption spectrometry detection of heavy metal in different fish organs

Skills

Analysis and purification of the active ingredients from Natural products (HPLC, LC-MS, GC-MS)

Software: Star work station (Varian), Chemdraw

Languages: Chinese (Mother language) English French

Publications

Li Zhou, Julie Le Grandois, Eric Marchioni, Minjie Zhao, Saïd Ennahar, Françoise Bindler. Improvement of Total Lipid and Glycerophospholipid Recoveries from Various Food Matrices Using Pressurized Liquid Extraction. *Journal of Agricultural and Food Chemistry.* 2010, *58*, 9912-9917.

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List of Common Abbreviations

ACN:	acetonitrile
APCI:	atmospheric pressure chemical ionization
CAR/PDMS:	carboxen-polydimethylsiloxane/ polydimethylsiloxane
DAD :	diode array detector
DESI:	desorption electrospray ionization
DHA:	docosahexaenoic acid
EFA:	essential fatty acid
EPA:	eicosapentaenoic acid
ESI:	electrospray ionization
FA:	fatty acid
FFA:	free fatty acid
GC:	gas chromatography
GPL:	glycerophospholipid
HPLC:	high-performance liquid chromatography
HS-SPME:	headspace solid-phase microextraction
LAESI:	laser ablation electrospray ionization
LC-FA:	long chain fatty acid
LC-PUFA:	long chain polyunsaturated fatty acid
LLL:	trilinolein
LPE:	lysophosphatidylethanolamine
LPC:	lysophosphatidylcholine
LPI:	lysophosphatidylinositol
MALDI-TOF:	matrix-assisted laser desorption/ionization-time of flight
MRM:	multiple reaction monitoring
MS:	mass spectrometry
MSI:	mass spectrometry imaging
MUFA:	monounsaturated fatty acid
NL:	neutral lipid
NP:	normal phase
OOO:	triolein
PA:	phosphatidic acid
PC:	phosphatidylcholine
PDMS:	polydimethylsiloxane
PDMS/DVB:	polydimethylsiloxane-divinylbenzene
PE:	phosphoatidylethanolamine
PG:	phosphatidylglycerol
PI:	phosphatidylinositol
PL:	phospholipid
PLE:	pressurized liquid extraction
PS:	phosphatidylserine
PUFA:	polyunsaturated fatty acid
RP:	reverse phase
SELDI:	surface-enhanced laser desorption/ionization
SFA:	saturated fatty acid
SIMS:	secondary ion mass spectrometry
SLE:	solid liquid extraction
SLPA:	1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphate
SLPE:	1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine
SLPC:	1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine
SLS:	1,3-stearoyl-2-linoleoyl-glycerol

SM: sphingomyelin
SOPA: 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphate
SOPE: 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
SOPC : 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SOS: 1,3-stearoyl-2-oleyl-glycerol
SPL: sphingolipid
SPE: solid phase extraction
SSS: tristearin
TAG: triacylglyceride
TIC: total ion chromatogram
TL: total lipid
TLC: tin-layer chromatography
UFA: unsaturated fatty acid
VOC: volatile oxidized compound

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GENERAL INTRODUCTION

Les lipides sont des molécules essentielles pour la vie. Il en existe un très grand nombre, ce qui a conduit à établir une classification. Les lipides sont séparés en deux grands groupes que sont les lipides simples et les lipides complexes. Les lipides simples sont uniquement constitués de carbone, d'oxygène et d'hydrogène. Ce sont des esters d'acides gras (AG) que l'on classe en fonction de l'alcool. Il s'agit des glycérides (l'alcool est le glycérol), des stérides (l'alcool est un stérol) et des cérides (l'alcool est à longue chaîne). Les lipides complexes contiennent notamment les phospholipides (PLs), qui sont des lipides polaires et qui contiennent, en plus des C, H et O, un atome de phosphore. On trouve deux types de PL: les glycérophospholipides (GPLs) et les sphingolipides (SPLs). La différenciation entre ces deux types se fait par la nature du squelette de base: soit le glycérol, soit la sphingosine. Les GPLs sont constitués de deux AG et d'un ester phosphorique estérifié en position *sn*-3. Les classes existantes sont les phosphatidylcholines (PC), les phosphatidyléthanolamines (PE), les phosphatidylsérines (PS), les phosphatidylinositols (PI) et les phosphatidylglycérols (PG).

Les AG sont attachés au squelette de glycérol à *sn*-1 et *sn*-2. Le groupe tête polaire-phosphate est attaché en position *sn*-3. La longueur de la chaîne alkyle des AG varie généralement de 14 à 24 atomes de carbone et le nombre de doubles liaisons de 0 à 6. La chaîne alkyle dans la position *sn*-1 est généralement saturée ou mono-insaturée, tandis qu'en position *sn*-2 elle est souvent polyinsaturée.

Parmi les PLs on peut également trouver des ether-PLs qui sont des PLs où l'AG en position *sn*-1 est remplacé par un alcool gras, lié au glycérol par une liaison ether. Les plasmalogènes sont un type d'ether PL caractérisé par la présence d'une liaison éther de vinyle à la position *sn*-1 et d'une liaison ester à la position *sn*-2. Les Plasmalogènes constituent environ 15-20% du total des PL des membranes cellulaires et parmi eux plus de 50% sont des plasmenylethalomines que l'on retrouve principalement dans le cerveau, le cœur, les neutrophiles et les éosinophiles (Braverman et Moser, 2012). Les Plasmanyl PL sont un autre type d'éther PL caractérisé par la présence d'une liaison éther de 1-O-alkyl.

Les SPLs quant à eux contiennent une molécule de sphingosine. Il s'agit d'un alcool aminé à longue chaîne insaturée dont le groupement amine est lié à un AG par une liaison amide. Cet ensemble est appelé céramide. Ces SPL sont classés en sphingomyélines (SM) et en glycosphingolipides. Contrairement aux GPLs, les SPL complexes ne sont composés que d'un seul AG estérifié à un squelette sphingoïde. Ils présentent un degré élevé de diversité structurelle due à des variations du degré de non-saturation, de méthylation et d'hydroxylation de la longue chaîne

de la base sphingoïde, de la nature de l'AG ainsi que de la nature de la tête polaire. La SM, qui est l'un des sphingolipides les plus répandus est constituée d'un céramide et d'un groupement phosphorylcholine.

Ces PLs, qui sont des composés majeurs des membranes cellulaires (en particulier les neurones), seraient également impliqués dans certains métabolismes, surtout en tant que précurseurs d'autres molécules biologiques. Des études ont notamment montré que les PLs joueraient un rôle dans la fonction et la croissance des cellules nerveuses, le maintien des fonctions cognitives de la mémoire et la réduction du niveau de cholestérol dans le sang en freinant son absorption au niveau intestinal et en augmentant son excrétion par les voies biliaires.

Parmi les informations disponibles à ce jour se rapportant à la structure moléculaire des PLs, très peu concernent les matrices alimentaires. Le plus souvent les études ont été menées sur des matrices biologiques. En ce qui concerne les matrices alimentaires, ce sont les PLs de soja et de jaune d'œuf (Kivini *et al*., 2004) qui ont été les plus étudiés, surtout parce qu'ils sont les constituants majeurs des lécithines utilisées industriellement comme additif. Pour ces substances les profils des familles et des espèces moléculaires ont déjà été établis (Pacetti *et al*., 2005). Certaines études font état d'analyses chromatographiques de lipides totaux (LT) par LC-MS sans étape d'isolement préalable des PLs. Toutefois, compte tenu de leur degré élevé de similitude structurale, les espèces moléculaires au sein d'une famille donnée, ne peuvent pas être complètement déterminées et les isomères ne peuvent être distingués. En outre, la quantification des différentes espèces moléculaires au sein de chaque famille de PL ne peut être effectuée par spectrométrie de masse qu'en mode single ion monitoring. Dans le cas d'analyses chromatographiques des LT alimentaires par LC-MS, la prédominance de lipides neutres dans les extraits de LT affecte l'ionisation dans les sources d'ionisation et la désolvatation des gouttelettes générées par électronébulisation. Aussi pour obtenir des informations plus précises sur les espèces moléculaires des PL et pour éviter la co-élution de différentes familles de PL, il est important de développer des procédures de purification des PLs et il est préférable d'analyser séparément chaque famille de PL.

Enfin, il est indispensable de s'intéresser à la stabilité des PLs et notamment à leur oxydation. En effet celle-ci est un mécanisme qui, d'une part, modifie beaucoup la qualité nutritionnelle des produits alimentaires riches en PLs et qui, d'autre part, conduit à la formation de molécules potentiellement toxiques (Peng and Taylor, 1998). Les molécules grasses les plus étudiées, en ce qui concerne les phénomènes d'oxydation, sont sans aucun doute les AG, les

triacylglycérides (TAG) et le cholestérol. Si l'analyse des produits d'oxydation des PLs par LC-MS a déjà fait l'objet de travaux scientifiques (Reis *et al*., 2005; Le Grandois *et al*., 2009 et 2010), aucune publication, à notre connaissance, ne s'est focalisée de façon plus spécifique sur l'étude par GC-MS des composés volatils issus de l'oxydation des PLs.

La première partie de ce travail avait pour objectif de mettre au point une méthode d'extraction des PL, alternative à la méthode d'extraction liquide-liquide communément appelée méthode de Folch (technique la plus couramment utilisée), si possible plus efficace, plus rapide et moins consommatrice de solvants. Parmi les nouvelles techniques disponibles, il en est une qui a retenu plus particulièrement notre attention c'est l'extraction liquide-solide assistée par la pression (PLE). En effet outre son efficacité cette technique a récemment fait l'objet d'un intérêt tout particulier en terme d'approche écologique.

La PLE a beaucoup d'avantages qui en font un excellent substitut aux méthodes traditionnelles. L'utilisation de température et de pression élevées lors de l'extraction ne permet pas seulement d'améliorer le rendement d'extraction mais diminue également le temps d'analyse et la consommation de solvants. En outre, la PLE, de par son principe de fonctionnement, est particulièrement adaptée à l'extraction de composés sensibles à la lumière et à l'oxygène.

Nous avons donc mis en œuvre un protocole d'analyse permettant de comparer ces deux méthodes d'extraction. Cette étude a été effectuée sur diverses matrices alimentaires, sources potentielles de PLs, à savoir : le jaune d' œuf (riche en matières grasses > 30%, m/m), le foie de bœuf et la cervelle de veau (tout deux à faible teneur en matière grasses < 10%, m/m), les graines de soja (contenant de 10 à 30% de matières grasses) et l'huile de krill qui est une huile animale d'origine océanique, particulièrement riche en PLs.

Pour chacune de ces matrices alimentaires les deux types d'extraction (Méthode de Folch et PLE) ont été réalisés et le profil phospholipidique a été établi par chromatographie liquide haute performance (CLHP) sur phase normale couplée à un détecteur évaporatif à diffusion de lumière (DEDL). La séparation des classes de PL a été effectuée sur un extrait de LT à partir de chacune des matrices alimentaires. Les différentes classes de PL présentes dans les matrices alimentaires ont été identifiées par comparaison de leurs temps de rétention avec ceux obtenus pour les standards. Les PLs du soja sont PE, PI et PC, en proportions relativement équivalentes. Le jaune d'œuf contient PE, PI, PC et SM avec PE et PC fortement majoritaires. Le foie de bœuf et la cervelle de veau contiennent chacun cinq classes de PL, PE, PI, PC, PS et SM. Enfin dans

l'huile de krill seules PC et PE ont été identifiées avec une forte proportion de PC.

Les matrices alimentaires analysées diffèrent à la fois par la nature des PLs mais également par leurs proportions. Si PC est prédominant dans le jaune d'œuf, le soja et le foie de bœuf pour la cervelle de veau ce sera PE qui sera majoritaire. Par contre quelque soit la matrice alimentaire PI et PS sont toujours en faibles quantités. Notre résultat est cohérent avec les études antérieures qui montrent que PC est habituellement le PL majoritaire et PE le second dans les matrices alimentaires (Pérez-Palacios *et al*., 2008; Avalli et Contarini, 2005).

Pour chaque méthode d'extraction des courbes d'épuisement ont été réalisées afin de déterminer le nombre d'extractions nécessaires à l'extraction maximale des PLs.

Dans un premier temps la méthode PLE fut mise au point avec un mélange de solvants identique à celui utilisé dans la méthode d'extraction solide-liquide (SLE) de Folch (chloroform/methanol, 88:12 v/v) en testant différentes températures (120°C à 150°C) et durées d'extraction (de 5 à 10 minutes). La récupération de PL augmente avec la température (120 à 140 °C) car une augmentation de la température diminue la viscosité d'un solvant liquide, renforçant ainsi sa pénétration à l'intérieur de la matrice, ce qui conduit à un procédé d'extraction amélioré (Mustafa et Turner, 2011). Mais, lorsque la température est portée à 150 °C, la quantité de PL extraite a légèrement diminué, ce qui est en accord avec les résultats obtenus par Boselli et concernant l'extraction des oxystérols d'aliments contenant du jaune d'œuf (Boselli *et al*., 2001). Cette diminution peut être expliquée par des phénomènes oxydatifs et de thermolyse qui se produisent à des températures plus élevées et qui transforment les PLs (Le Grandois *et al*., 2010). Il y a d'ailleurs un phénomène très remarquable lié à ces changements, c'est la modification de la couleur de l'extrait de LT qui passe du jaune au vert lorsque la température d'extraction est à 150 °C. Ce phénomène implique que certains changements se sont produits dans la composition chimique des matrices alimentaires à 150 °C. Par conséquent, 150 °C n'est pas une température convenant à l'extraction. En ce qui concerne la durée de l'extraction, aucune différence significative ($P > 0.05$) dans l'efficacité d'extraction n'a été observée, que l'extraction à 130 °C ait lieu pendant 5 min ou 10 min. Cela montre que l'équilibre de partage entre le solvant et la matrice alimentaire a été atteint dans les cinq premières minutes au cours d'une extraction statique, ce qui est en accord avec les résultats d'autres auteurs (Dunford et Zhang, 2003). Par conséquent, la température et la durée d'extraction optimale pour chaque extraction par la méthode PLE ont été fixées à 140 °C et 5 min, respectivement. Puis dans un deuxième temps nous avons montré que contrairement à la méthode SLE de Folch qui est longue, qui nécessite l'utilisation de grandes

quantités de solvants organiques (souvent dangereux, couteux et devant être éliminés une fois l'extraction terminée) et qui, en outre peut conduire à la dégradation des analytes, l'extraction PLE utilise de plus faibles quantités de solvants pour obtenir une extraction quantitative à partir d'échantillons solides et semi-solides en un temps relativement bref.

La méthode PLE, a permis en une seule étape d'extraire plus de 94% (±1) des LT et 96,4% (±0,5) de chaque famille (PE, PI et PC) de PLs. Par contre avec la méthode SLE de Folch, légèrement modifiée (Bligh and Dyer, 1959), quatre extractions successives ont été nécessaires pour récupérer l'ensemble des lipides totaux et chaque famille de PLs. Les résultats obtenus ont démontré que la PLE est une méthode rapide et efficace pour l'extraction des lipides neutre et polaires à partir de tissus végétaux et animaux. Cela peut s'expliquer par le principe même de l'extraction. Le procédé PLE utilise une température élevée, ce qui permet une grande solubilité et une grande vitesse de diffusion des substances lipidiques dans le solvant, et une pression élevée, ce qui conduit à une bonne pénétration du solvant dans l'échantillon. Ce processus permet ainsi d'améliorer la surface de contact entre la matrice et le solvant. Ainsi, la PLE a une efficacité d'extraction élevée. Pour la méthode SLE-Folch, les échantillons sont simplement mélangés à des solvants et aucun procédé ne permet de faciliter l'accès du solvant aux composants internes de l'échantillon. C'est la raison pour laquelle la méthode SLE-Folch est moins efficace que la méthode PLE et qu'elle nécessite un nombre d'extraction plus important.

En outre, la méthode PLE satisfait au principe de la technologie verte qui vise à préserver l'environnement naturel et à limiter l'influence négative de l'intervention humaine. La philosophie de la chimie verte est de développer et d'encourager l'utilisation de procédure qui permettent de réduire l'utilisation ou la production de substances dangereuses. Un principe de base de la chimie verte est de réduire l'utilisation de substances toxiques et de solvants organiques polluants et de faciliter et d'encourager l'utilisation de nouvelles techniques d'extraction qui sont connues pour être plus respectueuses de l'environnement. Les techniques traditionnelles d'extraction nécessitent généralement de longues durées d'extraction, et de grandes quantités d'échantillons et de solvants organiques, ce qui a une incidence négative sur l'environnement et sur la santé. L'intérêt croissant pour la méthode PLE est principalement dû au fait que c'est une technique automatisée qui permet des temps d'extraction courts, une consommation réduite de solvant et une protection des analytes de l'action de la lumière et de l'oxygène. Dans notre étude, le volume de solvant et le temps d'extraction nécessaire pour extraire tout les LT des échantillons étaient respectivement de 44 ml, 15 min pour la méthode PLE, et 175 ml, 150 min pour la méthode SFE-Folch. Par conséquent, les résultats montrent que la PLE est une méthode efficace

pour l'extraction des LT et des PL et ce sera cette technique qui sera utilisée pour la suite de nos travaux.

Dans une seconde partie nous nous sommes plus particulièrement intéressés au profil moléculaire de certains phospholipides afin de pouvoir notamment déterminer leur composition en acides gras. En effet les acides gras polyinsaturés (AGPI) jouent un rôle très important dans de nombreux aspects de la santé humaine, en particulier dans la réduction des risques de maladies cardio-vasculaires, des pathologies liées à l'hypertension, à l'inflammation, aux allergies, aux troubles immunitaires et rénaux. Des études ont montré que les AGPI, en particulier ceux à longues chaînes (LC)-AGPI sont surtout présents dans les GPLs des membranes cellulaires. En outre, les types d'AG présents dans le régime alimentaire détermine les types d'AG qui sont disponibles pour la composition des membranes cellulaires. Youdim *et al.* (2000) ont monté par ailleurs que les AG essentiels retrouvés dans les tissus proviennent, dans des proportions relativement élevées, des PL. En effet les PLs sont des transporteurs des AG beaucoup plus efficaces d'un point de vue de la biodisponibilité des AG que les TAG (Amate *et al*., 2001; Lemaitre-Delaunay *et al*., 1999; Wijendren, 2002), par conséquent, la supplémentation en PL riches en AGPI de produits alimentaires serait un moyen intéressant d'augmenter l'apport en AGPI à longue chaine carbonnée facilement assimilables dans le corps humain. La préparation de compléments alimentaires contenant des espèces moléculaires de PLs riches en acide linoléique (C18:2), acide linolénique (C18:3), acide docosahexaénoïque (DHA) et acide eicosapenténoïque (EPA) pourrait avoir une grande importance dans l'alimentation humaine.

Dans cette deuxième partie le but recherché était de déterminer le profil moléculaire des familles de PE des matrices sélectionnées (soja, œuf, foie de bœuf, la cervelle de veau et huile de krill) et des familles de SM (pour la cervelle de veau, le foie de bœuf, l'œuf et l'huile de krill) afin de connaitre la nature des AG présents sur le squelette des molécules. Parmi les différentes familles de GPLs, la PC est présente en quantités beaucoup plus grandes que la seconde famille de PL la plus abondante, qui est généralement la PE. Les espèces moléculaires de PC ont été examinées en détails dans diverses matrices alimentaires par Le Grandois *et al.* (2009). Mais à notre connaissance, aucune information n'est disponible à ce jour sur la présence d'espèces moleculaires de PE dans les matrices alimentaires. Quant à la SM, qui est le composé principal des sphingolipides chez les mammifères, aucune publication ne fait mention de sa purification avant analyse par LC-MS. Nous nous sommes donc plus spécifiquement intéressés à la détermination des espèces moléculaires de PE et de SM purifiées dans différentes matrices alimentaires. Pour ce faire, les LT alimentaires ont d'abord été extraits par la méthode PLE

présentée précédemment, la fraction lipidique polaire a été purifiée par chromatographie sur des cartouches de silice greffée et éluée avec des solvants choisis en fonction des caractéristiques de polarité des composés à séparer à chaque étape. La méthode est inspirée de celle de Hernàndez *et al*. (1999), à la quelle nous avons apporté quelques modifications [comme l'utilisation du mélange methanol / acide formique aqueux 1M (ajusté à pH = 3 avec de la triéthylamine) (98:2) pour éluer la fraction de PLs]. Ensuite, les classes de PLs ont été séparées par HPLC en phase normale avec une colonne semi-préparative, (Silice, 250 mm × 10 mm, 5 μm, Interchim, Montluçon, France) combinée avec un détecteur DEDL. Un split a été mis en place avant l'entrée dans le détecteur afin de pouvoir collecter les pics d'intérêt. Un quantième de la phase mobile (0,1 mL/min) pouvait pénétrer dans le détecteur alors que les 3,9 mL/min restants étaient dirigés vers un collecteur de fractions. Les pics de PE et SM ont été identifiés par comparaison de leurs temps de rétention avec ceux obtenus dans les mêmes conditions d'analyse avec les standards. La pureté et l'identité des fractions de PE et SM obtenues pour chacune des matrices sont vérifiées par CLHP en phase normale sur colonne de silice (150 x 3 mm, 3 µm, Luna, Phenomenex, Le Pecq, France), le débit de la phase mobile est de 0,5 ml / min et la séparation a été effectuée en utilisant un gradient linéaire de 20 min allant de chloroforme/methanol (88/12 v/v) à chloroforme/methanol/ acide formique aqueux 1M (ajusté à pH = 3 avec de la triéthylamine) (28/60/12 v/v). Les taux de pureté des PE et SM isolées à partir des matrices alimentaires étaient supérieurs à 98%.

Ensuite, nous avons mis au point une méthode chromatographique par couplage LC-ESI-MS^2 permettant la séparation des différentes molécules présentes dans les familles de PE et de SM (colonne Nucleodur C18HTec, 250 mm × 4,6 mm, 3 μm, Macherey-Nagel, Germany). La phase mobile était constituée de méthanol additionné de formiate d'ammonium (5 mM) et s'écoulait de façon isocratique. L'utilisation de la spectrométrie de masse en tandem (MS^2) a permis de déterminer la nature des AG liés au squelette de PL et avec cette méthode on a pu obtenir une bonne séparation et l'identification des espèces moléculaires présentes dans les familles de PLs.

Les principales espèces moléculaires de PE dans le jaune d'œuf ont été identifiées comme étant (C16:0-C18:1)PE and (C18:0-C18:1)PE, suivis de (C18:0-C18:2)PE et (C16:0-C18:2)PE. 11 espèces PE ont été identifiées dans des extraits de foie de bœuf, dont (C18 :0-C18:1), PE (C18:0-C20:4) PE, et (C18:0-C18:2) PE sont les plus importantes. Les principales espèces moléculaires de PE dans l'huile de krill ont été déterminées comme étant (C16:0-C22:6)PE, (C16:0-C20:5)PE, suivis de (C18:1-C22:6)PE, (C18:1-C20:5)PE, et (C16:0-C18:1) PE. Dans cette même huile de krill un pic majoritaire contenant l'ion *m/z* 750.7 normalement attribué à (C15:0-C22:6)PE a été trouvé à un temps de rétention de 17,2 min (temps nettement supérieur à celui du pic de (C16:0-

C22:6)PE) ce qui étant donné les conditions chromatographiques utilisées ne pouvait pas être en accord avec l'identification supposée. Les résultats de GC et HRMS nous ont permis d'élucider la structure de cette substance: il s'agissait d'une plasmanylethanolamine (Pake), qui est une PE dans laquelle l'un des deux AG est lié au glycérol non pas par une liaison ester mais par une liaison éther saturé, -O-alkyle, (alkylacyle-Pake).

La cervelle de veau est plus complexe que les quatre autres matrices alimentaires. Nous avons trouvé 14 espèces moléculaires de PE mais malheureusement seules 10 d'entre elles ont pu être identifiées. Pour les 4 restantes nous avons rencontré les mêmes difficultés que précédemment mais nous n'avons pas encore réussi à les résoudre.

Bien que les profils d'espèces moléculaires de PE aient été déterminés, les positions des AG dans les espèces moléculaires ne sont pas connues. De nombreuses études ont été menées pour tenter de comprendre la distribution des AG dans les espèces moléculaires de PL et ont montré que les acides gras saturés (AGS) ou monoinsaturés (AGMI) sont généralement en position *sn*-1, tandis que les AGPI sont généralement situés en position *sn*-2 (O'Donnell, 2011; Wood R, 1969). On peut donc raisonnablement penser que les AGPI des PE des cinq matrices étudiées sont en position *sn*-2.

En outre, il existe d'autres cas où deux AGPI étaient estérifiés sur la PE. Par exemple, (C18:2-C18:2)PE et (C18:2-C18:3)PE ont été identifiés uniquement dans la PE de soja et y étaient présents en proportions relativement importantes. Comme nous le savons, l'acide linoléique (18:2) et α-linolénique (18:3) sont considérés comme des acides gras essentiels (AGE) dans l'alimentation humaine. Ils sont les précurseurs des LC-AGPI, en particulier l'EPA et le DHA, qui sont essentiels au développement du cerveau et de la rétine. Donc le soja, riche en AG essentiels, pourrait être utilisé lors de supplémentation alimentaire ou dans des compléments alimentaires.

Si l'on compare nos résultats obtenus pour les espèces moléculaires de PE avec ceux de Le Grandois *et al.* (2009) pour les espèces moléculaires de PC pour les mêmes matrices alimentaires on a constaté que l'on retrouvait les même AG. Ceci est la preuve que les AG sont uniformément répartis dans les classes principales de PL (PE et PC). Il serait intéressant d'effectuer le même travail pour les autres classes de PL telles que les PS et PI.

Parmi les cinq sources de PE étudiées dans cette partie, nous avons trouvé deux sources intéressantes d'AGPI. Tout d'abord l'huile de krill dont les principales espèces moléculaires de PE sont riches en AGPI de 20 atomes de carbone ou plus, tel que le DHA (20:5) et l'EPA (22:6). Il est à noter que *m/z* 750,7 considéré comme C16:0(alkyl)-C22:6 (acyl)Pake, était le plus abondant dans les PE de l'huile de krill. Cette espèce est donc riche en EPA. Ainsi,il a été démontré que l'huile de krill est une source potentielle d'AGPI pouvant être utilisée sous forme de complément alimentaire par exemple. Une autre source d'AGPI est la PE de soja qui, elle, est riche en AGE. Parmi les principaux constituants de la PE de soja, non seulement on trouve (C16:0-C18:2) PE mais aussi (C18:2-C18:3)PE et (C18:2-C18:2) PE, ce qui fait du soja, une source importante des AGE c'est pourquoi le soja est largement utilisé comme complément nutritionnel dans l'alimentation humaine.

En ce qui concerne l'analyse des SM, la procédure de purification et les méthodes de détection sont identiques à celles utilisées pour l'analyse des PE. Après l'obtention de SM pur, les différentes espèces moléculaires ont été identifiées en LC-ESI-MS. 5 espèces moléculaires de SM ont été mises en évidence dans le jaune d'œuf mais une seule est très largement majoritaire, il s'agit de (d18:1-16:0)SM. Par contre les SM du foie de bœuf et de la cervelle de veau sont beaucoup plus complexes puisque on a pu identifier jusqu'à 21 espèces moléculaires. L'espèce majoritaire dans le foie de bœuf est la (d18:1-16:0)SM, comme dans le jaune d'œuf, alors que pour la cervelle de veau c'est la (d18:1-18:0)SM. Il faut également noter que pour la cervelle de veau on retrouve des proportions relativement importantes de SM contenant des acides gras mono-insaturés à longue chaine comme par exemple (d18:1-24:1)SM.

Trois sources de SM ont été déterminées dans cette partie, le jaune d'œuf et la cervelle de veau dont les SM ont déjà été étudiés précédemment, mais à notre connaissance, les données bibliographiques concernant les SM du foie de bœuf sont limitées. En outre, les compositions en AG des SM de ces trois sources alimentaires sont différentes. On peut donc dire que les différentes espèces moléculaires de SM sont caractéristiques des différentes sources. Comme indiqué précédemment, (d18:1-16:0)SM est l'espèce prédominante dans le jaune d'œuf et le foie de bœuf et le sérum humain, tandis que (d18:1-18:0)SM est l'espèce moléculaire la plus abondante dans le cerveau de veau et le cerveau humain.

La troisième partie de ce travail a consisté à étudier la sensibilité de certaines espèces moléculaires de PLs vis-à-vis des réactions d'oxydation. Nous nous sommes attachés à déterminer les cinétiques de formation de ces produits oxydés et de dégradation des produits précurseurs. L'objectif était de déterminer l'influence du traitement thermique sur la stabilité des PLs.

L'identification des produits de dégradation des PLs étudiés permet de savoir quels types de molécules sont produits lors du processus oxydatif. Nous avons également mis en évidence l'influence du constituant dans la position *sn*-3 sur la cinétique d'oxydation des AG. Pour cela les courbes de décroissance de PE, PC et acide phosphatidique (PA) ont été comparées à celle de TAG afin de déterminer quelle substance est la plus résistante au processus oxydatif. La chromatographie en phase gazeuse couplée à une détection par spectrométrie de masse (GC-MS), une injection en espace de tête et une micro-extraction en phase solide (ET-MEPS) a été utilisée pour analyser les composés volatils d'oxydation des PL. Les paramètres de SPME ont été déterminés après avoir testé différentes fibres (Polydiméthylsiloxane-divinylbenzène: PDMS/DVB, Polydiméthylsiloxane: PDMS et Carboxen-polydiméthylsiloxane/polydiméthylsiloxane: CAR/PDMS), différentes durées (10 min, 25 min et 40 min) et différentes températures (40°C, 50°C et 60°C) d'extraction. Les résultats obtenus montrent que les conditions optimales d'extraction par MEPS sont une température d'extraction de 50°C pendant 25 minutes avec la fibre PDMS/DVB.

En ce qui concerne les conditions d'oxydation des PLs, la température a été définie comme étant celle qui permette d'obtenir la plus grande concentration possible de composés volatils d'oxydation formés pour chaque PL précurseur étudié. Pour cela les échantillons ont été chauffés à différentes températures pendant 30 minutes et les composés volatils d'oxydation formés ont été analysés par GC-MS avec une colonne DB-wax (60 m x 0,25 mm, 0,15 µm, Agilent, USA). Il apparait que la production des composés volatils d'oxydation est maximale à 175°C pour 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) et à 125°C pour 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC). A partir de ces résultats, nous avons choisi de travailler à ces deux températures pour toutes les autres molécules étudiées [175°C pour 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (SOPE), 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphate (SOPA) et 1,3-stearoyl, 2-oleoyl-glycerol (SOS), 125°C pour 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (SLPE), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphate (SLPA) et 1,3-stearoyl, 2-linoleoyl-glycerol (SLS)].

En comparant les composés volatils obtenus lors de l'oxydation des PLs avec ceux obtenus lors de l'oxydation des TAGs correspondants, dans les mêmes conditions expérimentales, on a pu déterminer quelles étaient leurs origines. Un total de 35 composés volatils oxydés (CVOs) a été détecté dans cette étude. Ces composés appartenaient aux groupes chimiques suivants: des aldéhydes (15 composés), des alcools (6 composés), des cétones (13 composés) et des furanes (1 composé). 19 CVOs ont été obtenus lors de l'oxydation de SOPC. Les CVOs les plus abondants

étaient les aldéhydes, tel que (E)-2-décénal, nonanal, octanal et (E)-2-undécanal qui représentent 57.7% des CVO totaux. 20 CVOs ont été identifiés après oxydation de SLPC les aldéhydes étant également les principaux CVO formés. l'(E)-2-hepténal, l'(E)-2-octénal, l'(E, E)-2,4-décadiénal et l'hexanal comptaient pour plus de la moitié des CVO totaux détectés dans SLPC oxydé.

Par ailleurs une étude cinétique a été effectuée dans laquelle la dégradation de SOPC (SLPC) ainsi que la formation des produits d'oxydation à 175°C (125°C) ont été suivies au cours du temps, de 0 à 180 minutes de chauffage. Le développement des produits volatils de dégradation au cours du temps permet d'obtenir des informations sur la stabilité du précurseur étudié.

Les résultats ont montré que les quantités totales de CVOs provenant de SOPC oxydé, SOPE et SOPA atteignent leur maximum en 120 min, 90 min et 60 min, respectivement, et les quantités totales de CVOs provenant de SOS oxydé atteignent un équilibre en 90 min. Ceci permet de dire que les hydroperoxydes de SOPC se décomposent plus difficilement que les autres à la même température. Les résultats ont également montré que les quantités totales de CVO provenant de SLPE oxydé étaient inférieures à celles de SLPC oxydé, SLPA et SLS. Cela pourrait être du au fait que les hydroperoxydes de SLPE auraient plus tendance à se décomposer en substances non volatiles.

Dans l'ensemble, le processus d'oxydation thermique de PL est en quelque sorte une compétition entre la formation et la décomposition des hydroperoxydes. Ces derniers, formés à partir de l'oxydation des lipides, sont instables et se décomposent rapidement pour former les CVOs et les non-CVOs. Lorsqu'il s'agit de CVOs ou de non-CVOs formés à basse température ces substances peuvent encore se décomposer en d'autres CVOs sous l'effet d'une augmentation de la température ou du temps d'oxydation. Il y a deux façons supplémentaires pour augmenter la teneur en CVOs: soit les aldéhydes insaturés subissent une auto-oxydation, ce qui conduit à la formation d'autres composés volatils, soit les époxydes et les hydroperoxydes non volatils subissent une décomposition en produits volatils.

Quoiqu'il en soit, l'oxydation des lipides est un processus complexe et le mécanisme exact de la production de CVO n'est pas encore très clair et une étude plus approfondie de la formation des produits d'oxydation secondaires devrait être menée. En effet, la température et la durée de chauffage sont deux facteurs qui influencent largement la cinétique de formation des CVO de PL

au cours du traitement thermique, et par voie de conséquence les qualités nutritionnelles et organoleptiques des aliments transformés.

En résumé, il apparait que la cinétique de formation de la plupart des CVO à partir de PL (que ce soit PC ou PE mono-insaturés ou di-insaturés) est la même. On observe une augmentation plus ou moins rapide de la quantité de CVO présents dans le milieu jusqu'à atteindre un maximum puis après une période d'équilibre on observe une décroissance plus ou moins progressive de ces CVO. Par contre la cinétique de formation des CVO provenant des PA oxydés est différente de celle des PL mais similaire à celle des TAG. Ceci serait en faveur de l'influence de la présence ou non de la tête polaire.

En parallèle de l'étude de la cinétique de formation des produits de dégradation liés à l'oxydation, nous avons étudié la cinétique de dégradation des molécules de PLs soit SOPC, SLPC, SOPE et SLPE. Cette cinétique a également été comparée à celle des homologues triglycérides de ces PLs, SOS et SLS. La comparaison des résultats à chaque durée et température d'oxydation permet de montrer que les AG liés à PC ou PE sont plus résistants à l'oxydation que lorsqu'ils sont sous forme de TAG. En effet, il apparait que les teneurs en PC et PE décroissent moins vite que celles des TAG. En outre, les molécules de PE et de PC étaient également plus stables que leurs homologues sous forme de PA lors de l'oxydation thermique. Enfin les TAG étaient plus stables que les PA. En résumé, on peut classer les différentes substances analysées en fonction de leur stabilité décroissante à l'oxydation thermique de la façon suivante: PC > PE > TAG > PA. Ces résultats pourraient trouver leur explication dans le type de substituant se trouvant en *sn*-3 du glycérol (P-choline, P-ethanolamine, AGS ou PA). Enfin ces résultats montrent également que le type de tête polaire a son importance. Il serait intéressant de continuer ces études avec PS et PI.

Enfin, nous savons aujourd'hui qu'un certain nombre de nutritionnistes et d'industriels a envisagé une supplémentation de certains aliments en différents PLs non seulement pour prévenir tout déficit en ces substances, mais aussi pour réaliser une prévention nutritionnelle. Les données scientifiques obtenues au cours de cette étude pourront être exploitées dans l'objectif d'approfondir les connaissances scientifiques sur le comportement des PLs contenus dans divers aliments et de valoriser les PLs purifiés. L'étude d'oxydation de PLs permet d'offrir une aide pour l'identification des produits d'oxydation qui sont susceptibles d'être formés à partir d'aliments riches en PLs.

Chapter 1: Study context

1. Phospholipids

1.1 Structure and classification

Phospholipids (PLs) are vital components of biological cell membranes and play essential roles in cellular metabolism, signal transduction and membrane trafficking (Harrabi *et al*., 2009). PLs are divided into two groups: glycerophospholipids (GPLs) and sphingolipids (SPLs). GPLs are made of a polar headgroup with a phosphate moiety and two fatty acids (FAs) attached to the glycerol backbone. SPLs are characterized by a sphingosine backbone linked with a single FA. The most distinct difference between SPLs and GPLs is that the backbone of SPLs is not glycerol.

1.1.1 Glycerophospholipids

The membranes of eukaryote cells are highly complex structures consisting of hundreds of different lipid molecules as well as numerous different proteins. The lipids of eukaryote membranes can be divided into three main groups, i.e. GPLs, SPLs and sterols. The major classes of GPLs are phosphatidylcholine (PC), phosphoatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). PC is usually the predominant PL in animal tissues, amounting to almost 50% of the total PLs (Pérez-Palacios *et al*., 2007). PE is generally the second most abundant PL in animals, while PS usually constitutes less than 10% of the total PLs and the proportion of PI is often below 4% in animal tissues (Genge *et al*., 2003). PC and PE are neutral PLs, while PS is a weak acidic one and PI is strongly acidic.

FAs are attached to a glycerol backbone at *sn*-1 and *sn*-2. A phosphate and polar headgroup is attached at *sn*-3. The length of the alkyl chain typically varies from 14 to 24 carbons and the number of double bonds from 0-6. The alkyl chain in the *sn*-1 position is typically saturated or monounsaturated, while that in the *sn*-2 position is often polyunsaturated (Figure 1 and Table 1).

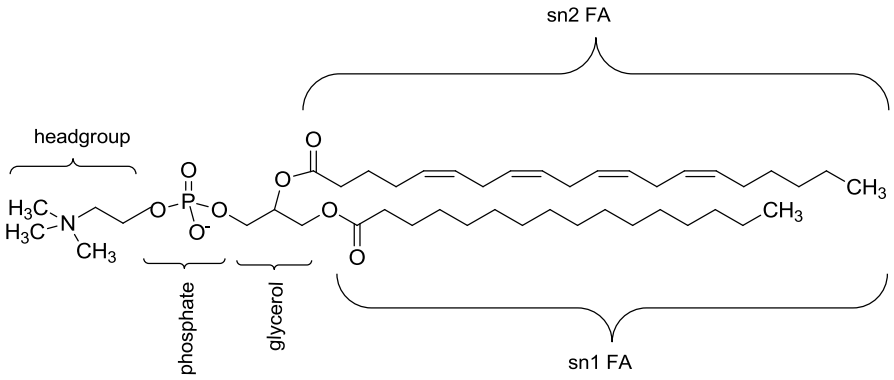
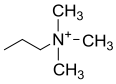
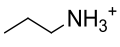
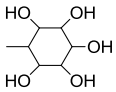
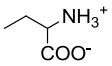
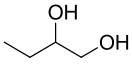


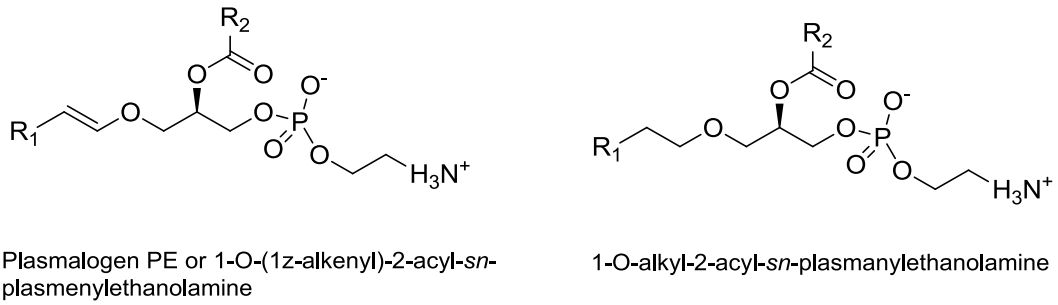
Figure 1: Structure of one GPL (O'Donnell *et al*., 2011).

Table 1: Classification of GPLs (O'Donnell *et al*., 2011)

Headgroup	Name	Family of glycerophospholipid
H		Phosphatidic acid (PA)
	Choline	PC
	Ethanolamine	PE
	Inositol	PI
	Serine	PS
	Glycerol	Phosphatidylglycerol (PG)

Ether PLs are lipids containing a fatty alcohol, rather than a FA at the *sn*-1 position. Plasmalogens are a type of ether PL characterized by the presence of a vinyl ether linkage at the *sn*-1 position and an ester linkage at the *sn*-2 position. The majority of ether PLs are plasmalogens. Plasmalogen constitute about 15-20% of total PL in cell membranes, more than

50% of plasmalogens are ethanolamine (designated plasmenylethalomines), which present mainly in brain, heart, neutrophils and eosinophils (Braverman and Moser, 2012). Plasmanyl PL is another type of ether PL linked by a 1-O-alkyl ether bond. Examples of a plasmenylethalomine and a plasmalogen PE are shown in Figure 2.



Plasmalogen PE or 1-O-(1z-alkenyl)-2-acyl-*sn*-plasmenylethanolamine

1-O-alkyl-2-acyl-*sn*-plasmanylethanolamine

Figure 2: Ether PE: plasmanylethanolamine and plasmenylethanolamine (plasmalogen) (Braverman and Moser, 2012).

1.1.2 Sphingolipids

According to Van Echten-Deckert *et al.* (2006), SPLs were first characterized by Thudichum, 1884. while studying the chemical constituents of brain. SPLs are classified as ceramides, sphingomyelins (SMs) and glycosphingolipids. In contrast to GPLs, complex SPLs are composed of only a single FA linked to a sphingoid backbone. They present a large degree of structural diversity due to variations in the degree of unsaturation, hydroxylation and methylation of long-chain sphingoid base, as well as in the nature of the polar headgroup (x in Figure 3) (Fischbeck *et al.*, 2009). D-erythro-sphingosine is the prevalent sphingoid base of most mammalian SPLs when linked with phosphocholine, the SPL is named as SM, which is major in SPL. But there are more than 60 different sphingoid base back bones (Karlsson, 1970) that vary in alkyl chain lengths (from 14 to 22 carbon atoms), degree of saturation and position of the double bonds, presence of a hydroxyl group at position 4 and branching of the alkyl chain (Figure 3) (Vesper *et al.*, 1999).

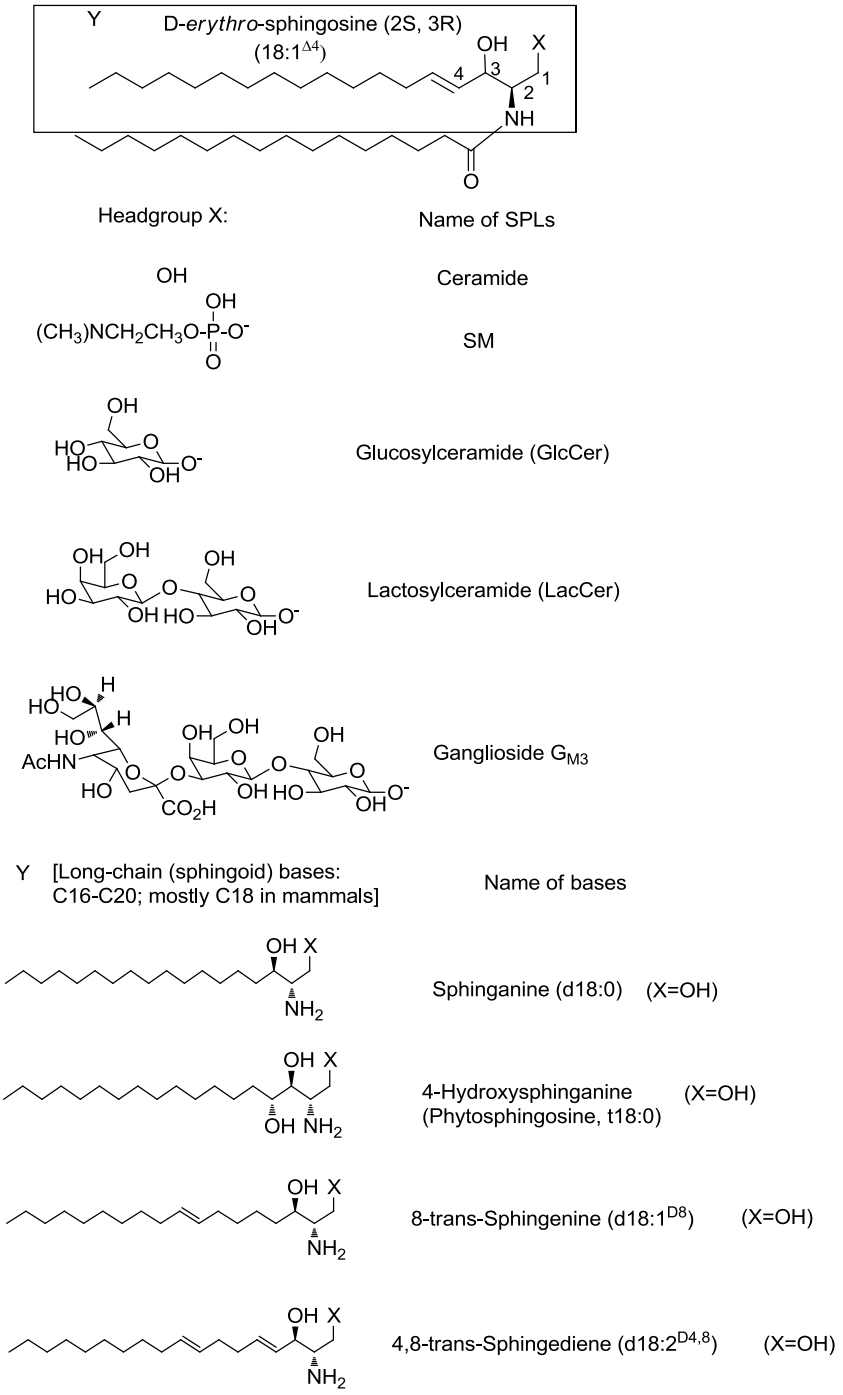


Figure 3: Structure of SPL (Vesper *et al.*, 1999; Bielawski *et al.*, 2006). Complex SPLs are elaborations of long-chain (sphingoid) bases by the addition of long chain fatty acids (LC-FAs) in amide linkage and polar headgroups. Sphingoid bases are abbreviated by citing (in order of appearance in the abbreviation) the number of hydroxyl groups (d and t for di- and tri-hydroxy, respectively), chain length and number of double bonds, as shown in the figure. Five common SPLs are shown: ceramide, SM, glucosylceramide (Glc-Cer), lactosylceramide (LacCer) and ganglioside G_{M3}.

1.2 Biological functions of phospholipids

1.2.1 Glycerophospholipids

Most GPLs are present in all cellular membranes and serve as key structural components of membranes. However, their relative abundances vary significantly from one organelle to another (Van Meer, 2008). PS is abundant in the inner leaflet of the plasma membrane, where it may serve to activate membrane-associated enzymes, such as protein kinase C, as well as assist in membrane fusion (Zhang, 2009). PE has a relatively small headgroup which can not only accommodate the insertion of proteins within the membrane while still maintaining the integrity of the membrane, but also form non-bilayer structures. This is important in the formation of new membranes and vesicles, as well as membrane fusion and budding processes.

GPLs are amphiphilic lipids found in all plant and animal cell membranes. The amphiphilic character enables GPLs more efficiently incorporated into cellular membranes of different organs than triacylglycerides (TAGs), thus affecting positively the membrane microstructure and its function. It has been shown that the supplementation of FAs in form of GPLs are much more efficient than TAGs (Amate *et al*., 2001; Lemaitre-Delaunay *et al*., 1999; Wijendran *et al*., 2002). And different types of dietary GPLs vary in their FA-composition and polar headgroup, and therefore may have different effects (Küllenberg *et al*., 2012). In other words, the joint actions of FAs and polar headgroups make different PLs possess different biological functions. For instance, PE is essential for the growth and stability of these energy-producing organelles. PI is part of the GPI-anchors which mediate the association of certain proteins to the outer leaflet of the plasma membrane (Guan and Wenk, 2008; Orlean and Menon, 2007). Positive effects of PC containing PUFAs as therapeutics have been verified by some experimental and clinical results, including, enhancement memory performance of the aging brain, promotion of gastroinstestinal function by mucosa restoration, and activation of the liver metabolism and detoxication (Kisel *et al*., 2001; Szuhaj, 2003; Hosokawa, 1999; Eibl and Unger, 1988). PS is found in highest concentration in the inner layer of neuronal cell membranes, it has the functions of cell-to-cell recognition and communication, inhibition of tumor necrosis factor, and influence over levels of neurotransmitters (Pepping, 1999). Clinical trials suggest that PS may have applications for the prevention and treatment of age-associated cognitive decline, Alzheimer's disease, depression, and other cognitive disorders (Crook *et al*., 1922).

1.2.2 Sphingolipids

SPLs are located in cellular membranes, lipoproteins and other lipid-rich structures (Vesper *et al*., 1999). SPLs protect the cell surface against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer. Certain complex SPLs were found to be involved in specific functions, such as cell recognition and signaling. Cell recognition depends mainly on the physical properties of the SPLs, whereas signaling involves specific interactions of the glycan structures of glycosphingolipids with similar lipids present on neighboring cells or with proteins (Holleran *et al*., 1991).

SM is a major SPL constituent of all mammalian cell membranes and plasma lipoproteins (Tomiuk *et al*., 1998). SM helps maintain the integrity of the plasma membrane by protecting PC against oxidative damage and phospholipase degradation. And SM is considered as an endogenous inhibitor of inflammation (Daniel, 2004). The catabolic metabolism of SM generates several lipid intermediates with potential second messenger functions, such as ceramide and sphingosine that induce growth arrest, differentiation and apoptosis; spingosine 1-phosphate stimulates growth and inhibit apoptosis (Vesper *et al*., 1999).

1.3 Phospholipids digestion and absorption

The normal dietary intake of GPL is 2-10 g per day, which represents 1-10% of total daily fat intake (Le grandois *et al*., 2009). GPL as a part of lipid, its digestion is synchronized with TAGs digestion. Lipid digestion needs the help of different lipases, which are the enzymes secreted by various parts of the digestive system. Lipid digestion begins with chyme, an acidic fluid mixture of gastric juice and partly digested food, passes from the stomach into the initial section of the small intestine, the duodenum. Gastric lipases are secreted in the stomach, but their contribution is ancillary to the enzymatic events that occur in conjunction with bile salts in the small intestine (Jackson *et al*., 2009; Ramírez *et al*., 2001). In fact, very minor digestion occurs in mouth and stomach which is negligible. Actually, the digestion of lipids begins in the small intestine, which specialized enzymes from the pancreas, and bile salts from the liver play critical roles in lipid digestion. The action of pancreatic lipases breaks the TAGs in the micelles down to FFAs, glycerol, and some monoglycerides. Van den Bosch *et al*. (1965) demonstrated that pancreatic tissues contains two phospholipases (phospholipase A1 and phospholipase A2) and each attack different FA positions of GPLs resulting in the formation of two isomeric lyso-PLs and free FAs (Figure 4). But chemical breakdown of GPLs is carried out primarily by

phospholipase A2, and other lipases secreted by the pancreas in response to food intake (Cohn *et al*., 2010). The lyso-PLs and FAs are absorbed by the intestinal mucosal cells and are resecreted within chylomicrons as newly-formed GPL or TAG, and enter the bloodstream incorporated in chylomicrons in a small proportion of very low density lipoproteins. However, it has been assumed that almost 20% of intestinal PLs are absorbed passively and without hydrolysation, and preferentially incorporated directly into high-density lipoproteins, which occurs relatively rapidly, i.e, within 5-6 hours of PL ingestion (Cohn *et al*., 2010).

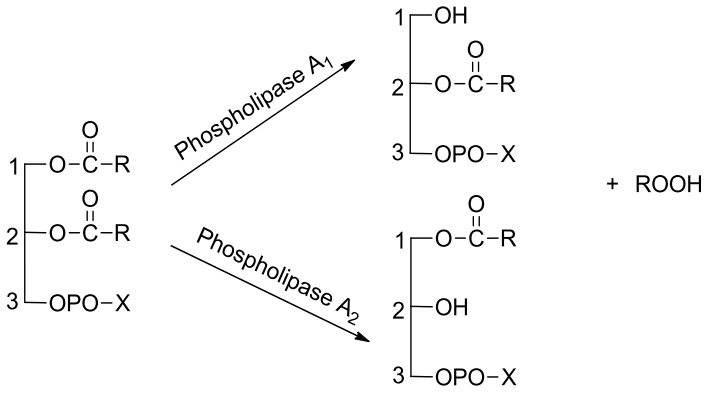


Figure 4: Pathways for the digestion and absorption of GPL (Van den Bosch *et al*., 1965).

SM is ingested at a level of 0.3-0.4g per day (Duan *et al*., 2009). Very little dietary SM directly contributes to plasma SM pools. The mechanism of the digestion and absorption of SM is different from GPL. It is not degraded by pancreatic enzymes. The major enzymes responsible for SM degradation in the intestinal lumen and mucosa are alkaline sphingomyelinase and neutral ceramidase. These enzymes are located in the surface membrane of mucosal cells with catalytic domains facing the outside of the cell. They are responsible for the conversion of SM to ceramide and subsequently to sphingosine free FAs (Cohn *et al*., 2010). The pathways for the metabolism of dietary SM in the gut are summarized in Figure 5.

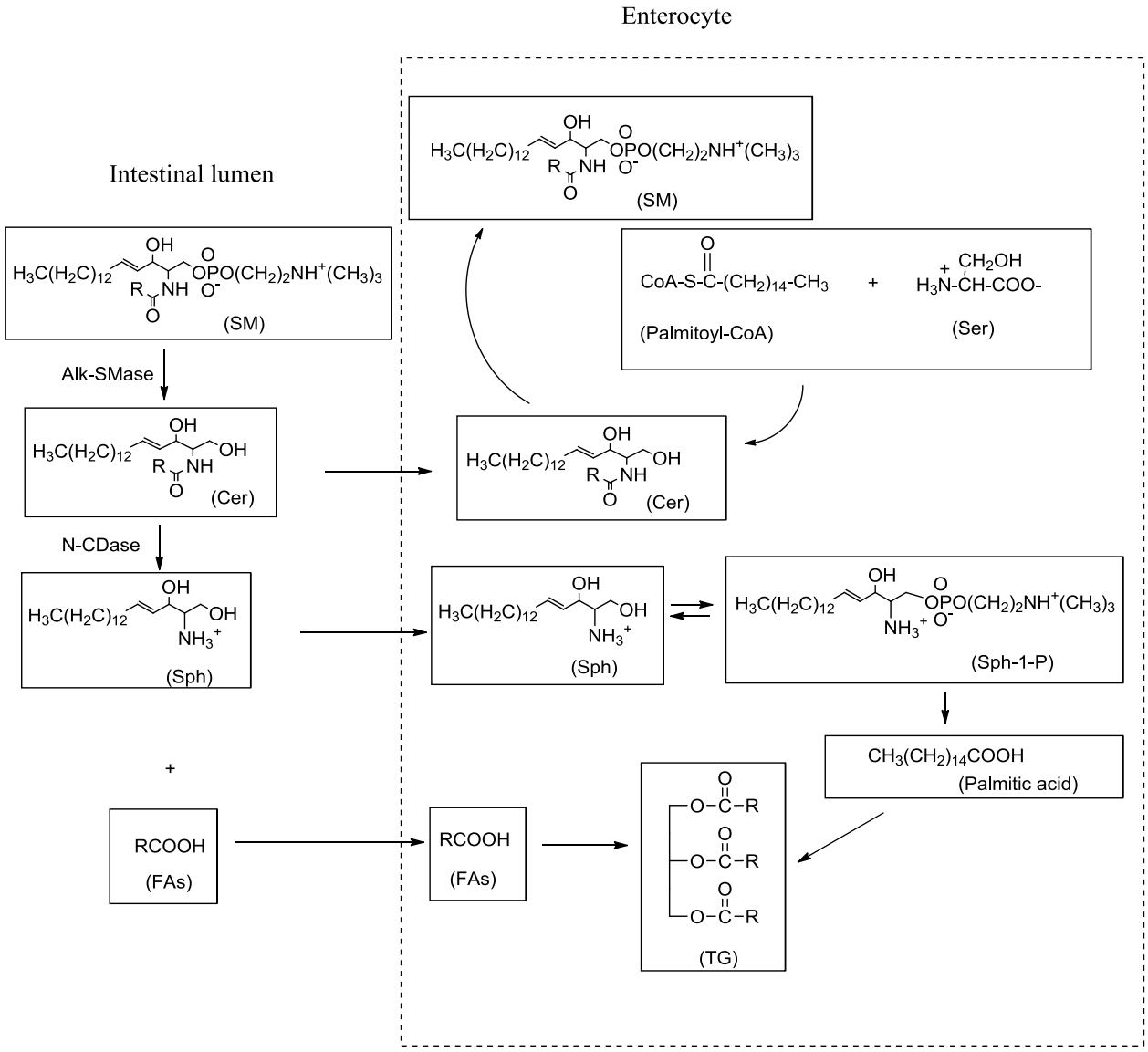


Figure 5: Pathways for the digestion and absorption of SM. Alkaline sphingomyelinase (Alk-SMase) and neutral ceramidase (N-CDase) sequentially hydrolyze SM to ceramide (Cer), sphingosine (Sph), and free fatty acid (FFA). Sphingosine is well absorbed and converted to chylomicron palmitic acid. A small part is incorporated into mucosa ceramide and more complex SPLs. The endogenous synthesis of SM, starting from serine (Ser) and palmitoyl-CoA, is also indicated (Nilsson *et al*., 2006; Duan *et al*., 2009).

1.4 Natural occurrence and food availability of phospholipids

1.4.1 Glycerophospholipids

The occurrence of individual species of PLs and their contents vary greatly in different sources (Table 2). PC and PE are abundant in food matrices, especially in marine PL, PC account for 87.5%. And PS and SM present mainly in animal sources. Previous studies demonstrated that conditions, such as temperature, pressure, pH, nutrient supply, light and age could influence the occurences of GPLs in different sources (Weihrauch and Son, 1983; Padley *et al*., 1994). The response of the PLs composition to the environmental stresses could be explained by the biosynthetic and metabolic pathways of lipids. As previously mentioned, PLs are highly effective in delivering their FA residues for incorporation into the membranes of cells involved in altering the membrane composition of the cells, furthermore, their FAs have a major impact on certain PL species. Pacetti *et al*. (2005) showed that feeding different levels of seal blubber oil resulted in a significant increase of PE, PC and PI molecular species carrying EPA, DPA (docosapentaenoic acid) and DHA. In fact, these changes on membrane composition are realized through the changes of the encoding genes of enzymes in lipid synthesis or metabolism (Dowhan and Bogdanov, 2002).

GPLs are currently of commercial importance and are beneficial to human health. Commonly, commercially available PLs are soybean lecithin, which contains around 20% PC, 15% PE, 20% PI, 5% PA, 5% carbohydrates and sterols, and 35% TAGs of total PL (Guo *et al*., 2005; van Nieuwen-huyzen, 1976). Eggs, organ meats, fishes, and oilseeds are also rich sources of PLs, especially for PC. Meats and tissues from different sources but performing similar functions have similar relative PL distribution (Guo *et al*., 2005). Because the PL composition of mammalian organisms are easily affected by environmental factors which are discussed previously. Whereas leafy vegetables, fruits and roots are relatively poor sources of PLs.

1.4.2 Sphingolipids

SPLs from mammals have a wide spectrum of SM, cerebrosulfatide, and cerebroside (Bell *et al*., 1993), while in plants the predominating SPL is cerebroside (Sperling and Heinz, 2003), and in bacteria, sphingolipid occurrence is mainly restricted to anaerobes (Olsen and Janzen, 2001).

The naturally occurring SPL species vary depending on the sources. SM is a main SPL class and present predominantly in animal sources. Organ meats show high content of SM, followed by muscle meats, eggs, soybeans, cream and fish. Vesper *et al*. (1999) has collected the related data about the SPL content in dietary foods and estimated SPL consumption per capita in the United States to be on the order of 115–140 g/year (or 0.3–0.4 g/day).

Table 2: Representative presentation of the natural occurrence of PLs from different food sources (% of total PL)

PLs	Soy PL (Wang *et al*., 1997)	Egg yolk PL (Ternes *et al*., 2003; Fuchs *et al*., 2007)	Milk PL (Avalli *et al*., 2005)	Butter PL (Avalli *et al*., 2005)	Marine PL (Küllenberg *et al*., 2012)
PC	55.3%	60.0-73.0%	36.8%	24.7%	87.5%
PE	26.3%	15.0-26.0%	31.6%	31.0%	5.8%
PI	18.4%	0.6%	5.3%	11.9%	2.2%
PS	nd	nd	nd	15.3%	nd
SM	nd	2.5-4.8%	26.3%	17.1%	nd

1.5 Impact on PLs during food processing

Food processing is the transformation of raw ingredients into food, or of food into other forms. Several common food-processing and preparation methods are used in food industry. These include heating, drying, freezing and irradiation. Different processes would have different influences on PLs. These processes impact on PLs during food processing are summarized briefly below.

1.5.1 Heating

Heating is the most widely used unit operation during food processing and preparation. However, the most common change in lipid structure is oxidation, which is often caused by heat treatment. PLs contain long-chain PUFAs, which are easily oxidized. In addition, as membrane components, PLs are in close contact with lipid oxidation catalysts in the cytoplasm (Timon *et al*., 2001). Therefore, the main reasons for the loss of PLs include thermolysis, PL oxidation, and the non-enzymatic browning stain reaction of PLs since most enzymes are destroyed by heating.

Wang *et al.* (2011) studied the changes of PLs in duck muscle by different heating methods. They showed that all the PL classes studied including PE, PI, PC, PS, SM and lysophosphatidylcholine (lyso-PC), decreased during heating. The roasting (90 °C, 60 min) caused a greater lost of PLs than boiling (90 °C, 30 min). Furthermore, the loss of PE was greater than PC, and PC decreased more slowly than PE. PI and PS decreased quickly, while SM and lyso-PC decreased more slowly. Demchenko *et al.* (1980) showed that a large decrease in PI and small decreases in PE and lysophosphatidylethanolamine (lysoPE) on the composition of sunflower oil PLs during heat treatment. Takagi *et al.* (1999) showed that the greatest rate of PL losses was observed in PE, followed by PC and PI during microwave heating for 6, 12 or 20 min at a frequency of 2450 MHz comparing with unheated samples.

1.5.2 Freezing

Freezing is one of the most important preservation methods for food matrices. Compared with other methods, it leads to a minimal loss of quality during long-term storage (Soyer *et al.* 2010). Despite the oxidation of PLs in the freezing condition is slow, some researchers have contributed on the effects of freezing and cold storage on PLs. Wilson and Rinne (1976) reported that the soy PE and PC were diminished while PA increased during freezing process. Yoshida and Sakai (1974) also found the same phenomenon. Little change occurred in total polar lipid of polar cortical tissue from -15 to -196 °C, while major change occurs within the lipid classes. PC and PE were in lower levels, but PA accounted for larger amounts. These changes in PL content are probably a result of increased phospholipase D activity caused by dispersal of the enzyme during cell disruption, the hydrolyzes of PC or PE by phospholipase D lead to the increase of PA (Wilson and Rinne, 1976).

1.5.3 Drying

Drying is a traditonal method of food preservation that works by removing water from the food, which inhibits the growth of microorganisms. There are many different methods for drying, each with their own advantages for particular applications. These include sunlight, drum drying, freeze drying, spray drying, and so on. During drying process, besides food shape is changed, food property may be changed. Thus, some researchers studied the effect of drying process on PL in various food matrices. Morin *et al.* (2007) investigated that spray-drying of buttermilk had a significant effect on PL content and composition. After spray-drying, the PL contents decrease by

38.2% and 40.6%, respectively in buttermilk from raw or pasteurized cream when compared with initial buttermilks. Dussert *et al*. (2006) showed that PE levels decreased very rapidly while PC and PI decreased slowly, and they demonstrated that this rapid and selective loss of PE was not due to FA de-esterification, which would have led to the formation of lyso-PE in significant amounts, but due to the formation of N-acylphosphatidylethanolamine (Chapman *et al*., 1999; Rawyler *et al*., 2002) which is synthesized from PE (Chapman *et al*., 1999).

1.5.4 Irradiation

Food irradiation is the process of exposing food to ionizing radiation to destroy microorganisms, bacteria, viruses, or insects that might be present in the food. Food irradiation acts by damaging the target organism's DNA beyond its ability to repair in order to terminer their malignant or pathogenic activities. However, meanwhile, irradation process would have effect on PLs of food matrices. Sopin *et al*. (1968) showed that the content of PLs (PE, PC and SM) of animal organisms decreased after irradation process. And Schwarz *et al*. (1965) demonstrated that the increase of PG took place in fractions with lysosomal and microsomal properties. Furthermore, the irradiation of PLs would lead to form series of volatiles, such as *n*-alkanes, 1-alkenes, *n*-alkanals, 2-alkanones, 3-alkanones, 4-alkanones, methyl and ethyl esters (Handel and Nawar, 1981). The formation of these volatiles can be explained by the cleavage in proximity to carboxyl group. Besides, a cleavage of amino group is also contributed to the formation of volatiles. Moreover, it is thought to compete with ionization at the carbonyl oxygen and amino nitrogen, that was the reason why volatile products formed from PE was significantly less than that from corresponding TAG (Handel and Nawar, 1981).

2. Phospholipids oxidation

The oxidation of lipids has long been a topic of interest in biological and food sciences because of the remarkable implications of their oxidative damage (Kanner and Rosenthal, 1992). At the biological level, the oxidation of lipids means damage to membranes, hormones and vitamins (McBrien and Slater, 1982). At the nutritional level, the oxidation of fatty constituents is the major chemical factor in the loss of food quality by deterioration of flavour and aroma, as well as in decay of nutritional power (Eriksson, 1982).

Numerous studies have been carried out on measuring the oxidation products of FAs or TAG (Chan and Levett, 1977; Neff and Byrdwell, 1998) while few studies have been done on PL, which also contains FAs. The different molecular structure between TAG and PL is in *sn*-3 position of glycerol. In fact, oxidation of fatty acyl residues esterified in PLs appears to proceed in a similar manners with the oxidation of FFAs (McIntyre *et al*., 1999). The UFA chains present in PLs are the main targets of oxidation (Spiteller, 2006; Niki, 2005). Oxidation of FA linked to *sn*-1 and *sn*-2 position of GPLs leads to many different reaction products, depending on chain length and degree of unsaturation (Fruhwirth *et al*., 2007) and the polar headgroup which may also have influence on the oxidation of FAs.

2.1 Oxidation of fatty acids

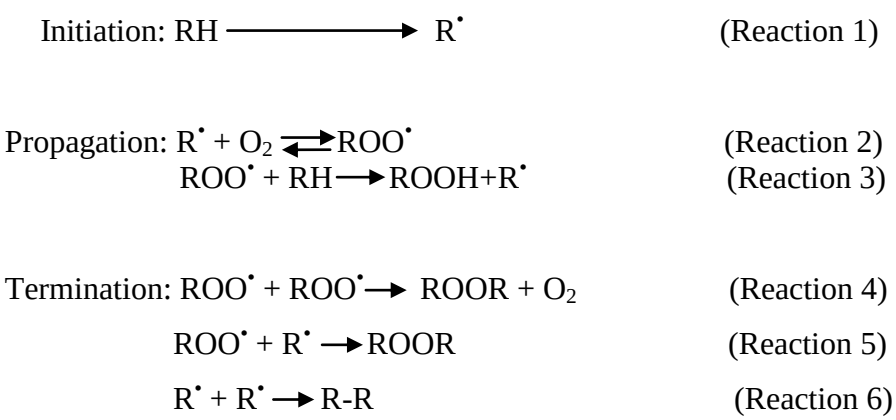
2.1.1 Mechanisms of lipid oxidation

Hydroperoxides are the primary products of lipid oxidation. The hydroperoxide group may be attached to various lipid structures, such as free FAs, TAGs, PLs and sterols.

FA hydroperoxides are formed through three different mechanisms, which are autoxidation, photo-oxidation and enzymatic oxidation. Autoxidation is a free radical chain reaction between unsaturated lipids and oxygen to form hydroperoxides, which then undergo further reactions with or without the participation of other compounds (Chan, 1987). During photosensitized oxidation, hydroperoxides are formed in the presence of oxygen, light energy and a photosensitizer (Bradley and Min, 1992; Frankel, 2005). As for enzymatic oxidation, the presence of enzymes, such as lipoxygenase, control the synthesis of hydroperoxides in biological activity (Gradner, 1996).

2.1.1.1 Autoxidation

Autoxidation is the direct reaction of molecular oxygen with organic compounds under mild conditions. Oxygen has a special nature in behaving as a biradical by having two unpaired electrons (•O-O•) in the ground state and is said to be in a triplet state. The oxidation of lipids proceeds like that of many other organic compounds by a free radical chain mechanism, which can be described in terms of initiation, propagation, and termination processes. These processes often consist of a complex series of sequential and overlapping reactions.

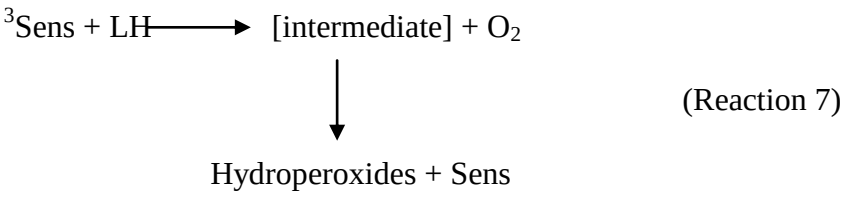


During the initiation step an alkyl radical (R•) is formed by an UFA (RH) (Reaction 1). Once formed, R reacts very rapidly with oxygen to form a peroxyl radical (ROO•) (Reaction 2). The second step of propagation, the abstraction of a hydrogen atom from a UFA (RH) by peroxyl radical (ROO•) to generate hydroperoxide (ROOH) and another free radical (R•) (Reaction 3). Finally, these radicals react with each other, forming more stable products that are not capable of propagating the chain reactions (Russell, 1957) (Reaction 4-6).

2.1.1.2 Photooxidation

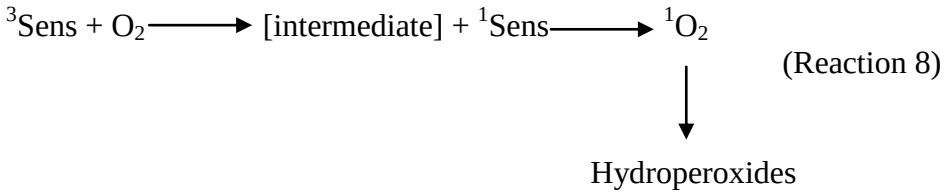
• Photosensitized oxidation (singlet oxygen oxidation)

Two types of sensitizers are recognized for photosensitized oxidation. These sensitizers proceed by two pathways. The Type I sensitizer serves as a photochemically activated free radical initiator. By light absorption, the singlet state (^{1}Sens) is converted to the triplet state (^{3}Sens). The sensitizer in the ^{3}Sens reacts with the lipid substrate by hydrogen atom or electron transfer to form radicals, which can react with oxygen (Reaction 7) (Frankel, 2005).



(Reaction 7)

The type II sensitizer in the triplet state interacts with oxygen by energy transfer to give non-radical singlet oxygen ($^1O_2$), a highly reactive species of molecular oxygen, which reacts further with unsaturated lipids (Reaction 8) (Frankel, 2005).



(Reaction 8)

• Hydroperoxide formation by singlet oxygen

$^1O_2$ is highly electrophilic, and it reacts very rapidly with the double bonds UFAs by a different mechanism from free radical autoxidation. Accordingly, oxygen is inserted at either end carbon of a double bond, which is shifted to an allylic position in the *trans* configuration. The resulting hydroperoxides have an allylic *trans* double bond (Figure 6).

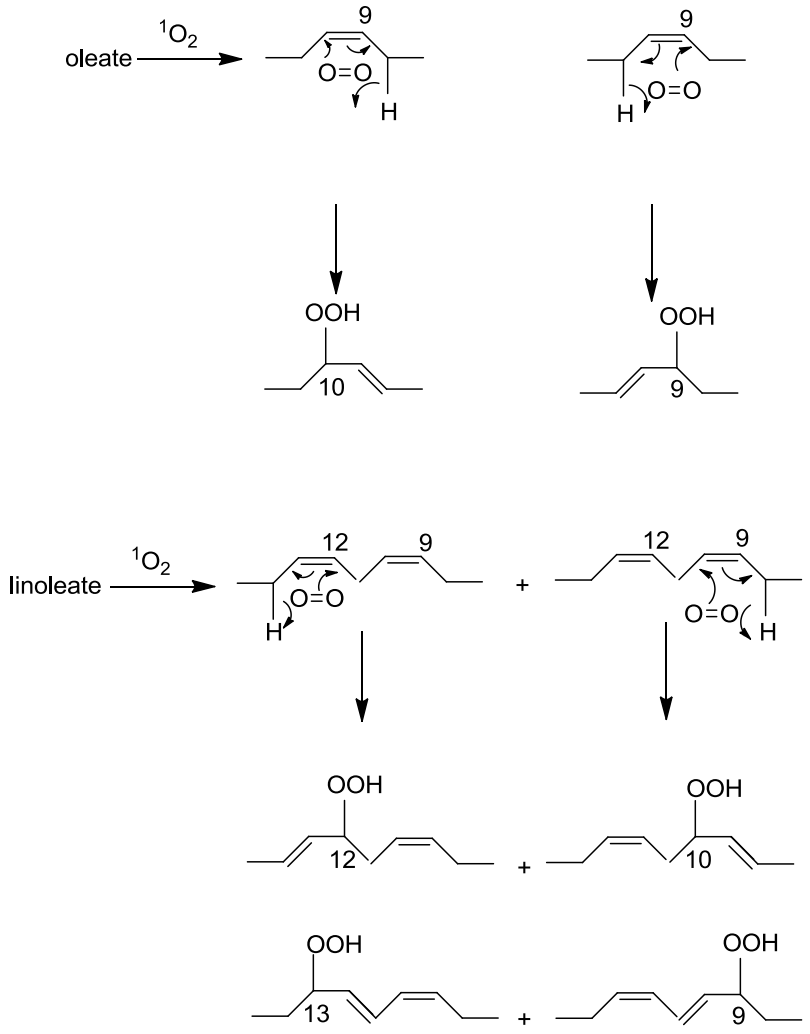


Figure 6: Oxidation of oleate and linoleate by singlet oxygen (Frankel, 2005).

2.1.1.3 Enzymatic peroxidation

Lipoxygenase comprise a family of lipid peroxidation enzymes that oxygenates free and esterified PUFAs to the corresponding hydroperoxy derivatives. They are widely distributed in both plant and animal kingdoms (Kuhn and Thiele, 1999). Arachidonic acid is the key precursor of potent physiological effectors (Prigge *et al.*, 1997). Until recently, lypoxygenases have been categorized to three major lipoxygenase isoforms with respect to their positional specificity of arachidonic acid oxygenation: 5-lipoxygenase, 12-lipoxygenase, and 15-lipoxygenase.

The mechanism of the lipoxygenase reaction involves two steps: substrate activation and oxygen addition. Two general mechanisms have been proposed (Figure 7). Both pathway A and B (Figure 7) begin with the active Fe^{3+} form of lypoxygenase (Nelson *et al.*, 1990).

In the mechanism A shown in Figure 7, Fe^{3+} oxidizes the 1, 4-diene moiety of the substate to form a pentadienyl radical. Reaction with molecular oxygen produces a peroxyl radical with reoxidizes Fe^{2+} and forms hydroperoxide product.

In the second B mechanism (Figure 7), the Fe^{3+} assists in the deprotonation of the diene by making a direct bond with the resulting carbanion. The path continues with insertion of dioxygen into the Fe-C bond followed by cleavage of the Fe-O bond.

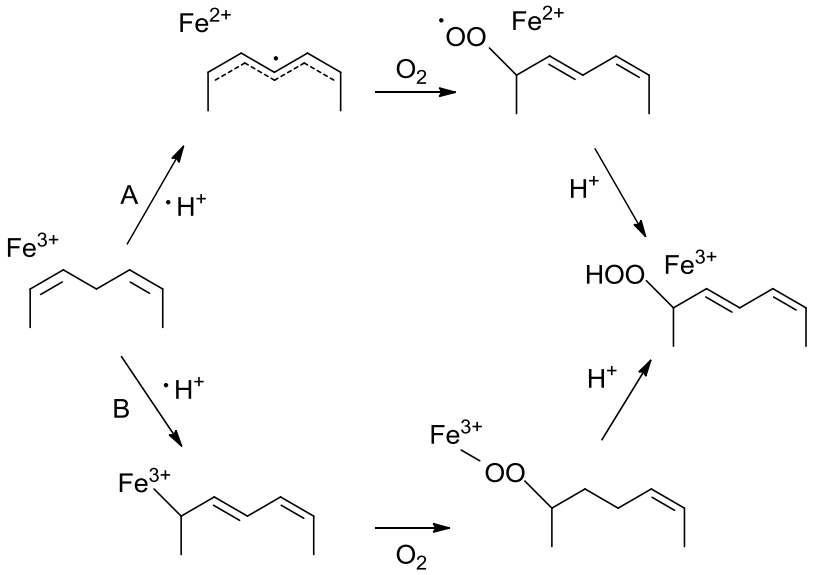


Figure 7: Mechanism of enzymatic peroxidation (Nelson *et al*., 1990).

2.1.2 Hydroperoxide decomposition

Hydroperoxides are relatively stable compounds under favorable conditions, such as low temperature, dilute solution, the presence of antioxidants and the absence of catalyst. Normally such conditions are not encountered, and the hydroperoxides become susceptible to chemical changes (Gruger and Tappel, 1970; Benzie, 1996). A complex mixture of monomeric, polymeric and small molecular weight volatile materials is produced when lipid oxidation is carried out to higher levels than 5-10%, especially with polyunsaturated fats containing more than two double bonds.

2.1.2.1 Monomeric products

Hydroperoxides may be converted to monomeric decomposition products containing functional groups such as hydroxy, keto and epoxy groups (Gardner *et al.*, 1974). Both mechanisms through peroxyl and alkoxyl radicals have been suggested for the formation of these products (Gardner, 1989). Two alkoxyl radicals combine to yield a hydroxy and a ketodiene compound (Figure 8a). Epoxides are formed by intermolecular addition of hydroperoxide oxygen across a double bond (Figure 8b).

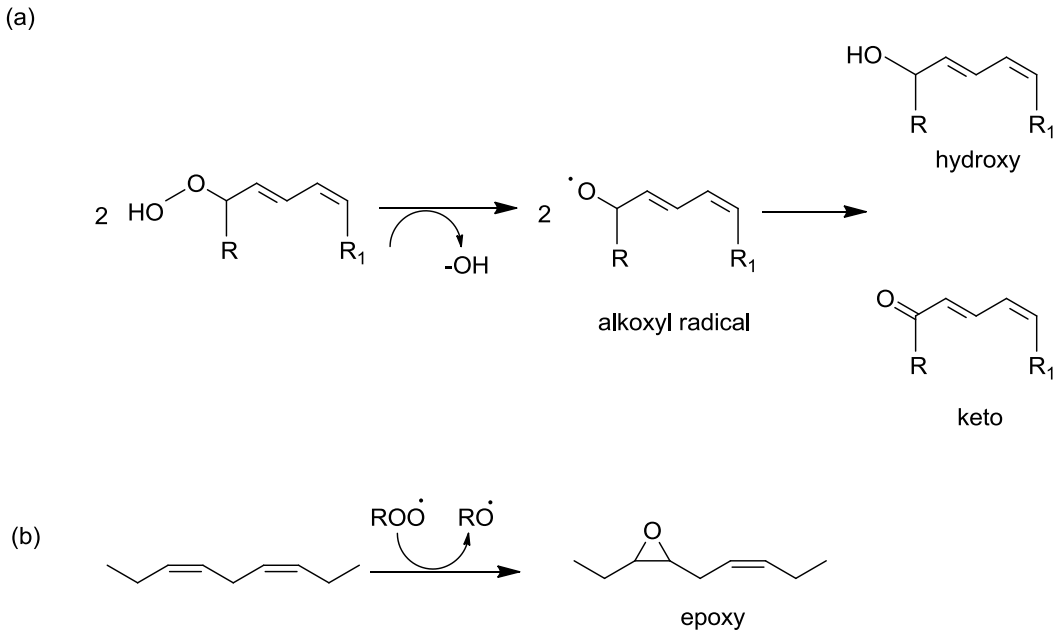


Figure 8: (a) Formation of hydroxyl and keto products from alkoxyl radicals; (b) Formation of epoxy products from peroxyl radicals.

2.1.2.2 Volatile products of lipids

A variety of volatile and nonvolatile secondary products are formed from hydroperoxides when lipid oxidation is carried out to high conversion or at elevated temperatures. The decomposition products includes: aldehydes, ketones, alcohols, acids, hydrocarbons, lactones, furans and esters. Alkyl hydroperoxides, allyl hydroperoxides and fatty ester hydroperoxides decomposition are accompanied by the formation of a lot of products such as carbonyls compounds, alcohols and acids, esters. The mechanism suggested for the formation of these products involves the homolytic cleavage of the hydroperoxide group to yield an alkoxyl and a hydroxyl radical (Frankel *et al.*, 1981; Grosch, 1987). The further decomposition of an alkoxyl radical involving carbon-carbon bond scission is the major pathway for the formation of volatile

products (Frankel, 2005). The α-scission and β-scission of an alkoxyl radicals lead to different decomposition products (Figure 9). The distribution of the volatile products is dependent both on the composition and isomeric distribution of the monohydroperoxides as well as the nature of the oxidative cleavage (Varlet *et al.*, 2007). In addition to monohydroperoxides, nonvolatile secondary products such as dimers, epoxyhydroperoxides and dihydroperoxides may undergo further decomposition into volatile products. Table 3 shows the possible origin to some aldehydes obtained from the oxidation of oleic, linoleic and arachidonic acids and the respective hydroperoxides formed as intermediaries.

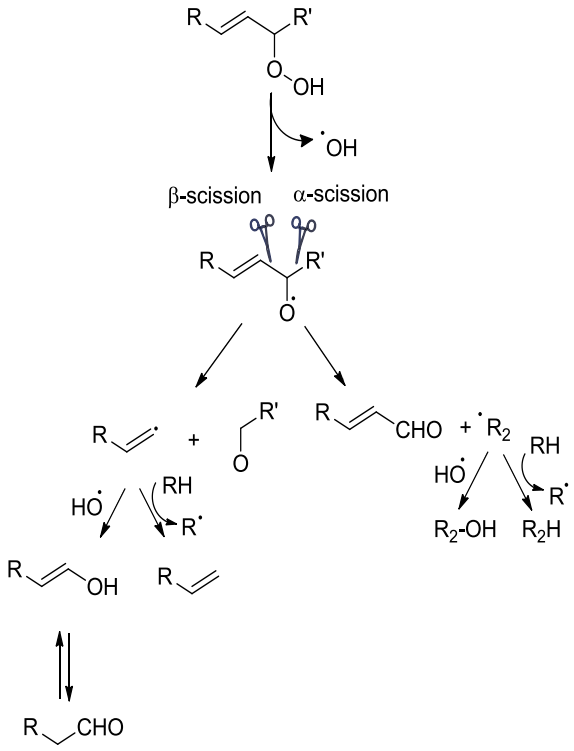


Figure 9: Homolytic-heterolytic mechanism of the decomposition of hydroperoxides (Varlet *et al.*, 2007; Frankel, 2005).

Table 3: Possible origin to several aldehydes deriving from UFA (Varlet *et al*., 2007)

UFA	Hydroxide position on hydroperoxides	Aldehyde
Oleic	C11	Octanal
	C10	Nonanal
	C9	2-Decenal
	C8	2-Undecenal
Linoleic	C13	Hexanal
	C11	2-Octenal
	C9	2,4-Decadienal
Linolenic	C16	Propanal
	C14	2-Pentenal
	C13	3-Hexenal
	C12	2,4-Heptadienal
	C11	2,5-Octadienal
	C9	2,4,7-Decatrienal
Arachidonic	C15	Hexanal
	C13	2-Octenal
	C12	3-Nonenal
	C11	2,4-Decadienal
	C10	2,5-Undecadienal
	C7	2,5,8-Tridecatrienal

2.1.2.3 Polymeric oxidation products

Polymeric oxidation products are hydroperoxide decomposition products containing two or more FA units cross-linked together either through peroxide, ether or carbon-carbon linkage. The peroxide and ether linkages are characteristic of polymerization carried out under mild reaction conditions (Miyashita *et al*., 1985). According to Neff *et al*. (1998), peroxide and ether linked dimers have been found during the autoxidation of linoleic and linolenic acids at low temperatures. Whereas dimers and higher oligomers are typical reaction products of heated lipids, which are produced under higher temperature are mainly linked through carbon-carbon bonds.

2.2 Biological effects of oxidized phospholipids

Research on oxidation of PLs goes back more than century, with very early studies by Thunberg in 1910 observing "respiration" of PL suspensions and noting that iron salts accelerated the uptake of oxygen in tissues (Elliot and Libet, 1944). The term "oxidized PLs" was reported for the first time in 1939 by Frederik and Mary Bernheim, following the studies on the action of vanadium salts as catalysts in the oxidation of PLs from heart and brain tissues (Bernheim, 1939; Reis, 2012). And then evidence is growing that oxidized PLs play a key role in the development of several chronic diseases and show a great variety of biological effects in vivo and in vitro (Fruhwirth, 2007). The main roles are following:

2.2.1 Role of oxidized PL in atherogenic inflammation

Atherosclerosis is a chronic inflammatory disease, characterized by specific infiltration of monocytes and T-cells (Leitinger, 2005). Vitro studies demonstrated that PL oxidation products can cause effects on vascular wall cells that could contribute to all stages of atherosclerosis from the fatty streak to thrombosis. There is also vivo evidence for a role of PL oxidation products including their accumulation in lesions, modulation of atherosclerosis by antibodies to oxidized PLs, increased myocardial infarction in individuals with alterations in enzymes that degrade PL oxidation products, and a decrease in fatty streak formation in animals given an inhibitor of the actions Platelet-activating factor (PAF) and PL oxidation products (Berliner and Subbanagounder, 2001).

2.2.2 Role of oxidized PL in endothelial cell

Two specific oxidized PLs, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC), were identified as abundant products in oxidized low density lipoprotein and were shown to have major roles in the activation of endothelial cells and induction of leukocyte binding (Berliner and Gharavi, 2008). Moreover, oxidized PLs modulate transcription factors such as peroxisome proliferator-activated receptors alpha and gamma, nuclear factor of activated T cells and early growth response protein 1 (Egr-1). Oxidized PLs also stimulate angiogenesis in human endothelial cells via induction of autocrine mediators such as vascular endothelial growth factor which works through activating transcription factor-4 (ATF4) (Oskolkava *et al*., 2008).

2.2.3 Role of oxidized PL in apoptosis

Apoptosis of various cell types was due to the oxidation of all major PL classes by reactive oxygen species, which were generated by nicotinamide adenine dinucleotide phosphate oxidase (Leitinger, 2003). The externalization of oxidized PLs was shown to be required for macrophage clearance of apoptotic cells (Kagan *et al*., 2002), and oxidized PL epitopes of apoptotic cells were recognized not only by specific antibodies (Chang *et al*., 1999) but also by C-reactive protein (Chang *et al*., 2002). Furthermore, membrane parts shed from apoptotic cells (apoptotic blebs) contained biologically active oxidized PLs that activated endothelial cells to bind monocytes (Huber *et al*., 2002). Apoptotic cells are thus an additional source of oxidized PLs and may actively contribute to inflammation. Several studies proved the role of oxidized PL in the apoptosis of cells. Fruhwirth (Fruhwirth *et al*., 2006) demonstrated that the oxidized PLs (POVPC and PGPC) inhibited growth and induce apoptosis in vascular smooth muscle cells. Kinnunen *et al*. (2012) and Fruhwirth *et al*. (2007) showed that oxidized PLs control the role of membrane binding of cytochrome c, a key protein in the control of the apoptosis.

3. Methods of analysis of phospholipids

PLs are a kind of functional lipids which are located in biological samples, such as blood, brain, liver, and so on. More and more studies have been done on PLs. Overall, the analysis of PLs involves different steps: total lipids (TLs) extraction of biological samples, isolation of PLs fraction from the other lipid classes and separation of the different PL classes. In this part, we will introduce analytical technologies of extraction, isolation and separation of PLs.

3.1 Extraction of total lipids

Extraction of TLs based on the Bligh and Dyer (Bligh and Dyer, 1959) and Folch methods (Folch *et al*., 1957) are routine procedures preceding analysis of FAs, sterols, PLs and other lipids (Phillips *et al*., 2008). Other traditional extraction techniques including Soxhlet method (Pérez-Palacios *et al*., 2008), ultrasound-assisted extraction (Seng *et al*., 2009) have been also employed to quantitatively extract TLs. These techniques require long extraction times, and large amounts of samples, sorbents and organic solvents, which have negative environmental impact and other human health issues. Moreover, one of a drawback of these traditional extraction methods is the obtained final extracts that often require subsequent concentration and clean-up prior to analysis. Furthermore, when considering the extraction of bioactive compounds that are sensitive, thermolabile and are found in low concentrations, traditonal extraction techniques would not be the most suitable option. Therefore, a green and efficient extraction approach is required.

Pressurized liquid extraction (PLE) is a green approach that has received particular attention recently (Mustafa and Turner, 2011). PLE has many merits that make it an excellent substitute to traditional methods. Using elevated temperature and pressure during the extraction does not only improve the extraction yield, but also decreases time and solvent consumption. Furthermore, the set-up in the PLE equipment provides protection for oxygen and light sensitive compounds.

Several studies has used PLE method for lipids extraction. White *et al*. (2009) compared PLE method with Bligh and Dyer method to extract soil PLs and neutral lipids. The results showed that PLE method was more efficient than Bligh and Dyer method for the extraction of soil lipids. Dunford and Zhang (2003) compared the efficiency of PLE and Soxhlet extraction methods for the extraction of wheat germ oil. The results indicated that PLE method could reduce solvent consumption and extraction time with no adverse effect on the extraction yield and FA

composition of the oil. Dodds *et al*. (2004) extracted TLs from fish tissue by PLE with different solvents, and showed that chloroform/methanol was a most effective solvent system for the extraction of polar lipids. Moreau *et al*. (2006) used PLE method to extract polar and nonpolar lipids in corn and oats with hexane, methylene chloride, isopropanol and ethanol at two temperatures (40 and 100 °C). The results showed that, for both corn and oats, methylene chloride extracted the highest levels of each nonpolar lipid class and increasing solvent polarity resulted in increasing yields of polar lipids, and for each solvent, more of each lipid class was extracted at 100 °C than at 40 °C.

In addition, there is another method (supercritical fluid extraction) for lipid extraction, Carbon dioxide (CO_2) is the most used superitical fluid, it is regarded with interest being safer than organic solvents such as hexane, offering mild operating conditions, negligible environmental impact and a short extraction (Andrich *et al*., 2005). Sometimes, it is modified by methanol (Patil *et al*., 2010).

3.2 Isolation of phospholipids from total lipids

PLs represent an overall minor part in the TLs which consist mainly of neutral lipids. In order to isolate PL fraction from neutral lipids, solid phase extraction (SPE) have been widely applied.

Chromatographic methods have been usually used for the isolation, separation, and quantification of PL along with other lipids including aliphatic hydrocarbons, sterol esters, glycerides and FFAs. SPE is considered as being one of the most powerful techniques currently available for rapid and selective sample preparation. It has elicited growing interest because it is rapid and reliable, requires less solvent, and can be easily automated (Bodennec *et al*., 2000). Silica gel (Si) and aminopropyl-bonded silica gel (NH_2) SPE cartridges have been usually applied for the isolation of PL from other lipids and for the separation of PL compound classes (Bondia-Pons *et al*., 2006; Avalli and Contarini, 2005). Lipids can be divided into neutral lipids, sugar lipids and PL using sequential elution with chloroform, acetone and methanol, respectively. But this method is normally applied to separate “total PL” from other lipids. Further fractionation of “total PL” into compound classes need use mixtures of chloroform and methanol (Bodennec *et al*., 2000).

3.3 Separation of phospholipid classes

Traditional technique for separation of PL classes included thin-layer chromatography (TLC), SPE and high-performance liquid chromatography (HPLC). Gas chromatography (GC) can also be used in separation of PLs, but it needs derivatization of PLs before injection. We will discuss it later.

3.3.1 TLC

TLC is a chromatography technique used to separate mixtures. TLC is performed on a glass, plastic plate, or aluminum foil, which are coated with a thin layer of absorbent material, usually silica gel, aluminum oxide, or cellulose. Among them, silica gel was the most widely used adsorbent for PL classes separations (Ruiz-Gutiérrez and Pérez-Camino, 2000).

An advantage of TLC is the ability to run a number of standards at the same time as your sample and it can separate PL classes as long as suitable solvents are chosen. For example, Pérez-Palacios *et al*. (2007) succeed to separate different PL classes, such as PE, PI and PS from animal muscle using TLC. PC, PE, PS and PI were sequentially eluted with 30 mL of acetonitrile:n-propanol (2:1, *v/v*), 10 mL of methanol, 7.5 mL of isopropanol:methanolic 3N HCl (4:1, *v/v*) and 17.5 mL of chloroform:methanol:HCl 37% (200:100:1, *v/v/v*), respectively. Furthermore, there are some reports of methods using a TLC hyphenated with matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Fuchs *et al*., 2007; Leβig *et al*., 2004), high-performance chromatography (HPTLC) (Miwa *et al*., 1996) and fluorometric microdetermination (Heyneman *et al*., 1972), which can not only one-line monitor the separation of PLs, but also quantify and identify the molecular species of PL.

3.3.2 SPE

SPE is one of conventional methods for separation of PLs and it is widely used today. The most common stationary phase is silica and aminopropylsilica column (Peterson and Cummings, 2006). SPE can not only isolate PLs from neutral lipids, which are inferred in 3.2, but can also separate PL classes by different solvent. The mobile phases most frequently used for the separation of PLs are chloroform, methanol and H_2O, and sometimes other solvents like hexane, isopropanol, ethanol and triethylamine are also used to modify the elution procedure.

3.3.3 HPLC

The HPLC detection of PL classes is accomplished commonly by UV, ELSD or MS detectors. HPLC-UV analyses is used extensively. However, some major problems result from its sensitivity of gradient conditions and result in baseline drift and high background noise (Helmerich and Koehler, 2003), besides, the optical adsorption by PLs in the range 200-210 nm make it difficult to use common chromatographic solvents, which are not transparent in this region. Whereas, ELSD detector is not much affected by gradient conditions and can have stable baselines through rapid changes in eluent composition. Thus, it has received particular attention recently (Kang and Row, 2002; Avalli and Contarini, 2005; Néron *et al*., 2004).

3.4 Separation of phospholipids molecular species

HPLC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. The major advantage of MS is its high selectivity which can be realized by monitoring the signals in the multiple reaction monitoring (MRM) mode when the analyte has an access to tandem MS-MS. The detector can be programmed to select certain ion fragments and selective transitions. Therefore, numerous studies on the separation of PLs by HPLC-MS method (Hayakawa and Okabayashi, 2004; Chen *et al*., 2007; Sommer *et al*., 2006) have been developed.

The chromatography is performed either on normal phase or on reverse phase. For the normal phase separation, the silica phase is the most commonly used (Rombaut, 2005). The silica normal phase chromatography allows the separation of PL classes using adsorption mechanisms of the different polar headgroups. Separation of molecular species of each PL classes, even the isomers of them on the basis of different chain lengths and the degree of unsaturation of the acyl chain should be done by reverse phase HPLC since it can separate different molecular species within one PL class, such as PE and SM. Quantification of the different molecular species within each PL class can be only carried out by MRM mode.

In order to identify the molecular species of PL classes, HPLC is always coupled with mass detector. With the invention of soft ionization techniques, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) and surface-enhanced laser desorption/ionization (SELDI), nearly all lipid classes can be analyzed very sensitively with only minimal fragmentation (Fuchs *et al*., 2007). Le Grandois *et al*. (2009) has used HPLC-ESI-MS^2 to

identify the molecular species of PC of various food matrices in our laboratory. In addition, a emerging technology, mass spectrometry imaging (MSI) is a remarkable technology that enables analysts to visualize the spatial distribution of biological molecules present in tissue sections by direct ionization and detection. Various ionization methods that can ionize the analytes on the surface, such as MALDI (Sparvero *et al*., 2012), desorption electrospray ionization (DESI) (Manicke *et al*., 2010), laser ablation electrospray ionization (LAESI) (Shrestha *et al*., 2010) and secondary ion mass spectrometry (SIMS) (Sostarecz *et al*., 2004). Among them, MALDI-MSI is currently one of the only methodologies enabling simultaneous visualization of various lipids, such as PLs, NLs and FAs (Goto-Inoue *et al*., 2011).

Table 4 summarized analytical technologies for analysis of PLs from several biological matrices, such as soybean, human blood, egg yolk… Different detectors are applied for separation and identification of PL classes, even molecular species of PL classes.

Table 4: Common analytical techniques for the analysis of PL

Samples	PL classes or molecules	Separation	Detection	References
Soybean	PE, PI, PC, LPC, LPI	RP- HPLC	ESI-MS	Lee et *al*., 2010
	PC	RP- HPLC	ESI-MS	Bang *et al*., 2006
	PC, LPC, PE	NP-HPLC	UV	Balazs *et al*., 1996
	PC, PE	RP-HPLC	ELSD	Wang *et al*., 1999
Human blood	PE, PG, PI, PS, PC, SM, LPC	NP-HPLC	ESI-MS^3	Pang *et al*., 2008
	PE, PS, PC, LPC, SM	NP-HPLC	ESI-MS^2	Wang *et al*., 2004
	PE, PI, PS, PC	NP-HPLC	ESI-MS^2	Malavolta *et al*., 2004
	PS	NP-HPLC	ESI-MS^2	Hvattulm *et al*., 1998
Human milk	PE, PI, PS, PC, SM	NP-HPLC	ELSD	Sala-Vila *et al*., 2005
	PE, PC, PI, PS, SM	NP-HPLC	ELSD	Sala-Vila *et al*., 2003
Fish	PC, PI, PE, PS	NP-HPLC	ESI-MS^2	Boselli *et al*., 2012
Rat liver	PE, PI, PS, LPE, PC, SM, LPC	NP-HPLC	ELSD	Juaneda *et al*., 1990
Rat brain	PC, PE	-	SIMS	Benabdellah *et al*., 2010
	SM	-	DESI	Manicke *et al*., 2010
	PE, PI, PS, PA, PC, SM, PG	NP-HPLC	MALDI-MSI	Sparvero *et al*., 2012
Egg yolk	PC, SM, PE	NP-HPLC	UV	Yoon and Kim, 2002
	PE, PI, PC, SM, LPC	NP-HPLC	ESI-MS^2	Pacetti *et al*., 2005
	PE, PC, PI, LPE, LPC, SM	TLC	MALDI-TOF-MS	Fuchs *et al*., 2007

-: column not used

Molecular species are identified by MS^n (n=1, 2 and 3), SIMS and DESI.

3.5 Separation of fatty acids of phospholipids by GC

GC is a common method for the analysis of FAs composition of PLs and volatile products of oxidized PLs, but it doesn't allow the analysis of the intact molecules because of the very low volatility of PL due to the polar headgroup. In this study, GC has been used to analyse FAs of PLs and volatile products of oxidized PLs. Therefore, the method of GC couldn't identify the molecular structure of PL without derivatization. If the analysts want to determine the composition of the FAs in a PL class, they should firstly isolate the PL class before derivatization and GC analysis.

3.5.1 Derivatization in GC

To become GC compatible, PLs requires firstly hydrolysis and derivatization of lipid molecules into volatile components before separation (Bang *et al*., 2006). PLs have been usually hydrolyzed by phospholipase C and derivatized with tert-butyl-dimethylsilyl (TBDMS) (Oda *et al*., 1985), trimethylsilyl (TMS) (Yon and Han, 2000) and pentaflurobenzoyl (PFB) (Wang *et al*., 2003). Another method for the derivatization is the transesterification of a PL class using KOH in CH_3OH Unfortunately, the process of the derivatization would lead to the loss of information about the chemical structure of the whole PL.

3.5.2 Determination of volatile compounds from fatty acid of oxidized phospholipid

GC-MS has been widely used for the instrumental analysis of volatile compounds from various food matrices, and nowadays, headspace solid-phase microextraction (HS-SPME) is a rapid, sensitive and solvent-free sampling technique that is gaining popularity for sample preparation hyphenated GC-MS (Beltrán *et al*., 2011). Several fibers are frequently used, polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB), and carboxen/polydimethylsiloxane (CAR/PDMS), for the separation of volatile compounds. Many studies have been performed to determine volatile oxidation compounds of different oils by HS-SPME-GC-MS (Iglesias *et al*., 2009; Lee *et al*., 1995; Morales *et al*., 1997; Beltrán *et al*., 2011). In fact, lipids contain many classes, such as cholesterols, triglycerides, PLs, fat-soluble vitamins and so on. The effect of thermal treatment on different lipid classes may be different. And nowadays, several studies were contributed on the volatile compounds of complex PLs mixtures. Meynier *et al*. (1998 and 1999) analyzed volatile oxidized compounds (VOCs) of oxidized pork and turkey PLs. Lin and Blank (2003) identified the VOCs generated by thermally treated egg PLs.

4. Position of the work

This overview of literature shows that PLs are rich in polyunsaturated fatty acid (PUFA) and contribute to the health benefits for human. PLs can be divided into GPLs and SPLs. GPLs are the main PL class found in cell membranes. Different types of dietary GPLs vary in their FA composition and headgroup, and therefore have different effects. The most representative SPL is SM, primarly exists in brain and neural tissue. PL supplementation has health benefits and has received more and more attention. Therefore, it is worth charactering potential sources of PUFA-PLs. It is however well known that food process such as heating, drying, freezing and irradiation, may influence the stability of PLs, and the most common change in lipid structure is oxidation, which is often caused by heat treatment, the most widely used unit operation during food processing and preparation. Therefore, another focus of this study is to investigate the oxidative stability of PLs.

This study aims to determine molecular species of PE and SM in various food matrices, and investigate oxidative stability of different molecules of PC and PE and compare with their corresponding TAGs. Five potential sources, ox liver, calf brain, egg yolk, soy and krill oil, are chosen. PLE method is used to extract TLs and PLs from various food matrices and the extraction efficiency is determined by comparing with Folch method. The molecular species of PE and SM were identified by LC-ESI-MS^2. The identification of molecules can give the information of FA composition of molecular species of PLs which is conducive to explore potential sources of PUFA-PLs, especially, Docosahexaenoic acid (DHA)-PLs and Eicosapentaenoic acid (EPA)-PLs. The volatile oxidized products of PLs are detected by HS-SPME-GC-MS during heat treatment as a step towards understanding the behaviour of PL with regard to thermal oxidation, and the comparation of oxidative stability of PLs with TAGs could provide information on the choice of PUFA-PL as food supplementation.

Chapter 2: Development of methods for the analysis of PE and SM molecular species

Introduction

PUFAs play very important roles in many aspects of human health, in particular in reducing risks of cardiovascular diseases, inflammation, hypertension, allergies, and immune and renal desorders (Le Grandois, 2009).

Two types of PUFA are essential fatty acids (EFA): linoleic acid (18:2ω6) and α-linolenic acid (18:3ω3). These two FAs cannot be synthesised by humans, so they must be supplied in the diet. In addition, they are the precursors of long-chain PUFAs (LC-PUFAs) (18:3ω3 is a precursor of 20:5 and 22:6; 18:2ω6 is a precursor of 20:3 and 20:4), in particular EPA and DHA, which are essential for brain and retina development (Smit *et al*., 2004; Nakamura and Nara, 2003). Thus, these PUFAs are important in human diet and nutrition.

Studies have shown that PUFAs, especially LC-PUFAs are mostly found in GPLs of cell membranes. There is a consistent pattern of distribution of individual FAs within different PL subclasses and within different tissues (Leef, 1996). Additionally, the types of FAs in the diet determine the types of FAs that are available to the composition of the cell membranes. And Youdim *et al.* (2000) showed that the EFAs taken up by tissues are recovered in relatively high proportions as components of PLs. The result was in line with the fact that PLs are much more efficient carriers than TAGs according to FA biodisponibility (Amate *et al*., 2001; Lemaitre-Delaunay *et al*., 1999; Wijendren, 2002), despite TAGs are the major carrier of FAs, with 50-100 g/day for an adult, followed by PLs, with 2-10 g/day (Parmentier *et al*., 2007). Therefore, supplementation of food products with PUFA-rich PLs has recently emerged as an interesting way of increasing the assimilation and the health benefits of LC-PUFAs in the human body, the preparation of food supplements containing PLs molecular species (rich in 18:2, 18:3, DHA and EPA) has great significance in human nutrition.

Therefore, data regarding PL content is highly important when it comes to the nutritional value of foods. Incomplete extraction of TL can cause serious errors in the PL determinations. Therefore, effective extraction methods need to be developed for various food matrices in order to investigate their TL and PL contents.

Various procedures have been employed to quantitatively extract lipids, such as soxhlet method (Manirakiza *et al*., 1997), Bligh and Dyer (Bligh and Dyer, 1959), as well as the Folch method (Folch *et al*., 1957), based on chloroform/methanol extraction. However, these extraction

methods often rely on the use of high amounts of organic solvents, most of them being hazardous or expensive and must be properly handled and disposed of, once the extraction is finished (Boselli *et al*., 2001). In addition, these extractions are often time consuming, which in turn may lead to degradation of active ingredients (Cho *et al*., 2007).

Five potential sources of PLs were studied in this chapter, which are egg yolk, calf brain, soy, ox liver and krill oil. Among them, egg yolk and soy are common as raw materials used to manufacture lecithin and some information of them have been provided in previous studies, while the information of other three sources was relatively less. Moreover, they represent various levels of fat content. Therefore, we hope that through this study, a comprehensive information of PLs can be provided.

The purpose of the first part of this chapter was therefore to develop a method to improve the recoveries of TLs and PLs from these food matrices. Among new alternative techniques that are available, there is PLE, which has received particular attention recently and gained wide acceptance for the extraction of organic contaminants (Wang *et al*., 2010). This method uses small amounts of conventional solvents at elevated temperatures and pressures to achieve quantitative extraction from solid and semi-solid samples in a short time (Coute *et al*., 1997; Macnaughton *et al*., 1997; Jansen *et al*., 2006). We used this method to extract TLs and PLs from various food matrices and determine the extraction efficiency by comparing with a standard liquid extraction method, a modified Folch method.

In the second part, the aim was to determine the molecular species of PE and SM. Usually, the analysis of PL is aimed at the simultaneous determination of PL classes in a specific sample. Yet, few literatures are available when it comes to the determination of molecular species within a specific GPL class. Among the different GPL classes, PE and PC are predominant in most foods. PC molecular species have been thoroughly investigated in various food matrices by Le Grandois *et al*. (2009) using LC-ESI-MS^2, but to our knowledge, no information is available as of today about the occurrence of PE molecular species in food matrices. As far as SPLs are concerned, SM is a major member which is characterized by a sphingosine backbone linked with a single FA. This study describes a series of method allowing purification of PE and SM from different food matrices. TL was first extracted by PLE, the polar lipid fraction was purified by SPE, and the PL classes were separated by semi-preparative HPLC hyphenated with ELSD. PE and SM were

collected via a split system placed ahead of the ELSD detector, and then PE and SM molecular species were determined by RP-HPLC coupled with ESI-MS^2.

Part A: Extraction and analysis of phospholipids

In this part, a new method, based on PLE, was applied for TL (including PL) extraction and compared with a standard liquid extraction method, a modified Folch method. The extraction efficiency of TL and PL by these two methods was discussed.

1. Material and method

1.1 Material

Solvents used for HPLC analyses were of HPLC-grade and those used for extraction were of analytical grade. Chloroform was purchased from VWR (Strasbourg, France) and methanol was purchased from Carlo Erba (Val de Reuil, France). Washing solution was prepared from analytical grade sodium chloride (1%, w/v; VWR) and ultrapure water (Synergy, Millipore S.A.S, Molsheim, France). Sand (Fontainebleau) was provided by VWR.

The PL content in four food matrices was studied. They are hen's eggs, soybeans, calf brain and ox liver which were purchased from a local retailer. The calf brain and ox liver were stored in -20°C, the eggs were stored in 4°C, and the soybeans were stored in the room temperature. Krill oil was kindly provided by Nestec (Lausanne, Switzerland).

Standard solutions: solutions of soy lecithin mix standard (certified: 14.41% PC, 12.06% PE and 9.64% PI) from Spectral Service GmbH (Cologne, Germany) were prepared at six concentrations between 0.1 to 1.1 mg/mL.

1.2 Sample Preparation

1.2.1 Cryogenic conditions

Cryogenic conditions exist at temperatures of below approx. -195.8°C (approx. < 77.4 K). The temperature is therefore far below the glass transition temperature of water. No further re-crystallisation of ice takes place and no further ice crystals form (Burden, 1999). The liquid nitrogen can quickly block the energe transfer, cause rapid freezing on contact with samples and make them enter the freezing process rapidly. This ensures that chemical processes in the samples are reduced to a minimum and morphological change, e.g. through further growth of ice crystals, are prevented. Cryogenic grinding could make sample solidified and softened, which permits the

grinding process easier and more thoroughly. During storage in the gas phase over liquid nitrogen, an inert-gas atmosphere is created by the nitrogen evaporating in the sample-storage container. This also prevents any changes in the samples caused by oxygen from the atmosphere (oxidation processes).

Preparation was different for each sample depending upon its physical state. With the exception of egg yolk, other samples were ground using a 6870 Freezer/Mill (Spex CertiPrep, Stanmore, U.K.) (Figure 10). The grinding method consists of three cycles as follows: 3 min cooling in liquid nitrogen, grinding 5 min and then 1 min of cooling again.

Figure 10: Freezer/Mill

1.2.2 Freeze-drying

Freeze-drying (also known as lyophilization or cryodesiccation) is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport and storage. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase.

The obtained frozen powder (cryogenic conditions) was placed in a freeze-drying flask in a bath for 10 min, which is cooled by liquid nitrogen, and then was freeze dried in a freeze-drier (Alpha 2-4, Bioblock Scientific, Illkirch, France) (Figure 11) during 12 hours. At the end of the lyophilization, the product has less than 5% water and bio-chemical properties of the treated sample are retained. The powders obtained were transferred in amber glass bottles of 30 mL sealed with a stopper and stored in the freezer (-20°C).

Egg yolk powder was obtained by manually removing egg white from fresh eggs, homogenizing and freeze drying. All the food samples were stored at -20°C until use.

Figure 11: Freeze-drier Alpha 2-4

1.3 Extraction of total lipids

1.3.1 Modified Folch method

TL was extracted by a solid-liquid extraction method (SLE) according to Folch *et al.* (1957), with minor modifications (the volum of solvents had a little changed). Briefly, a total of 1 g of the prepared food sample was suspended in 30 mL of a $CHCl_3$/CH_3OH (2/1, v/v) mixture and shaken mechanically by orbital shaker (Bioblock Scientific, Illkirch, France) at room temperature and 350 rpm for 20 min. The suspension was centrifuged at 8500 rpm for 10 min. The supernatant was removed and the pellet was re-extracted 4 times (total of 5 extractions) following the same procedure until total exhaustion of lipids from food matrices. Supernatant of each extract were washed with 5 mL of sodium chloride aqueous solution (1%, w/v). The organic phase containing the lipid fraction was collected, evaporated under vacuum using a rotary evaporator (40℃, 300 kPa) and dried under a gentle stream of N_2. The TL extract was weighed (expressed in mg/g of fresh food).

1.3.2 Pressurized Liquid Extraction

PLE is similar to Soxhlet extraction, except that the solvents are used near their supercritical region where they have high extraction properties. In that physical region the high temperature enables high solubility and high diffusion rate of lipid solutes in the solvent, while the high pressure, in keeping the solvent below its boiling point, enables a high penetration of the solvent in the sample. Thus, PLE permits high extraction efficiency with a low solvent volume and a short extraction time (Figure 12).

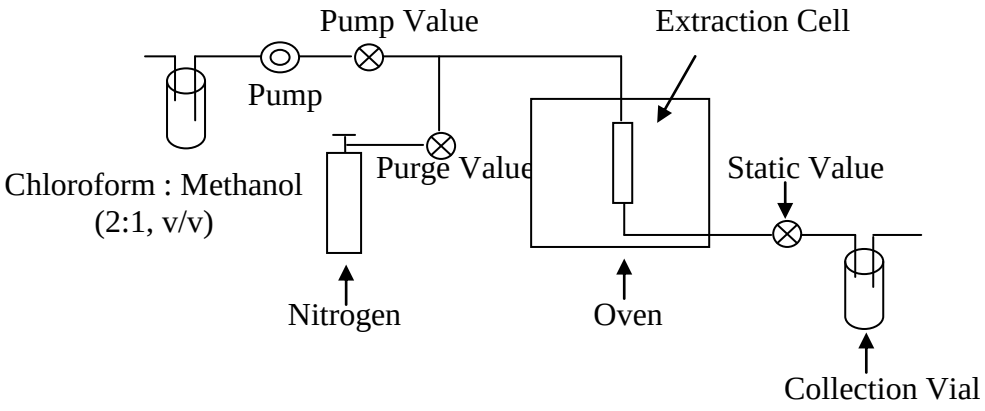


Figure 12: The schema of PLE (Mustafa and Turner, 2011)

A Dionex PLE 350 (Dionex, Sunnyvale, CA, USA) system (Figure 13) was used to extract TL. Sample powder, 1.0 g, was mixed homogenously with sand so as to fill the 10 mL stainless steel extraction cell (L × Ø: 52 mm × 15 mm). The use of a dispersion agent, such as sand used for the extraction, is recommended in order to reduce the solvent volume by filling the empty part of the cell (Salces *et al*., 2001). The solvent mixture employed was the same as that used for the SLE-Folch procedure, chloroform/methanol (2/1, *v/v*). Different extraction temperatures (120 to 150°C) were investigated. The extraction time and pressure were set at 5 min and 10.34 MPa, respectively according to Zhou *et al* (Zhou *et al*., 2010). One to three extractions (successive extractions on the same sample and collection in three independent vials) were performed on each food matrix. Each extract (17 mL) was collected in a 60 mL glass vial and washed with 5 mL of sodium chloride aqueous solution (1%, w/v). The organic phase containing the lipid fraction was collected and dried as previously described (1.3.1).

Figure 13: ASE 350

1.4 Determination of phospholipid classes by HPLC-ELSD

A chromatographic system, made of a 616 controller, a 2424 ELS detector and a 717 Plus autosampler (Waters, Saint-Quentin-Fallavier, France) controlled with Empower 2 software (Waters), was used to analyse PL classes. High-purity nitrogen from a nitrogen generator (Domnik Hunter, Villefranche-sur-Saône, France) was used as a nebulizing gas at a pressure of 310 kPa. The drift tube temperature was set at 45°C. PLs were separated into their classes using a 150 x 3 mm, 3 µm Luna normal phase silica column (Phenomenex, Le Pecq, France). The flow-rate of mobile phase was 0.5 mL/min and separations were performed at room temperature using a 20 min linear gradient ranging from $CHCl_3/CH_3OH$ (88/12, v/v) to $CHCl_3/CH_3OH$/1M aqueous formic acid (adjusted to pH=3 with triethylamine) (28/60/12, v/v/v) (Rombaut et *al*., 2005). Each extract was dissolved in a mixture chloroform/methanol (2/1, v/v), filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter (Macherey-Nagel, Hoerdt, France) to eliminate particles and injected (20 µL) in the chromatographic system. PL classes were identified by comparison of their retention times with those obtained under the same analytical conditions with certified standard soy lecithin. Quantification of each PL class was performed based on a quadratic model of external calibration obtained using standard solutions. PL contents were expressed in mg/g of fresh food.

1.5 Analysis of results

Three determinations were performed for each food matrix (n = 3). All results are given as mean ± standard deviation (SD). The Student *t*-test was used to determine significant differences between extraction efficiencies. Significance level was considered at $P < 0.05$.

2. Result and discussion

2.1 Determination of optimal pressurized liquid extraction for phospholipid

The extraction temperature, time and number of extractions were considered to be important for effective extraction (Zhuang *et al*., 2004). Number of extractions means that extraction was repeated several times on a sample in order to extract TLs as much as possible. To establish an appropriate PLE method for extracting PL from food matrices, it was necessary to determine the effect of these extraction conditions on the extraction yield. First, the effects of extraction temperature and duration on the extraction efficiency were investigated. Soybean was chosen as a model food matrix. As shown in Figure 14, the recovery of PL gradually increased with the increase of temperature from 120 to 140 °C. Because an increased temperature decreases the viscosity of a liquid solvent, thereby enhancing its penetration inside the matix particle, which results in an improved extraction process (Mustafa and Turner, 2011). Moreover, according to Arrhenius equation:

$$K = Ae^{-Ea/RT}$$

K: reaction rate constant; A: pre-exponential factor; Ea: activation energy; R: universal gas constant; T: temperature.

it can be seen that increasing the temperature will result in an increase in rate of reaction (Rodríguez-Aragón *et al*., 2005). However, when the temperature was raised to 150 °C, the recovery of PL slightly decreased, which is in line with the results obtained by Boselli on the extraction of oxysterols from egg-containing food (Boselli *et al*., 2001). This decrease can be explained by an oxidative losses on thermolysis which occur at higher temperatures (Le Grandois *et al*., 2010). And there is a very noteworthy phenomenon related to these changes, which was that the TL extract turned green when the extraction temperature was at 150 °C. The phenomenon implied that some changes occurred in the chemical compositon of food matrices at 150 °C. Therefore, 150 °C was not a suitable temperature for extraction. Another point was that no significant difference ($P > 0.05$) in extraction efficiency was observed when extracting at 130 °C for 5 min or for 10 min (Figure 14). This showed that the partition equilibrium between solvent and food matrix was achieved within the first five minutes during a static extraction, which was consistent with previous reports (Dunford and Zhang, 2003).

Therefore, the optimal extraction temperature and time for each extraction by PLE method were set at 140 °C and 5 min, respectively.

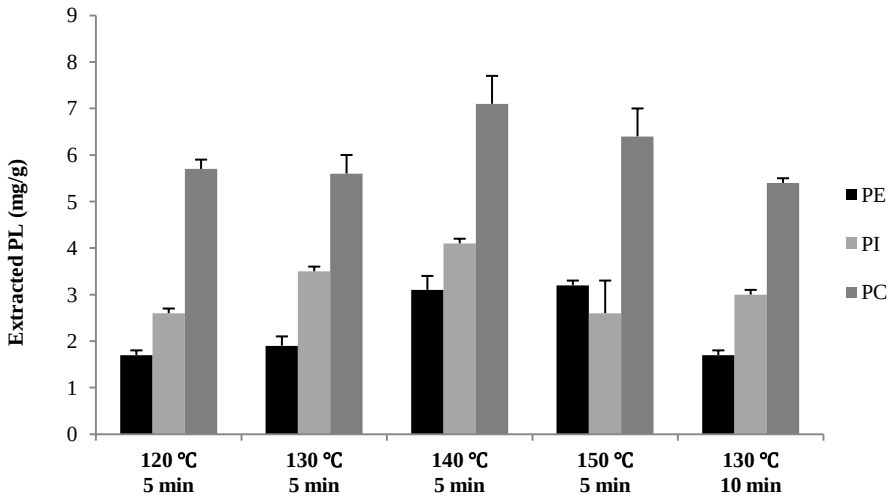


Figure 14: Amounts of PL recovered from soybean after one extraction by PLE method. Standard deviation (n = 3) is shown as error bars.

2.2 Determination of optimal extraction number (total lipid)

TLs were extracted by PLE from four food matrices with different fat content: soy (10-30% w/w of the fresh food), egg yolk (> 30% *w/w* of the fresh food). while ox liver and calf brain contain low fat (< 10% *w/w* of the fresh food). As for krill oil, it represents a feature of marine oils, and it has already been lipid, so it doesn't need PLE extraction. Therefore, we chose solid state matrices, egg yolk, soy, calf brain and ox liver to study the optimal extraction condition of TL and PL.

2.2.1 Extaction numbers (TL)

The amount of extracted TL was therefore monitored according to the number of successive extractions for each selected food matrix using SLE-Folch and PLE methods (Figure 15). With the SLE-Folch method, three successive extractions could recover 98% of TL, and a fourth extraction was necessary to achieve total recovery. On the other hand, PLE method was more efficient. Indeed, only two successive extractions were needed to recover the whole TL content of each food matrix (Figure 15).

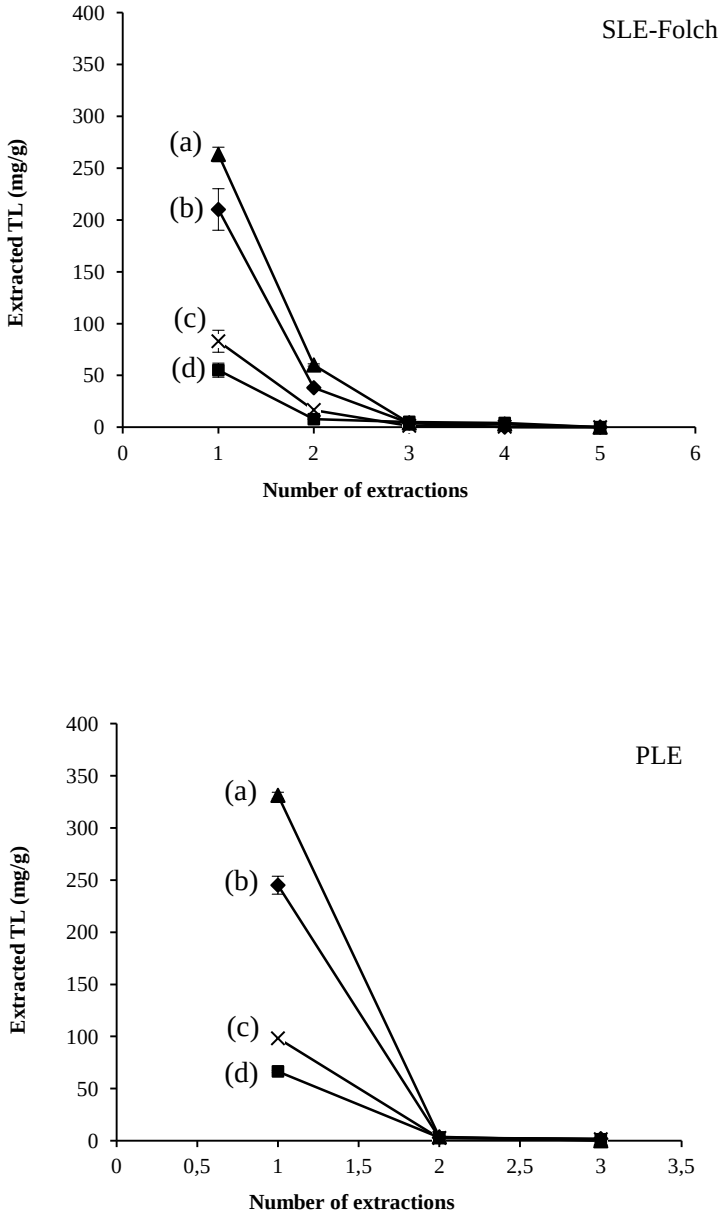


Figure 15: Amounts of TL recovered in successive extractions: a, egg yolk; b, soybean; c, calf brain; d, ox liver.

2.2.2 Total lipid contents in food matrices

Using PLE and SLE-Folch method, the amounts of TL (mg/g of fresh food) recovered from each food matrix was similar (Table 5). Egg yolk contained the highest amount of TL which was in accordance with food composition tables (Table 6), while ox liver and calf brain contained the lowest amounts, but they were higher than values reported in food composition tables. Soybean nevertheless contained about 250-255 mg/g of TL, which was also higher than values reported in food composition tables. The method used for sample preparation, which consisted in

cryogenic grinding probably allowed higher TL recovery for soybean, ox liver and calf brain, the matrices which are very hard to deal with using conventional methods. Because the samples can be mashed as thoroughly as possible by the use of cryogenic grinding, which can increase the extraction efficiency. Moreover, the use of liquid nitrogen can freeze samples rapidly in order that chemical processes in the samples are reduced to a minimum. So lipids in the samples suffered damage were greatly reduced. While the grinding method used in food composition tables was probably tranditional methods. These methods was usually conducted at ambient temperature. As we known, the process of grinding would produce heat, which can lead to the damage of chemical composition. That was the reason why the content of TL obtained by cryogenic grinding was higher than values reported in food composition tables. In order to prove the statement, a test performed with soybean was used by ultraturrax grinder and the result showed that the recovered TLs were less than half of TLs obtained by cryogenic grinding. It demonstrated that cryogenic grinding was indeed more efficent than ultraturrax grinder for sample preparation.

The total amounts of TL could be recovered after 4 successive extractions using the SLE-Folch method (no recovery of TL in the fifth extraction) and 2 extractions using PLE (Table 5). A single extraction however resulted in a TL recovery of 68 to 327 mg/g, and 55 to 263 mg/g by the means of PLE and SLE-Folch method, respectively (Table 5).

Table 5: Total amounts of TL extracted from egg yolk, calf brain, soybean and ox liver[a]

Extraction method	Egg Yolk (mg/g) [b]	Ox Liver (mg/g) [b]	Calf brain (mg/g) [b]	Soybean (mg/g) [b]
PLE (two successive extractions)	331 ± 3	72 ± 4	102 ± 3	255 ± 10
PLE (single extraction)	327 ± 3	68 ± 2	98 ± 3	251 ± 5
SLE- Folch (four successive extractions)	329 ± 7	72 ± 7	101 ± 11	250 ± 21
SLE- Folch (single extraction)	263 ± 4	55 ± 5	83 ± 8	208 ± 20

[a]Results (n = 3) are expressed as mean ± SD. [b]TL contents are expressed in mg/g of fresh food.

Table 6: The amounts of TL reported in food composition tables (Souci et *al*., 1994)

Food matrices	TL (mg/g)
Egg yolk	331
Ox liver	37
Calf brain	76
Soy	180
Krill	34

2.3 Separation of phospholipid classes

The separation of PL classes was performed on the TL extract from each food matrices. The different PL classes presented in food matrices were identified by comparison of their retention times with standards. Soy PL contained PE, PI and PC (Figure 16a) and their proportions were relatively close. Egg yolk PL contained PE, PI, PC and SM, among them, PE and PC were major (Figure 16b). Both ox liver PL (Figure 16c) and calf brain PL simultaneously contained five PL classes. Krill oil PL only contained PE and PC (Figure 16d) and PC was predominant.

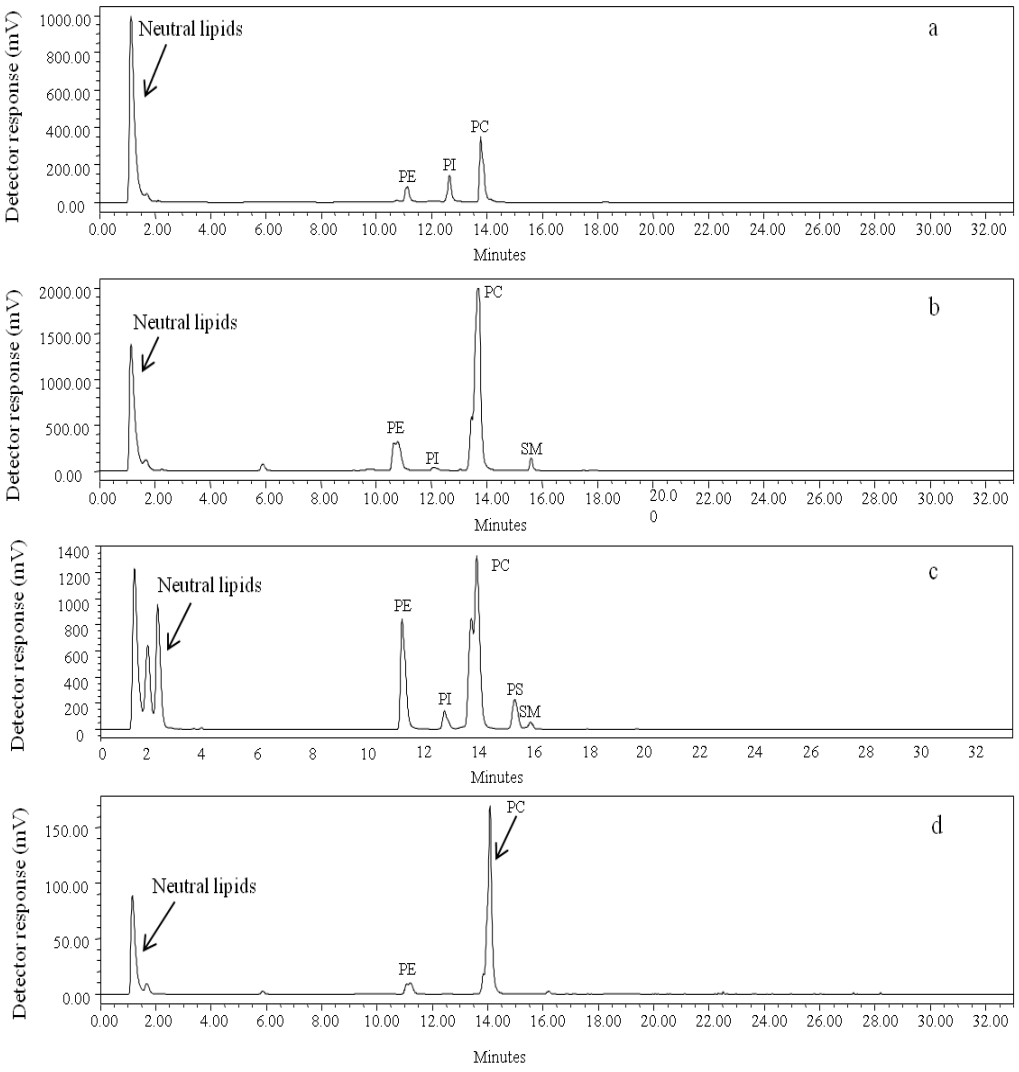


Figure 16: Chromatogram of PLs classes identified in soy (a); egg yolk (b); ox liver (c); krill oil (d). Separation was performed using a Luna normal phase (150 mm x 3 mm, 3 µm) and a linear gradient ranging from $CHCl_3/CH_3OH$ (88/12, v/v) to $CHCl_3/CH_3OH$/1M formic acid adjusted to pH=3 with triethylamine (28/60/12, v/v/v) at a flow rate of 0.5 mL/min. Detection was performed using an ELSD detector.

2.3.1 Extraction numbers (PL)

As shown above, five PL classes (PE, PI, PC, PS and SM) were investigated. Table 7 shows the recovery of TL and PL of each food matrix with a single extraction using PLE and SLE-Folch method. A comparison between the two methods showed that there was a significant difference ($P < 0.05$) in extraction efficiency. With SLE-Folch method, one extraction resulted in the recovery of more than 80% of PE and PC (PE: from 80 to 91%, PC: from 82 to 94%), but not more than 89% of PI, PS and SM (PI: from 71 to 78%, PS: from 76 to 81%, SM: from 81 to 89%). The quantity extracted was then plotted against the number of extractions, which showed that four

successive extractions were necessary to recover all the PL content (Figure 17). However, by PLE, one single extraction was sufficient to extract more than 95% of PL (PE: from 97 to 99%, PI: from 96 to 98%, PC: from 98 to 100%, PS: 95%, SM: 97 to 98%), and two successive extractions could achieve total recovery of the three studied PL classes (Figure 18).

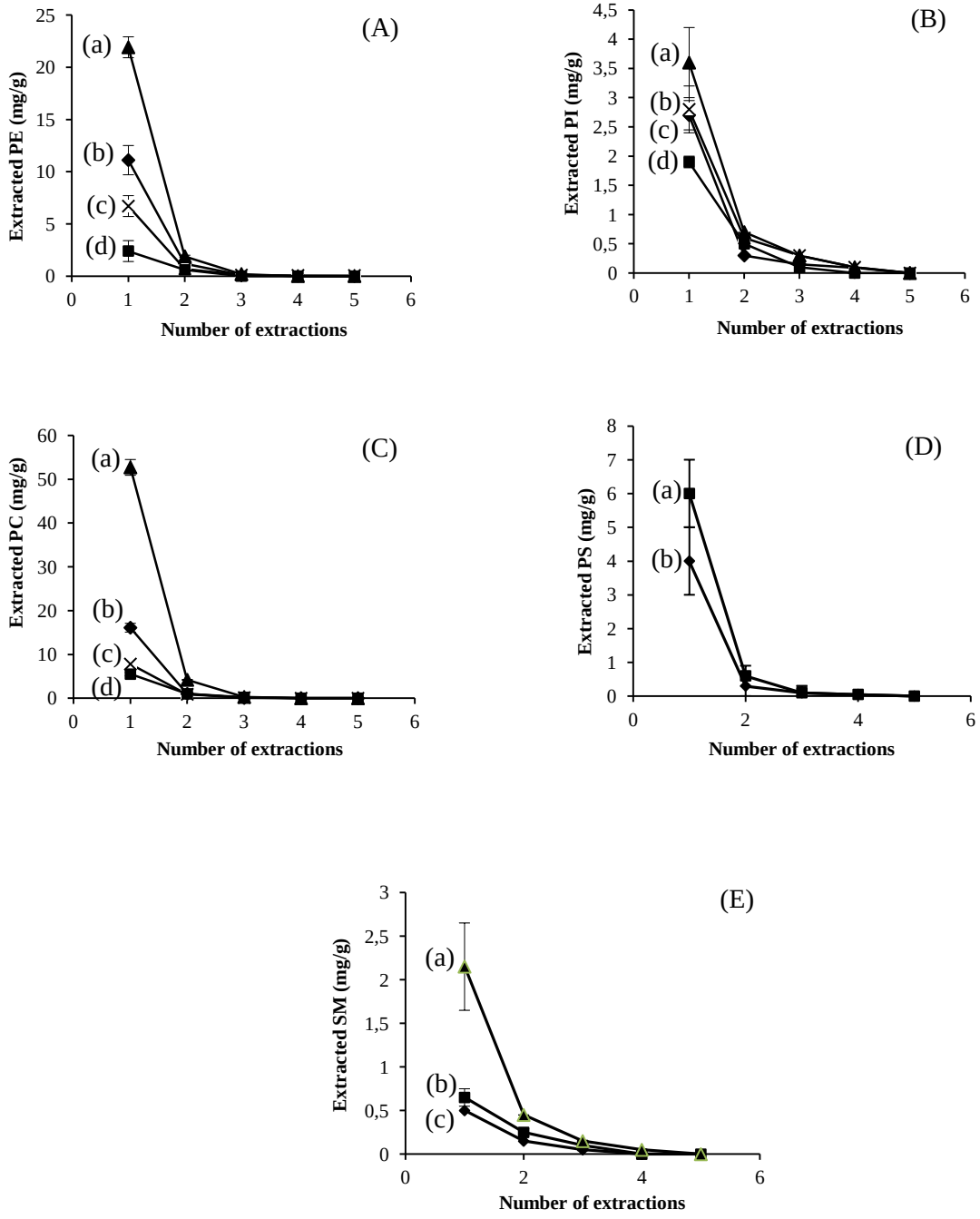


Figure 17: Amounts of PL recovered in successive extractions using SLE-Folch method: (A) PE (a, egg yolk, b, calf brain, c, ox liver, d, soybean), (B) PI (a, soybean, b, calf brain, c, ox liver, d, egg yolk), (C) PC (a, egg yolk, b, ox liver, c, calf brain, d, soybean), (D) PS (a, calf brain, b, ox liver), (E) SM (a, calf brain, b, ox liver, c, egg yolk)

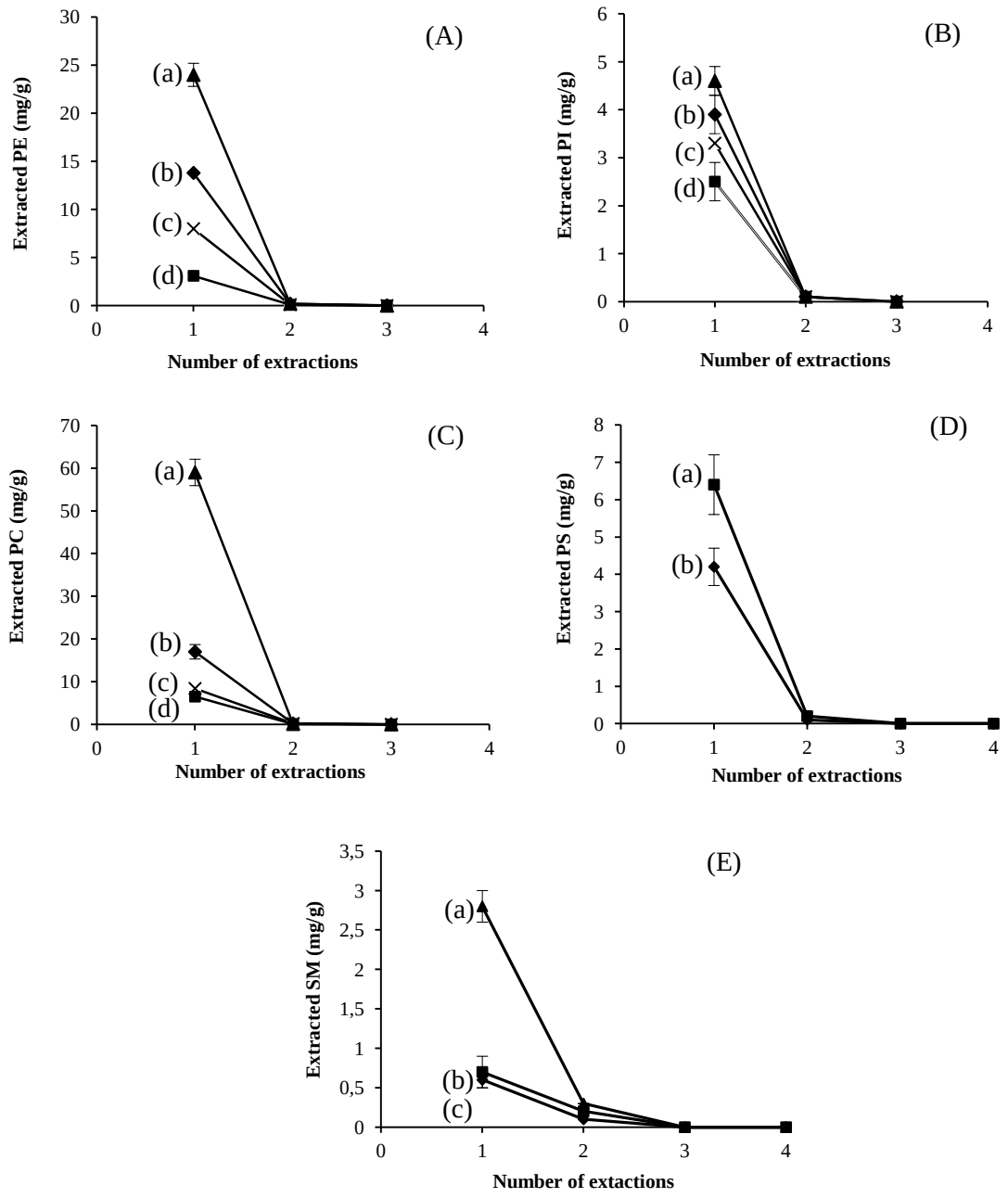


Figure 18: Amounts of PL recovered in successive extractions using PLE method: (A) PE (a, egg yolk; b, calf brain; c, ox liver; d, soybean), (B) PI (a, soybean; b, ox liver; c, calf brain; d, egg yolk), (C) PC (a, egg yolk; b, ox liver; c, calf brain; d, soybean), (D) PS (a, calf brain; b, ox liver), (E) SM (a, calf brain; b, ox liver; c, egg yolk)

Table 7: Recovery rates of a single extraction of TL and PL from egg yolk, calf brain, soybean and ox liver[a]

Food matrix	Method	TL (%)[b]	PE (%)[b]	PI (%)[b]	PC (%)[b]	PS (%)[b]	SM (%)[b]
Egg Yolk	PLE	98.88± 0.02	98.96 ± 0.01	96.4 ± 0.5	99.92 ± 0.02	nd	97.88 ± 0.03
	SLE Folch	80 ± 1	91.3 ± 0.9	75.8 ± 0.9	92 ± 2	nd	88 ± 4
Calf Brain	PLE	96.1 ± 0.2	98.6 ± 0.3	97.33 ±0.06	98 ± 1	95.3 ± 0.2	98 ± 3
	SLE Folch	82.0 ± 0.4	90.3 ± 1.0	78.3 ± 0.8	88 ± 2	81.3 ± 0.6	89 ± 6
Soybean	PLE	98.4 ± 0.6	97.2 ± 1.0	98 ± 1	97.6 ± 0.6	nd	nd
	SLE Folch	83 ± 1	80 ± 1	77 ± 1	81.7 ± 0.8	nd	nd
Ox liver	PLE	94 ± 1	99 ± 1	98 ± 1	99.0 ± 0.8	95± 2	97.3 ± 1.5
	SLE Folch	77 ± 2	88 ± 2	71 ± 2	93.9 ± 0.9	76 ± 3	81± 6

[a]Results (n = 3) are expressed as mean ± SD. [b]Percentage of the recovery of one extraction of TL and PL (mg/g, in fresh food).

2.3.2 Phospholipid contents in food matrices

Five classes of PL were identified in the four food matrices studied, with PC and PE being the major ones and PI, PS and SM being less abundant. The total quantity of these PL classes (mg/g of fresh food) within the error range was almost identical whatever the extraction method used (Table 8). To determine the quantities of PL in the krill oil, it was injected three times directly without prior preparation by the means of PLE, but according to Table 6, the quantities of PL classes in fresh food (Table 8) was done with conversion (1 g krill can be extracted 34 mg krill oil). It is necessary to know that it is only the oil of krill which is consumed and not krill fresh.

Egg yolk was the food matrix which contained the highest quantity of PL (sum of the five PL classes) per gram among the four food matrices, with PC and PE being the main classes. PI and SM were also identified but as a minor class. Similar patterns for egg yolk have also been reported in previous studies (Souci *et al.*, 1994; Kivini *et al.*, 2004; Shahidi, 2006).

As far as soybean is concerned, the total content of PL was higher than contents found in the literature (Shahidi, 2006). It was noteworthy that the proportions between the PL classes were very close. PC was the main PL class, accounted for 45.2%, following PI and PE accounted for 32.2% and 21.9%, respectively. Furthermore, it has been reported that PL composition of soybean depends on seed maturity (Shahidi, 2006). The more mature the seeds are, the higher are the

amounts of PE, PI and PC, compared with other lipids. In this study, the seeds used were intended for human consumption, and therefore highly mature. Unsurprisingly, our results are in agreement with a high maturity pattern of seeds (Shahidi, 2006).

Organ meats, particularly brain and liver, are among the major source (such as meat and milk products) of dietary PL. In terms of PL classes diversity, these two matrices are the most complex among the foods investigated in this study. They contained PE, PI and PC, but also PS and SM. In ox liver, the PL content was about 34 mg/g, with PC (17 mg/g) and PE (8mg/g) being the two predominant classes, followed by PI and SM as minor class (Table 8). No previous data is available for ox liver PL classes in food composition tables. However, when compared to data on pork liver, our results showed a similar pattern (Souci *et al*., 1994). As opposed to the other foods investigated, the most abundant PL class of calf brain was not PC, but PE instead. Calf brain SM content was highest among the food matrices studied. This is consistent with the fact that SM is an important constituent in nervous tissue and mainly exists in the animal brain (Mano *et al*., 1997; Murphy *et al*., 2001; Isaac *et al*., 2003). PS, however, was slightly less concentrated in the calf brain, but at higher levels than in ox liver. This is in agreement with previous studies which showed that the highest levels of PS were found in brain, particularly in myelin or grey matter (Mozzi *et al*., 2003).

Krill oil is an important marine oil and is rich in UFAs, thus it attracts more and more attention currently. According to the PL content of krill oil in Table 8, it showed clearly that krill oil was the matrix richest in PL (317.5 mg/g) and the major class was PC (294.0 mg/g) accounted for 93% of total PL content. The remaining PL class was PE, which was much less than PC, but it can be observed that the PE content of krill oil like egg yolk was relatively high among the five PL sources. And the content of PC was highest, about 5-44 times of other four PC content. Therefore, krill oil was a potential source of PL and could be used as food supplementation in food nutrition.

In summary, it should be noted that the proportion of PL classes in different food matrices are very different. PC is predominant in egg yolk, soybean and ox liver, while in calf brain, PE is predominant, and the content of PI and PS in all the food matrices is relatively low. Our result is consistent with previous studies which demonstrate that PC is usually the major PL, PE is generally the second most abundant PL in food matrices (Pérez-Palacios *et al*., 2008; Avalli and Contarini, 2005).

Table 8: Total amounts of extracted PL classes from food matrices in mg/g of fresh food[a]

Food matrix	Extraction method	PE (mg/g) [b]	PI (mg/g) [b]	PC (mg/g) [b]	PS (mg/g) [b]	SM (mg/g) [b]	Total PL(mg/g) [b]
Egg Yolk	PLE	24 ± 1	2.6 ± 0.4	59 ± 3	nd	0.7 ± 0.2	86.3 ± 0.2
	SLE-Folch	24 ± 1	2.6 ± 0.1	57 ± 2	nd	0.7 ± 0.3	84.3 ± 0.1
Calf Brain	PLE	14.0 ± 0.1	3.4 ± 0.1	8.6 ± 0.2	6.6 ± 0.3	3.1 ± 0.1	35.7 ± 1.4
	SLE-Folch	13 ± 2	3.3 ± 0.3	9 ± 1	6.4 ± 1.8	2.8 ± 0.3	34.5 ± 0.5
Soybean	PLE	3.2 ± 0.3	4.7 ± 0.5	6.6 ± 0.5	nd	nd	14.5 ± 0.3
	SLE-Folch	3 ± 1	4.8 ± 0.6	6.7 ± 0.5	nd	nd	14.5 ± 1.1
Ox liver	PLE	8.1 ± 0.4	3.8 ± 0.4	17 ± 2	4.3 ± 0.3	1.0 ± 0.5	34.2 ± 1.0
	SLE-Folch	8 ± 1	3.8 ± 0.4	17 ± 1	4.3 ± 0.6	0.9 ± 0.3	34.0 ± 1.1
Krill oil	-	23.5 ± 1.2	nd	294.0 ± 1.8	nd	nd	317.5 ± 0.3
Krill (calculated)	-	≈8	nd	≈100	nd	nd	≈108

[a]Results (n = 3) are expressed as mean ± SD. [b]total quantity of PE, PI and PC. nd: not detected.

As far as the PL content of egg yolk in food composition tables (Souci *et al*., 1994) was concerned, the values of PC, PE, PI and SM were about 67.9 mg/g, 19.3 mg/g, 0.64 mg/g and 4.88 mg/g, respectively. The content of PC and SM were higher than that in our results, while the content PE and PI, especially PI, were much lower than our results. In fact, the difference in the PL composition between the results of our study and food composition tables could be explained, because it is well established that PL composition depends on factors such as animal species, their diet, and rearing conditions (Wang *et al*., 2009).

The study of this part showed that, compared to SLE-Folch method, a single extraction by PLE allowed a recovery of more than 94% of TL and 95% of each PL class. Two successive extractions could achieve a total recovery of the five studied PL classes. It demonstrated that PLE method is more efficient than SLE-Folch method. That can be explained by the principle of extraction. PLE method uses high temperature, which enables high solubility and high diffusion rate of lipid solutes in the solvent, and the high pressure, which enables a high penetration of the solvent in the sample. This process can improve the contact surface between the matrix and solvent greatly. Thus, PLE permits high extraction efficiency. However, as for SLE-Folch method, samples are mixed with solvents weakly, because it lacks external forces to improve solvent access to interior components. So TL couldn't be completely integrated into the solvent

during one or two extractions, that is the reason why SLE-Folch method is less efficient than PLE method and it needs more extractions than PLE method.

Moreover, PLE method followed the principle of green technology. The application of green technology aims to preserve the natural environment and its resource, and to limit the negative influence of human involvement. The philosophy of green chemistry is to develop and encourage the utilization of procedures that reduce and/or eliminate the use or production of hazardous substances. A basic principle of green chemistry is to reduce the use of toxic and pollutant organic solvents, and to facilitate and encourage the use of novel extraction techniques that are known to be more environmental friendly. Traditional extraction techniques usually require long extraction times, and large amounts of samples and organic solvents, which has negative environmental impact and health risks. PLE, as a green technology for the extraction of nutraceuticals has received particular attention recently. The increasing interest is mainly due to the fact that PLE is automated with reduced extraction time and solvent consumption and its set-up suits analytes that are oxygen and light sensitive. In our study, the volume of solvent and the extraction time necessary to extract all the TL content were respectively 44 mL, 15 min for the PLE method, and 175 mL, 150 min for SFE-Folch method. Therefore, the results show that PLE is a efficient method for TL and PL extraction and it will be used for further study in this thesis.

Part B: Determination of molecular species of PE and SM

The aim of this part is to determine molecular species of PE and SM in various food matrices (ox liver, soybean, calf brain, egg yolk and krill oil). Firstly, a series of methods, such as PLE, adsorption column chromatography, semi-preparative and analytical chromatographies, were described to purify PE and SM from different food matrices. PLE was used for the extraction of TL from these food matrices, adsorption column chromatrography was used for the isolation of PL from TL, and NP-semi-preprative HPLC was used for the separation of PL classes and collection. Finally, the purity of each PL classes was checked by analytical chromatography. Secondly, RP-HPLC coupled with ESI was developed to separate and identify molecular species of PE and SM from each food matrix.

1. Material and methods

1.1 Material

All solvents used for the liquid chromatography analyses were of HPLC-grade. Chloroform was obtained from VWR (Strasbourg, France) and methanol was obtained from Carlo Erba (Val de Reuil, France). Chloroform (Sigma-Aldrich, Steinheim, Germany) and methanol used for lipid extraction and SM purification were of analytical grade.

Hen's eggs, calf brain, soybeans and ox liver were freshly purchased at a local retailer. Krill oil has kindly provided by Nestec SA (Lausanne, Switzerland).

Different standards were used to identify the families of PL existing in these food matrices, such as PE, PC, (d18:1-18:1)SM and SM, were purchased from Sigma-Aldrich (St. Louis, MO, USA). PI and PS were purchased from Avanti Polar Lipids (Alabaster, AL).

1.2 Sample preparation

The food matrices investigated could be categorized into three groups according to their fat content: a low-fat group with ox liver and calf brain (< 10%, w/w of fresh food), an intermediate group with soybean (10-30%, w/w of fresh food) and a high-fat group with egg yolk (> 30%, w/w of fresh food), while krill oil is a typical marine oil.

Soy, ox liver and calf brain were ground under cryogenic conditions (3 steps of 5 min each) using liquid nitrogen and a 6870 Freezer/Mill (Spex CertiPrep, Stanmore, U.K.). The obtained frozen powder was freeze-dried (Alpha 2-4, Bioblock Scientific, Illkirch, France). Egg yolk powder was obtained by removing egg white from fresh eggs, homogenizing and freeze-drying. Krill oil was used for PL extraction and analysis without any preparation since it is already the lipid fraction. All food samples were stored at - 20 °C until use.

1.3 Extraction of total lipid by pressurized liquid extraction

PLE was performed as previously published (Macnaughton *et al.*, 1997) in a Dionex PLE 350 (Dionex, Sunnyvale, CA, USA) extractor using $CHCl_3$/CH_3OH (2:1, v/v) for each extraction. Two grams of each sample powder, was mixed homogenously with sand so as to fill the 10 mL stainless steel extraction cell (L × Ø: 52 mm × 15 mm). The use of a dispersion agent is intended to enhance solute exchange between phases and reduce the solvent volume used by filling the void volume in the cell. Single extraction was applied to extract TL of each sample. The extraction temperature, time and pressure were set at 140 °C, 5 min and 10.34 MPa, respectively according to Zhou *et al.* (2010). The mobile phase containing the lipid fraction was collected, evaporated under vacuum using a rotary evaporator (40 °C, 300 kPa) and dried under a gentle stream of N_2.

1.4 Purification of PE and SM

1.4.1 Adsorption column chromatography

TL was separated into NL and PL using silica cartridges as described by Hernàndez *et al.* (1999). A 150 mg aliquot of the TL chloroformic extract was subjected to preparative chromatography on a silica gel glass column (35 cm × 2 cm i.d., 15 g of Si 60 silica gel, particle size 40-63 μm, Geduran, Merck) preconditioned with chloroform. Elution was performed at a flow rate of 4 mL/min, first using 250 mL of chloroform for removing NLs, such as TAGs and carotenoids, then with 200 mL methanol/1 M aqueous formic acid (adjusted to pH = 3 with triethylamine) (98:2, v/v) mixture for eluting PLs (Figure 19). The column was then washed with 50 mL of methanol. The organic solvents in the fractions of interest were removed by a rotary evaporator under vacuum using a rotary evaporator (40 °C, 300 kPa) and dried under a gentle stream of N_2.

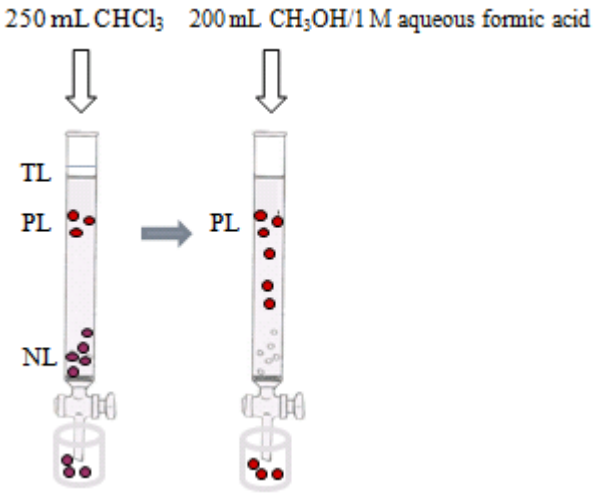


Figure 19: Schematic representation of the separation of PL from the examined samples

1.4.2 Semi-preparative and analytical chromatographies

PL classes were separated by semi-prepative chromatographic system which was made of a 616 controller, a 717 Plus autosampler and a 2424 ELSD detector (Waters, Saint-Quentin Fallavier, France). High purity nitrogen at a pressure of 310 kPa from a nitrogen generator (Domnik Hunter, Villefranche sur-Saône, France) was used as a nebulizing gas for the ELSD detector. The drift tube temperature was set at 45 °C (Le Grandois *et al*., 2009). Semi-preparative separation was achieved using a silica column (Uptisphere, 250 mm × 10 mm, 5 μm, Interchim, Montluçon, France). The mobile phase was made of chloroform/methanol (88:12, v/v) (solvent A) and methanol/1 M aqueous formic acid (adjusted to pH = 3 with triethylamine) (82.4:17.6, v/v) (solvent B). The gradient started at 100% A, then decreased to 80% A in 8 min, then to 60% A in 2 min and held for 3 min, and then decreased to 40% A (within 5 min). Finally, solvent A was increased to 100% in 2 min and delivered isocratically for 10 min. The flow rate was 4 mL/min. A split system was used ahead of the ELSD inlet, allowing 97.5% of the flow (3.9 mL/min) diverted to the collector, and 2.5% (0.1 mL/min) to the ELSD detector. The autosampler was set to inject 50 µL purified PLs solution in chloroform (about 200 mg/mL). PE and SM were identified in comparison of their retention time to the standard one, and collected.

The purity of collected PE and SM from different matrices was checked by the analytical HPLC which used a silica column (150 mm × 3 mm, 3 µm, Luna, Phenomenex, Le Pecq, France). The flow rate of mobile phase was 0.5 mL/min and separation was performed by using a 20 min

linear gradient ranging from chloroform/methanol (88:12, v/v) to chloroform/methanol/1 M aqueous formic acid (adjusted to pH = 3 with triethylamine) (28:60:12, v/v/v). Purity (*P*) of each collected PL class was calculated as follows:

$$P = \frac{A_t}{A_{t^\circ}} \times 100$$

Where A_t is peak area of each PL class and A_{t° is the total of peak areas on the chromatogram.

1.5 Separation and identification of PE and SM molecular species

Molecular species of PE and SM were determined using a Prostar HPLC system made of two 210 solvent delivery modules, a 410 autosampler, and a 1200 L triple quadrupole mass spectrometer fitted with an ESI source (Varian, Les Ulis, France). High-purity nitrogen (Domnik Hunter) was used as a nebulising gas, set at 317 kpa, and as a drying gas, set at 250 °C. Separation was performed on a Nucleodur C18HTec (250 mm × 4.6 mm, 3 μm, Macherey-Nagel, Duren, Allemagne) using an isocratic elution of 100% methanol containing 5 mM ammonium formate at a rate of 1 mL/min. A split system allowed the HPLC effluent to enter the mass spectrometer at a flow rate of 0.2 mL/min and the injection volume was performed in positive mode in a mass range between *m/z* 400 and 900. The mass range was set between *m/z* 200 and 800 for fragmentation products. The dwell time was set to 0.2 s for each *m/z*. Argon was used as a collision gas, and collision energy was set to 30 V. The percentage of each molecular substance was calculated as follows:

$$\% = \frac{A[\text{peak } (m/z)]}{\Sigma A(\text{peaks})} \times 100$$

Where A [peak (*m/z*)] refers to the peak area of selected *m/z* and ΣA (peaks) refers to the sum of all peak areas, each measured at its specific *m/z*. Analyses were performed in triplicate, with results expressed as mean ± standard deviation (SD).

1.6 GC analysis of fatty acid of the fraction *m/z* 750.7 (krill oil PE)

A 3400 Varian gas chromatograph equipped with a flamme ionization detector was used for the analysis of FA methyl esters (FAMEs) after transesterification of purified *m/z* 750.7 PE [fraction collected by analytical RP-HPLC according to its retention time (17.2 min)]. Separation

was made by a CP Sil 88 column [(88% cyanopropyl)-arylpolysiloxane, 100 m × 0.25 mm, 0.2 µm, Varian]. Samples (1 µL) were injected. Injector was set to 250 °C, detector was set to 270 °C and the column was set at 60°C, held for 5 min, and raised to 165 °C (rate of 15 °C/min). The temperature of 165 °C was held for 1 min and then raised to 225 °C (rate of 2 °C/min), the final temperature was maintained for 29 min. Then, the oven temperature was allowed to cool down within approximately 3 min and to equilibrate during 2 min before the next injection. Peaks were identified by a comparison to standards (FAME mix C4-C24, Sigma-Aldrich, Saint-Quentin-Fallavier, France). Purified *m/z* 750.7 krill oil PE (1 mg) was transesterified using 100 µL methanolic KOH (2M) by vortexing for 2 min at room temperature (Figure 20). Then, FAMEs were extracted using 200 µL n-heptane.

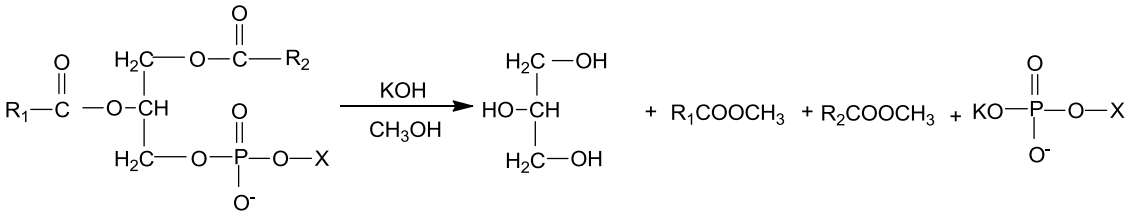


Figure 20: Hydrolysis scheme of GPL

1.7 High resolution mass spectrometry (HRMS) for identification of the molecular structure of MH^+ (*m/z* 750.7 krill oil PE)

Confirmation analysis was performed using a high resolution mass spectrometer (HRMS) (Agilent Technologies, Massy, France) [G1312B binary pump, G1367C AutoSampler, DAD SL G1315C detector, MS 6520 Accurate Mass Q-TOF]. A nebulising gas was set at 286 kPa. Chromatographic separation was carried out on a Thermo Hypersil Gold C8 analytical column (100 x 1.0 mm; 1.9 µm particle size). Mobile phase consisted of aqueous formic acid (0.05%) and ACN, at a flow rate of 0.1 mL/min. Gradient elution started at 100% aqueous formic acid maintained for 2 min, followed by raising the ACN concentration linearly to 95% within 6 min. ACN concentration was maintained for 6.5 min. Subsequently, the ACN concentration decreased to 0% in 0.5 min. A sample (0.4 µL) was injected and eluted with a flow rate of 0.5 mL/min. The detection was performed in positive mode and the mass range for fragmentation products was set between *m/z* 50 and 1700.

2 Result and discussion

2.1Separation of phospholipids and purification of PE and SM

As discussed in Part A previously, PL classes can be separated by selecting appropriate solvent and separation methods. NLs account for a large amount in TL, much more than PL. However, high NL contents would affect ionization in ESI sources and desolvation of the liquid chromatographic effluent droplets because MS signals and responses often undergo significant alteration when complex matrices are analyzed and when the analyses are performed without adequate sample preparation (Pucci *et al*., 2009). Thus, it is advisable to remove NL from TL by SPE using non-polar solvent (chloroform). PL was eluted by the mixture methanol/1 M aqueous formic acid (adjusted to pH = 3 with triethylamine) (98:2, v/v) and their purity was checked by analytical NP-HPLC-ELSD. PE and SM were collected by comparison of retention times with those of standards. Figure 21 shows the scheme followed for the purification of PE and SM from the examined samples.

RP-semi-preparative chromatograms of PL classes of soy, egg yolk, krill oil and ox liver were shown in Figure 22. The number of classes identified varied depending on the food considered. Three (PE, PI, and PC) in soy (Figure 22a), four classes (PE, PI, PC, and SM) were found in egg yolk (Figure 22b), two (PE and PC) in krill oil (Figure 22c). In ox liver and calf brain, five classes were identified, with PC and PE being the major ones and PI, PS and SM being less abundant (Figure 22d). And upscaling the HPLC to the semi-preparative level did not seem to reduce the quality of the separation of PL classes. This enabled adequate amounts of the fractions containing PE and SM to be collected. Figure 23 shows the analytical chromatogram of egg yolk PE (Figure 23a) and SM (Figure 23b) obtained after purification showing a sufficient purify of the classes to be used for the determination of their molecular content. For each food sample investigated, the purity of the collected PE and SM fraction, monitored by analytical HPLC-ELSD, was shown to be at least 95%.

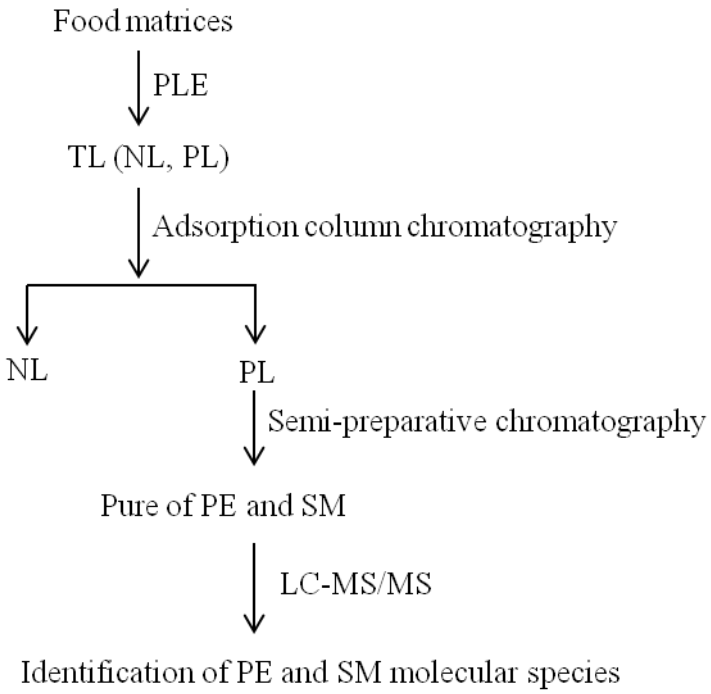


Figure 21: Flow chart for the purification of PE and SM from the examined samples and the determination of PE and SM molecular species.

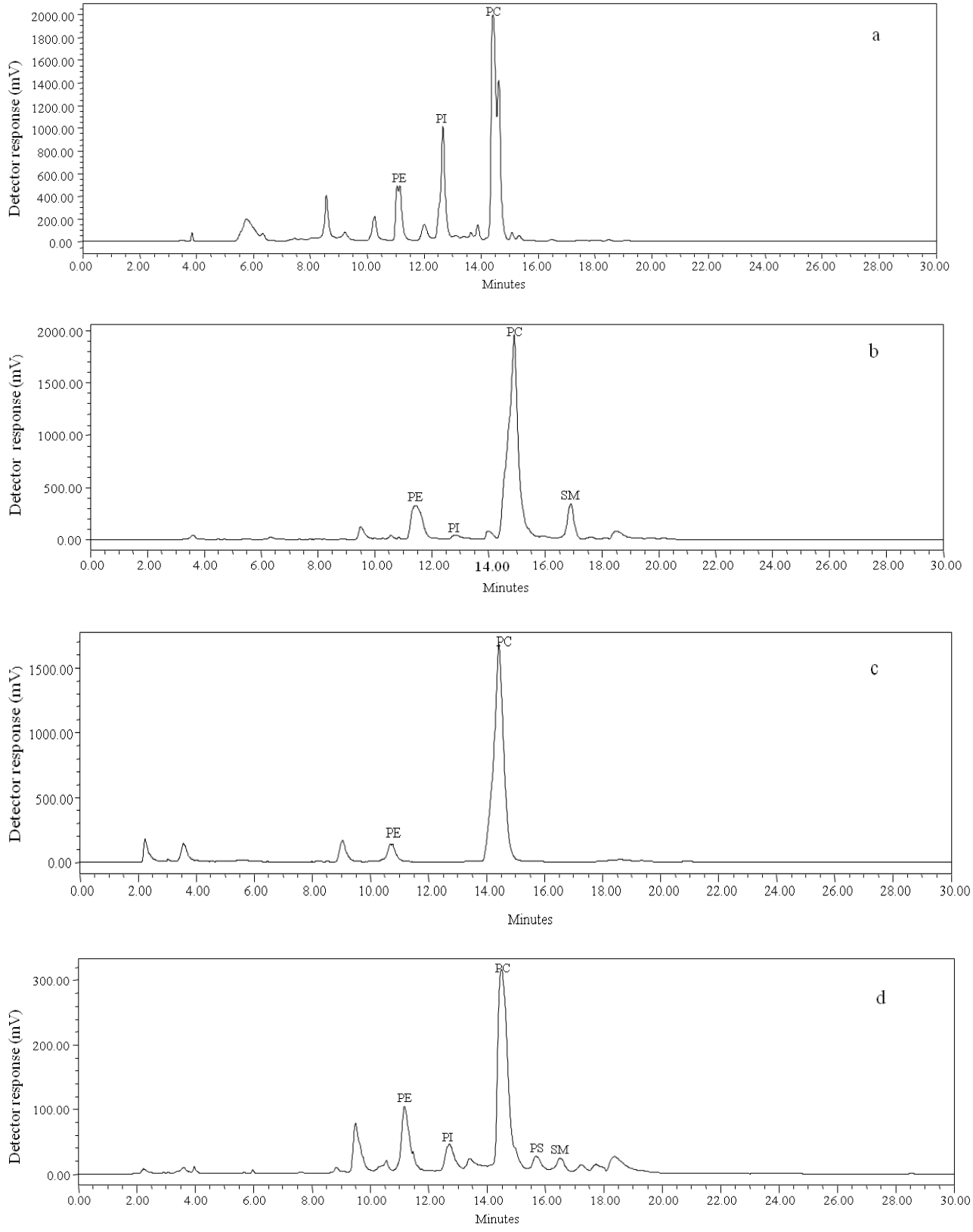


Figure 22: Chromatogram of purified PL classes identified in soy (a), egg yolk (b), krill oil (c) and ox liver (d). Separation was performed using a semi-preparative silica column (250 mm × 10 mm, 5 μm). The mobile phase was a gradient of solvent A [CH_3OH/1 M aqueous formic acid (adjusted to pH = 3 with triethylamine) (82.4/17.6)], and solvent B [$CHCl_3$/CH_3OH (88/12, v/v)] at a flow of 4.0 mL/min. Detection was performed using an ELSD detector.

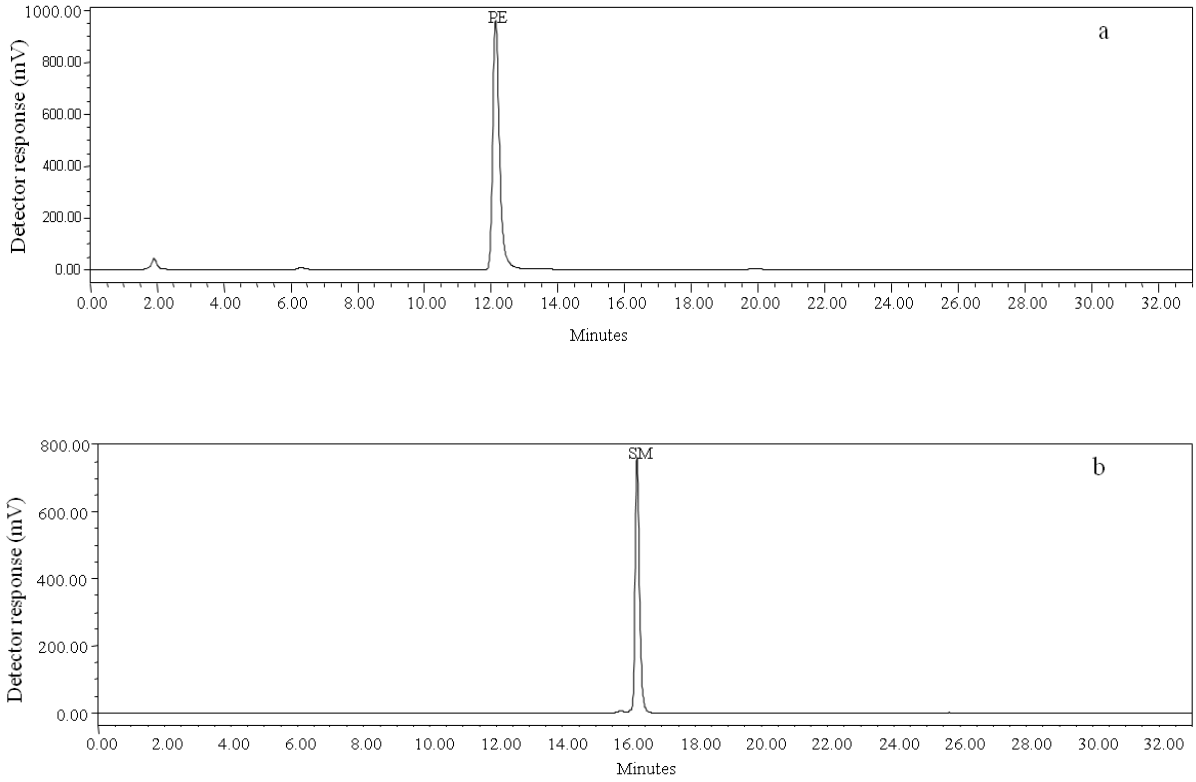


Figure 23: Chromatogram of PL classes identified in egg yolk extract. Separation was performed using a Luna normal phase (150 mm × 3 mm, 3 µm) and a linear gradient ranging from $CHCl_3/CH_3OH$ (88/12, v/v) to $CHCl_3/CH_3OH$/1 M formic acid adjusted to pH = 3 with triethylamine (28/60/12, v/v/v) at a flow rate of 0.5 mL/min. Detection was performed using an ELS detector.

2.2 PE molecular species

2.2.1 Separation chromatography

Four columns, RP-Nucleodur sphinx (3.0 mm × 150 mm, 3 μm,), RP-C8 Lichrospher RP Select B (Interchim, Montluçon, France), C18 Kinetex (150 mm × 2.1 mm, 2.6 μm, Phenomenex, Le pecq, France), and Nucleodur C18HTec (250 mm × 4.6 mm, 3 μm, Macherey-Nagel, Duren, Allemagne) were tested for the separation of molecular species. The results showed that the separation on the first three columns were not very resolutive enough, either the time of separation was long, or the peaks couldn't be separated completely. However, the separation performed on the Nucleodur C18HTec was better due to the best resolution of peaks. That was the reason why we chose this column for the separation of molecular species.

As far as the mobile phase was concerned, we tried different solvent mixtures, eg, methanol mixed with H_2O, formic acid, ammonium formate, final results showed that 100% CH_3OH containing 5 mM ammonium formate was most suitable for the separation.

PE in five food matrices (soy, egg yolk, ox liver, kill oil and calf brain) was investigated. Figure 24 shows TIC chromatogram for the PE molecular species of soy, egg yolk, ox liver, kill oil and calf brain. As for the PE species of calf brain (Figure 24E), we will discuss it latter. Soy PE (Figure 24A) was of simpler composition than egg yolk PE (Figure 24B), ox liver PE (Figure 24C), and krill oil PE (Figure 24D). By comparing the retention time, some peaks were present in different sources of PE, but their percentages were not the same. And it showed that representative species in different sources of PE were different. Moreover, it is noteworthy that the animal sources of PE were more complicated than plant sources of PE. Especially, ox liver PE, individual peaks were not completely separated, but their quantitative assesment can be carried out by SIM mode. Soy PE contained only six molecular species. Figure 25 shows the extracted individual ion chromatograms of them carried out by SIM mode. Method of identification will be explained in the 2.2.2.

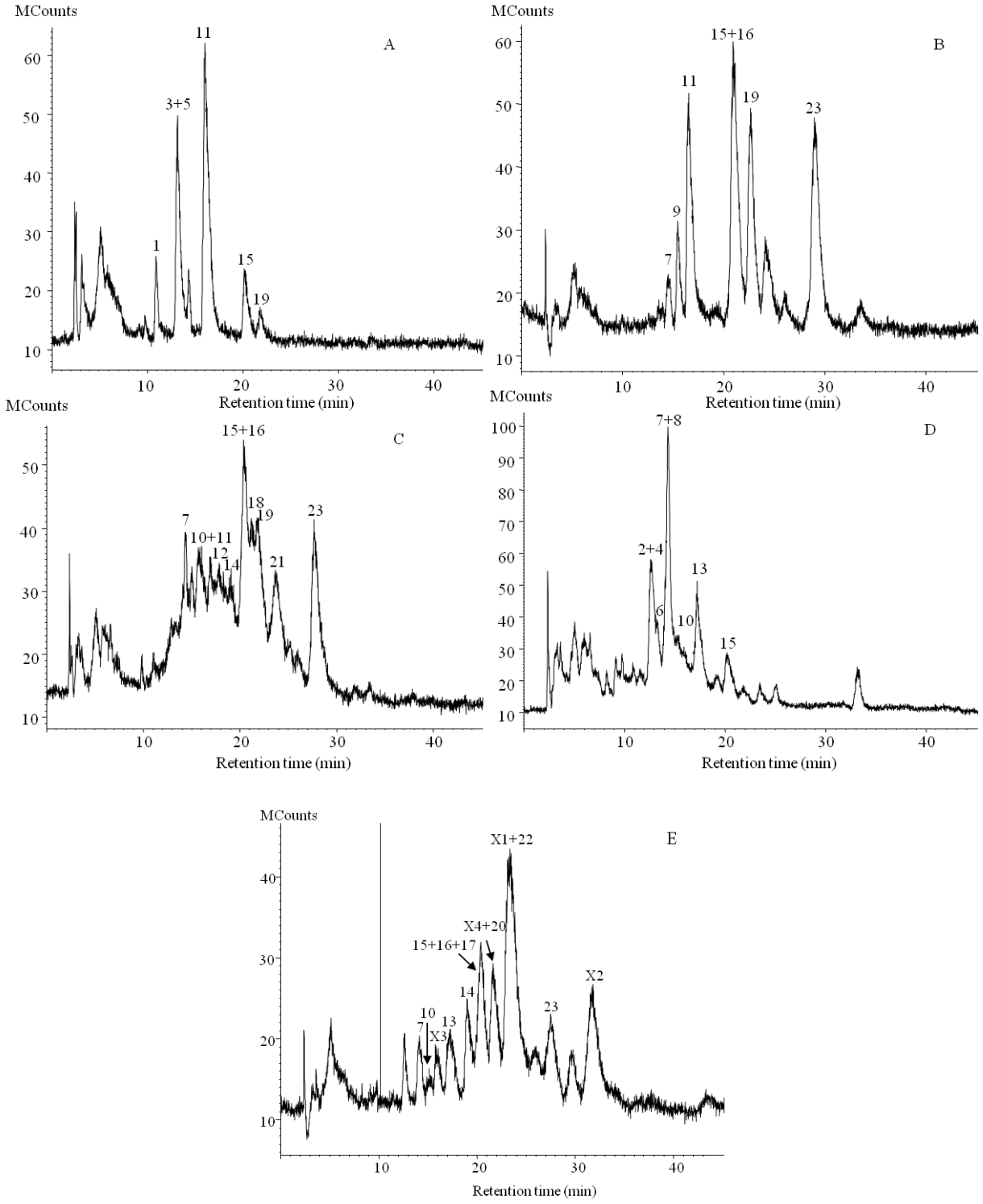


Figure 24: Chromatograms of purified PE: (A) soy, (B) egg yolk, (C) ox liver, (D) krill oil, (E) calf brain using LC-ESI-MS. Separation was performed using isocratic conditions: CH_3OH containing 5 mM ammonium formate at a rate of 1 mL/min on a Nucleodur, C18HTec (250 mm × 4.6 mm, 3 μm). 1, (C18:2-C18:3)PE; 2, (C16:0-C20:5)PE; 3, (C18:2-C18:2)PE; 4, (C18:1-C20:5)PE; 5, (C16:0-C18:3)PE; 6, (C18:1-C20:4)PE; 7, (C16:0-C22:6)PE; 8, (C18:1-C22:6)PE; 9, (C16:0-C20:4)PE; 10, (C16:0-C22:5)PE; 11, (C16:0-C18:2)PE; 12, (C18:0-C18:3)PE; 13, [C16:0(akyl)-C22:6(acyl)]PakE; 14, (C18:0-C22:6)PE; 15, (C16:0-C18:1)PE; 16, (C18:0-C20:4)PE; 17, (C18:1-C18:1)PE; 18, (C18:0-C22:5)PE; 19, (C18:0-C18:2)PE; 20, (C19:1-C20:5)PE; 21, (C18:0-C20:3)PE; 22, (C15:1-C20:2)PE; 23, (C18:0-C18:1)PE.
X1, *m/z* 702.7 ; X2, *m/z* 730.7 ; X3, *m/z* 748.7 ; X4, *m/z* 752.7.

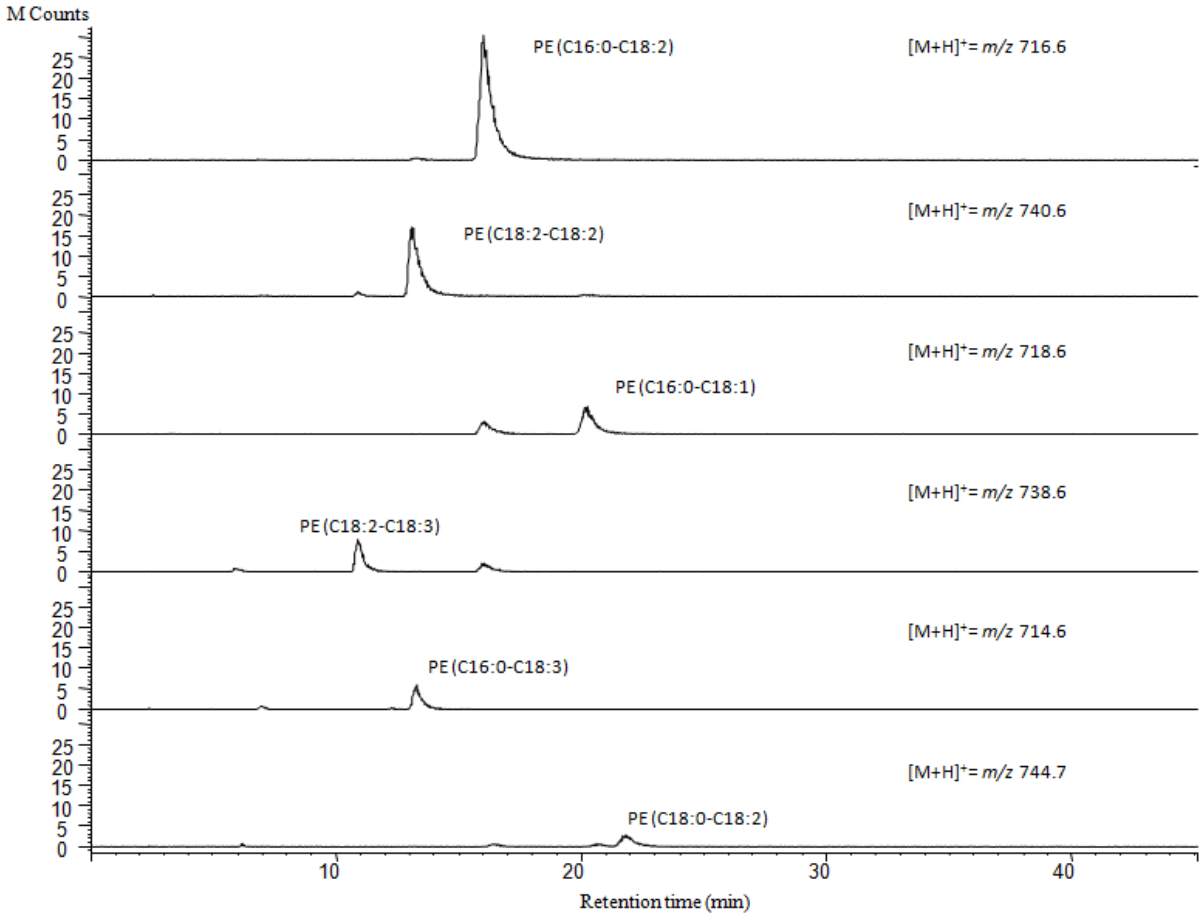


Figure 25: Extracted individual ion chromatograms for soy PE showing the following species: C16:0-C18:2; C18:2-C18:2; C16:0-C18:1; C18:2-C18:3; C16:0-C18:3; C18:0-C18:2. Separation was performed using isocratic conditions: CH_3OH containing 5 mM ammonium formate at a rate of 1 mL/min onto a Nucleodur C18HTec (250 mm × 4.6 mm, 3 μm).

2.2.2 Identification

Separation and identification of PE molecular species were performed by RP-HPLC coupled ESI-MS^2. On the basis of different chain lengths and the degree of unsaturation of the acyl chain, RP-HPLC can separate different molecular species within one PL class. To avoid the use of complex mixtures or highly toxic solvents, isocratic elution with methanol, containing 5 mmol/L ammonium formate was used as the mobile phase. This method is very simple and can separate PE molecular species rapidly.

Positive-ion and negative-ion modes were both tested for optimization of the MS signal. The result of test showed that full-scan mass spectra and MS-MS spectra were acquired to obtain the maximum number of available transitions for each compound in positive-ion mode. Thus, we chose positive-ion mode to identify molecular species.

Under the MS^2 conditions used, $[M+H]^+$ was fragmented in the collision cell to produce five main fragments:

- $[M+H-141]^+$, a specific fragment corresponding to the loss of the polar headgroup $[NH_3(CH_2)_2OPO_3H]^+$ and enabling distinction between PE and other PLs;
- two acyl groups $[RCO]^+$ corresponding to the detached FAs;
- two $[M+H-141-RCO]^+$, resulting from simultaneous loss of both the polar headgroup and one of the two acyl groups, as previously reported (Pacetti *et al*., 2007; Wang *et al*., 2009).

The last two fragments gave an extra proof of the identity of the two FAs. The fragmentation patterns obtained proved in fact to be effective in identifying various PE species, including symmetrical, asymmetrical, saturated and unsaturated ones.

A representative MS^2 fragmentation spectrum of the C16:0-C18:1 molecular species of PE from egg yolk (*m/z* 718.6, RT = 19.6 min) is presented in Figure 26. A major fragment ion corresponding to loss of the polar headgroup ($[M+H-NH_3(CH_2)_2OPO_3H]^+$) was obtained at *m/z* 577.5. Two other fragment ions corresponded to the simultaneous loss of both the polar headgroup and either an oleic acyl group (*m/z* 312.9) or a palmitic acyl group (*m/z* 339.2). Two final fragment ions at *m/z* 238.8 and *m/z* 265.1 corresponded to detached palmic and oleic acyl groups, respectively. This approach was applied to each *m/z* detected from the purified PE samples and enabled identification of the constitutive PE molecular species for each food investigated.

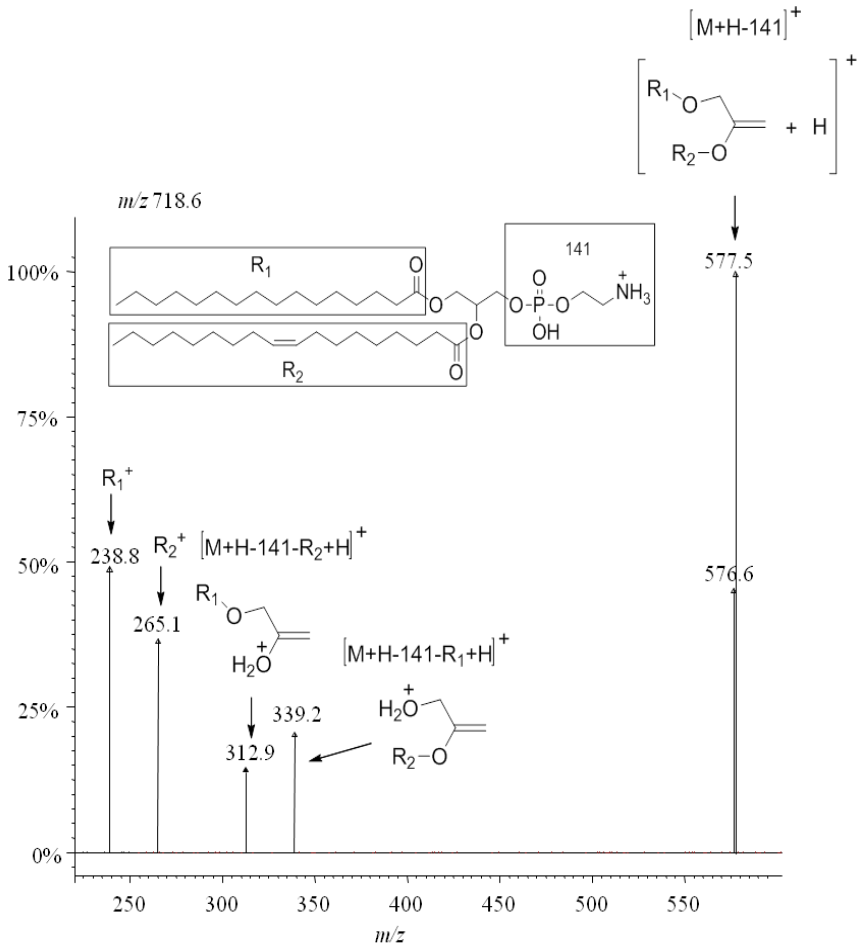


Figure 26: Product ions obtained in positive mode in ESI-MS^2 between *m/z* 200 and 600 from fragmentation of *m/z* 718.6 of egg yolk. $R_1 = C_{16}H_{31}O$; $R_2 = C_{18}H_{33}O$.

2.2.3 Profiles of PE molecular species

Table 9 gives the relative distributions of the various PE molecular species in the foods investigated. The relative intensity of MS^2 PE fragments is given in Table 10. The major soy PE species were (C16:0-C18:2)PE (47.0%), followed by (C18:2-C18:2)PE (24.0%) and (C16:0-C18:1)PE (10.4%), as reported for other oils, for example as corn oil (Harrabi *et al.*, 2009) and flaxseed oil (Herchi *et al.*, 2011). Minor species included (C18:2-C18:3)PE (8.0%), (C16:0-C18:3)PE (6.4%) and (C18:0-C18:2)PE (4.1%). As far as we are aware, this is the first report of systematic identification and the relative contents of individual molecular species of soy PE. Data available on soy PLs species is, in fact, focused on PC (Wang *et al.*, 1999; Bang *et al.*, 2006; Bang *et al.*, 2007; Dobson and Deighton, 2001).

Table 9: Molecular species profiles of purified PE[a] from each food matrix

m/z [M+H]$^+$	RT (min)	Structure [b]	(%)[c]				
			Soy PE	Egg yolk PE	Ox liver PE	Krill oil PE	Calf brain PE
702.7	22.6	X1	nd	nd	nd	nd	15.1± 0.1
714.6	12.9	C16:0-C18:3	6.4 ± 0.1	nd [d]	nd	nd	nd
716.6	16.0	C16:0-C18:2	47.0 ± 0.5	16.7± 0.3	3.5 ± 0.1	nd	nd
718.6	19.6	C16:0-C18:1	10.4 ± 0.3	26.6 ± 0.3	5.7 ± 0.1	9.8 ± 0.1	4.3± 0.3
728.7	23.2	C15:1-C20:2	nd	nd	nd	nd	19.8± 0.4
730.7	31.3	X2	nd	nd	nd	nd	15.4± 0.1
738.6	10.6	C18:2-C18:3	8.0 ± 0.1	nd	nd	nd	nd
	12.5	C16:0-C20:5	nd	nd	nd	17.6 ± 0.4	nd
740.6	12.7	C18:2-C18:2	24.0 ± 0.2	nd	nd	nd	nd
	14.6	C16:0-C20:4	nd	4.9 ± 0.1	nd	nd	nd
742.7	17.2	C18:0-C18:3	nd	nd	1.6 ± 0.1	nd	nd
744.7	20.2	C18:1-C18:1	nd	nd	nd	nd	4.9±0.2
	21.0	C18:0-C18:2	4.1± 0.1	20.0 ± 0.3	15.2 ± 0.2	nd	nd
746.7	26.7	C18:0-C18:1	nd	26.5 ± 0.5	24.7 ± 0.2	nd	6.4± 0.1
748.7	15.7	X3	nd	nd	nd	nd	2.9± 0.3
750.7	17.2	C16:0(alkyl)-C22:6 (acyl)PakE	nd	nd	nd	22.4 ± 0.3	3.9± 0.1
752.7	21.5	X4	nd	nd	nd	nd	7.4± 0.3
764.6	12.8	C18:1-C20:5	nd	nd	nd	13.1 ± 0.3	nd
	13.9	C16:0-C22:6	nd	2.2 ± 0.1	4.0 ± 0.1	18.1 ± 0.4	2.7± 0.1
766.7	13.3	C18:1-C20:4	nd	nd	nd	2.2 ± 0.1	nd
	15.6	C16:0-C22:5	nd	nd	6.6 ± 0.2	2.8 ± 0.1	1.1
768.7	19.7	C18:0-C20:4	nd	3.1 ± 0.1	19.6 ± 0.4	nd	6.7± 0.1
770.7	22.8	C18:0-C20:3	nd	nd	7.9 ± 0.2	nd	nd
778.7	21.8	C19:1-C20:5	nd	nd	nd	nd	3.9± 0.1
790.7	14.4	C18:1-C22:6	nd	nd	nd	14.0 ± 0.1	nd
792.7	18.5	C18:0-C22:6	nd	nd	3.7 ± 0.1	nd	5.6± 0.3
794.7	20.3	C18:0-C22:5	nd	nd	7.5 ± 0.2	nd	nd

[a]Results (n=3) are expressed as mean ± SD. [b]The position of FAs in the glycerol moiety has not been determined. [c]percentages of all identified molecular species. [d]nd = not detected. RT: Retention time. Four species were not identified: X1, X2, X3 and X4.

Table 10: Relative intensity of raw MS^2 transition of PE molecular species

m/z $[M+H]^+$	Relative intensity (%)				
	$[M+H-141]^+$	$[R_1CO]^+$	$[R_2CO]^+$	$[M+H-141-R_1CO]^+$	$[M+H-141-R_2CO]^+$
714.6	573.4 (100)	238.7 (14.7)	261.2 (10.7)	335.1 (4.8)	313.6 (17.2)
716.6	575.4 (100)	239.2 (18.5)	263.3 (7.0)	337.5 (7.7)	313.1 (10.2)
718.6	577.3 (100)	239.1 (59.5)	264.8 (13.0)	339.4 (20.1)	313.3 (18.1)
738.6	597.6 (100) 597.6 (100)	261.3 (12.5) 239.3 (11.6)	262.8 (38.2) 285.0 (23.3)	337.3 (21.8) 359.4 (12.8)	335.3 (40.0) 313.1 (86.0)
740.6	599.3 (100) 599.2 (100)	263.1 (17.5) 239.3 (45.7)	263.1 (17.5) 287.4 (30.1)	337.3 (39.2) 361.2 (11.5)	337.3 (39.2) 313.3 (10.7)
742.7	601.5 (100)	267.5 (71.8)	261.0 (64.7)	335.9 (51.8)	340.8 (21.1)
744.7	603.2 (100)	267.3 (35.1)	263.7 (33.8)	335.7 (48.3)	339.4 (77.0)
746.7	605.7 (100)	266.7 (33.3)	265.4 (65.8)	339.5 (41.7)	341.0 (16.7)
750.7	609.8 (100)	nd	311.5 (10.1)	nd	298.9 (42.4)
764.6	623.4 (100) 623.4 (100)	265.5 (10.7) 239.3 (76.8)	285.3 (43.7) 311.5 (39.1)	358.9 (22.0) 385.4 (14.5)	339.3 (11.1) 312.9 (26.1)
766.7	625.5 (100) 625.4 (100)	265.5 (41.3) 238.9 (33.0)	287.3 (17.8) 313.5 (47.8)	361.1 (26.8) 387.1 (15.7)	339.2 (37.9) 313.3 (62.3)
768.7	627.8 (100)	267.3 (70.4)	287.1 (8.9)	360.7 (30.0)	341.3 (66.7)
770.7	629.6 (100)	266.7 (23.1)	288.8 (5.7)	363.1 (42.6)	341.5 (20.0)
790.7	649.5 (100)	265.5 (37.8)	311.5 (12.0)	384.9 (27.0)	338.7 (45.9)
792.7	651.4 (100)	267.5 (29.3)	311.5 (14.6)	384.9 (13.3)	340.9 (28.0)
794.7	653.8 (100)	266.6 (35.0)	313.3 (40.8)	387.4 (32.5)	341.4 (61.7)

nd: not detected.

The major PE molecular species in egg yolk were identified as (C16:0-C18:1)PE (26.6%) and (C18:0-C18:1)PE (26.5%), followed by (C18:0-C18:2)PE (20.0%) and (C16:0-C18:2)PE (16.7%). Minor species included (C16:0-C20:4)PE (4.9%), (C18:0-C20:4)PE (3.1%), and (C16:0-C22:6)PE (2.2%). Egg yolk PE was determined by Zhao et *al.* (2011) and 10 PE species were found. The predominant species were (C18:0-C20:4)PE, (C16:0-C18:1)PE and (C16:0-20:4)PE.

However, the proportion of (C16:0-C20:4)PE and (C18:0-C20:4)PE were minor in our sample. This difference is not surprising, because it is well established that PL composition depends on factors such as animal species, their diet and rearing conditions (Wang *et al*., 2009). In particular, amounts of feed and its composition have a substantial effect on the molecular species profile. Pacetti (Pacetti *et al*., 2005) showed that feeding with different amounts of blubber oil markedly affected the molecular species composition of PE in hen egg. In this paper, the authors noticed that the most abundant molecular species was (C18:0-20:4)PE for samples obtained by feeding a diet that was not fortified with blubber oil, whereas the major species were (C16:0-22:6)PE and (C18:0-22:6)PE for samples obtained by feeding diets fortified with blubber oil; these species were increased significantly with increasing blubber oil supplementation.

Eleven PE species were identified in ox liver extracts, including (C18:0-C18:1)PE, (C18:0-C20:4)PE, and (C18:0-C18:2)PE as the major ones, with respective levels of 24.7%, 19.6% and 15.2% (Table 9). Minor species included (C18:0-C20:3)PE (7.9%), (C18:0-C22:5)PE (7.5%), (C16:0-C22:5)PE (6.6%), (C16:0-C18:1)PE (5.7%), (C16:0-C22:6)PE (4.0%), (C18:0-C22:6)PE (3.7%), (C16:0-C18:2)PE (3.5%), and (C18:0-C18:3)PE (1.6%). Such species, have on the other hand, been determined in rat liver by Houjou *et al*. (2005) and Brouwers *et al*. (1999). These studies showed that (C18:0-C20:4)PE, (C16:0-C20:4)PE were the major species. Whereas (C18:0-C20:4)PE was also predominant in ox liver, the two other species were either present at a low level or absent.

As far as krill oil is concerned, to our knowledge, its PE molecular species have so far not been studied, although FA profiles of PLs have been determined (Saito *et al*., 2002). The major PE molecular species in krill oil were determined as (C16:0-C22:6)PE (18.1%), (C16:0-C20:5)PE (17.6%), followed by (C18:1-C22:6)PE (14.0%), (C18:1-C20:5)PE (13.1%), and (C16:0-C18:1)PE (9.8%). Other species identified at lower levels included (C16:0-C22:5)PE (2.8%), and (C18:1-C20:4)PE (2.2%). Surprisingly, the mass spectrum of the major peak (22.4%) contained the ion *m/z* 750.7, which is normally attributed to (C15:0-C22:6)PE. However, such a high level of odd-chain FA is uncommon in natural foodstuffs. Moreover, the retention time of this peak (17.2 min) is longer than that of (C16:0-C22:6)PE (13.9 min), which is inconsistent with the principle of the reversed phase chromatography. GC profile of FAMEs contained only one peak corresponding to C22:6 methyl ester. No other peak was observed, which meant that there was only one esterified FA in this PE molecule.

To identify this PE molecular species (*m/z* 750.7) in krill oil, the corresponding HPLC fraction between 16.9 and 17.7 min was collected and analysed by HRMS using the conditions previously described. Figure 27 shows TIC chromatogram of purified krill oil PE (*m/z* 750.7000). Figure 28 shows MS^2 fragmentation spectrum of *m/z* 750.7000 by HRMS. *m/z* 609.5227 corresponded to the loss of the polar headgroup. An exact mass *m/z* 750.5437 $[M+H]^+$ was obtained at a resolution of 9207, suggesting $C_{43}H_{77}NO_7P$, so the molecular formula was $C_{43}H_{76}NO_7P$ with a mass of 749.5363. The theoretical mass of $C_{43}H_{76}NO_7P$ is 749.5369. Therefore the calculated mass error was -0.51 ppm (Table 11). According to this formula, a PE molecule containing an esterified C22:6 FA proved by GC would necessarily contain a C16:0 alkyl group linked to one of the two -OH groups of glycerol by an ether bond.

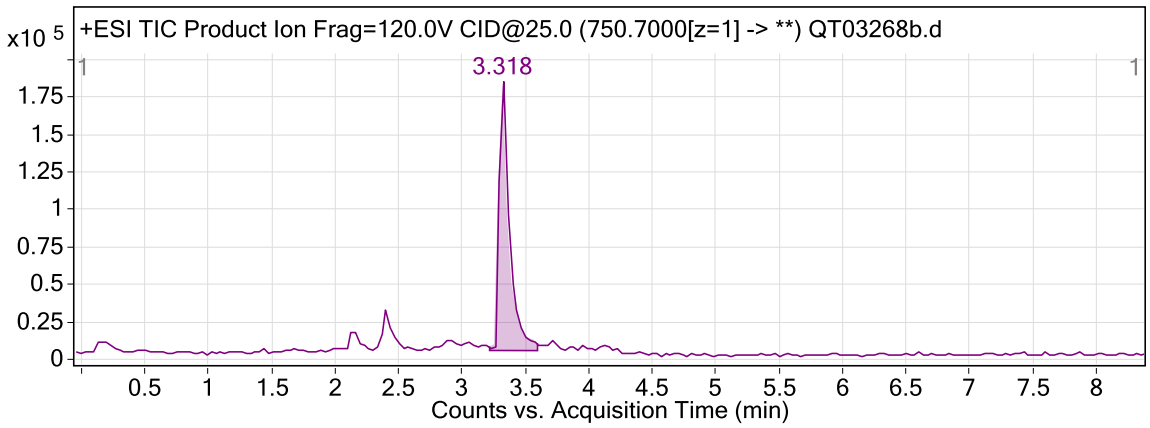


Figure 27: TIC chromatogram of purified krill oil PE (*m/z* 750.7000).

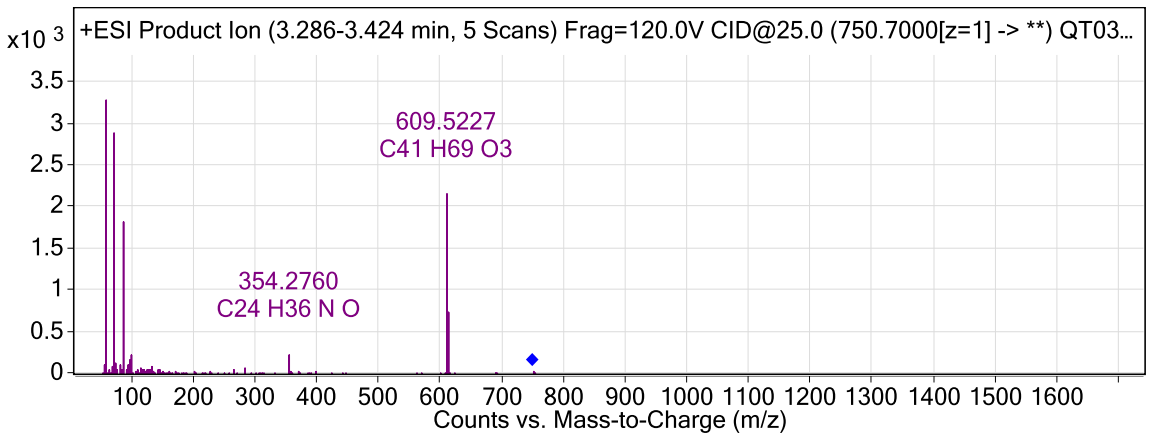


Figure 28: Product ions obtained in positive mode in HRMS between *m/z* 50 and 1700 from fragmentation of *m/z* 750.7 of krill oil.

The overall results suggest that the peak (*m/z* 750.5437) corresponds to a plasmanylethanolamine (PakE), which is a PE in which one of the two esterified FAs is replaced by a saturated ether linkage, -O-alkyl, (alkylacyl-PakE) (Magnusson and Haraldsson, 2011; Nagan

and Zoeller, 2011), in our case, C16:0(alkyl)-C22:6(acyl)PakE. PakE seems to be common among marine animals. Chapelle (Chapelle, 1987) revealed it accounted for 83% of the ethanolamine PLs (PE) in the sponge Halichondriapanicea.

Table 11: Exact mass of PE molecular species of krill (*m/z* 750.7, RT= 17.2 min) and proposed raw formulas for candidates within 5 ppm

Formula	Mass	Tgt Mass	Diff (ppm)	*M/Z*	Ion Species	Score
$C_{43}H_{76}NO_7P$	749.5363	749.5369	-0.51	750.5437	$C_{43}H_{77}NO_7P$	99.23
$C_{44}H_{72}N_5O_3P$	749.5363	749.5373	1.26	750.5437	$C_{44}H_{73}N_5O_3P$	98.22
$C_{46}H_{67}N_7O_2$	749.5363	749.5356	-0.96	750.5437	$C_{46}H_{68}N_7O_2$	96.53
$C_{34}H_{71}N_9O_9$	749.5364	749.5375	1.49	750.5437	$C_{34}H_{72}N_9O_9$	96.37
$C_{45}H_{71}N_3O_6$	749.5363	749.5343	-2.72	750.5437	$C_{45}H_{72}N_3O_6$	95.91
$C_{39}H_{72}N_7O_5P$	749.5363	749.5333	-4.13	750.5437	$C_{39}H_{73}N_7O_5P$	94.59
$C_{50}H_{71}NO_4$	749.5363	749.5383	2.66	750.5437	$C_{50}H_{72}NO_4$	92.41
$C_{38}H_{76}N_3O_9P$	749.5363	749.5319	-5.89	750.5437	$C_{38}H_{77}N_3O_9P$	88.81
$C_{41}H_{67}N_9O_4$	749.5364	749.5316	-6.34	750.5437	$C_{41}H_{68}N_9O_4$	88.30

Calf brain PE is more complex than other four food matrices. In generally, PC is a most abundant PL class in foods, but in calf brain, it is interesting that the proportion of PE is higher than PC in PL classes. We found 14 PE molecular species. Unfortunately, we could identify only 10 PE molecular species, other species were not identified, including X1, X2, X3 and X4 (Figure 24). Because by analyzing these ion fragments, we found the fracture modes of some peaks didn't follow the fragmentation patterns which we introduced in 2.2.2, and the retention times of these peaks were inconsistent with the principle of the reversed phase chromatography. Nevertheless, these compounds are for sure PE molecular species, because they contained a specific fragment $[M+H-141]^+$, corresponding to the loss of ethanolamine headgroup. Therefore, these molecular species contain certainly the same ethanolamine headgroup, but one or two FA chains which maybe linked to the glycerol backbone by other bonds, such as ether, acetal or alkenoxy. However, in our experimental conditions, we were unable to identify these molecular species. The identification of PE molecular species with different kinds of linkages needs further study. That will be an interesting and challenging work.

In addition, *m/z* 750.7 was not only present in krill oil PE, but also in calf brain PE and the same retention time at 17.2 min. For krill oil PE (*m/z* 750.7), we have analyse it by HRMS and we

speculated that it was C16:0(alkyl)-C22:6(acyl)PakE. Therefore, we assume that it could also the same molecule for calf brain PE, although we don't have enough time to collect it and analyse it.

2.2.4 Discussion

Although profiles of PE molecular species were determinated, the respective positions of FAs in the molecular species were not known. Many studies were conducted to try to understand the distribution of FA in molecular species of PL and showed that SFA or monounsaturated fatty acid (MUFA) was typically found in the *sn*-1 position, while PUFA was usually situated in the *sn*-2 position (O'Donnell, 2011; Wood R, 1969). It was clear that the one of FA chain of PE molecular species was mainly C18:0 or C16:0, the other FA chain was always MUFAs and PUFAs. And (C16:0-C18:1)PE existed in four food matrices. We can assume that molecular species such as (C16:0-C18:2)PE in soy PE, (C18:0-C20:4)PE in ox liver PE, (C18:0-C18:2)PE in egg yolk PE, or (C16:0-C20:5)PE and (C16:0-C22:6)PE in krill oil PE, found in relatively large proportions, have PUFA in *sn*-2 position. Moreover, it exists other case where two FAs of PE were PUFAs. For example, (C18:2-C18:2)PE and (C18:2-C18:3)PE which were identified in soy PE, accounted for a relatively large proportion. And they only existed in soy PE and were not found in other food matrices. As we are aware, linoleic acid (18:2) and α-linolenic acid (18:3) are considered as EFAs in human nutrition, because they have to be necessarily supplied by the diet and cannot by synthesized by the human organism. And they are the precursors of LC-PUFAs, in particular EPA and DHA, which are essential for brain and retina development (Amate *et al*., 2001; Heinemann *et al*., 2005; Horrocks and Yeo, 1999). Therefore, it provides an important information that soy which was rich in EFAs, can be used as a food additive and an nutritional complement.

In fact, the FAs composition of PE was similar with that of PC. Le Grandois *et al*. (2009) identified the molecular species of PC in the same food sources like ours (soy, ox liver, egg yolk, krill oil and calf brain). By the comparison of molecular species of PE and PC, we found that the major species present in PC class contain the same FAs as the major one contained in PE class. It indicated that FAs were evenly distributed in the main PL classes (PE and PC). For other PL classes, such as PS and PI, the distribution of FAs in them were not know. It would be a interesting work to continue to identify the molecular species of PS and PI in a further study.

In the five souces of PE studied in this part, we found two interesting sources of PUFA. One source is krill oil. The major species of krill oil PE was rich in PUFA of 20 carbons or more,

such as DHA (20:5) and EPA (22:6). It is noteworthy that *m/z* 750.7 considered as C16:0(alkyl)-C22:6(acyl)PakE, was most abundant in krill oil PE. This species was rich in EPA. Thus, krill oil was shown as a potential PUFAs source for food supplementation. Other PUFA source was soy PE which was rich in EFAs. Among the major constituents of soy PE, not only (C16:0-C18:2)PE contains a large proportion of EFA, but also (C18:2-C18:3)PE and (C18:2-C18:2)PE contain a relatively high proportion. Hence, soy is widely used as nutritional complement in human nutrition.

Moreover, it wellknown that TAG is a typical carrier of PUFA. As mentioned in 1.3 (chapter 1), the digestive mechanism of TAG is different from PL. Of the three ester functions of a TAG, only those which correspond to the *sn*-1 and *sn*-3 positions of the glycerol can be hydrolyzed by pancreatic lipase, and it does not hydrolyze the secondary ester function which corresponds to the *sn*-2 position of the glycerol (Brockherhoff and Jensen, 1974). Due to this, the TAGs are consecutively hydrolyzed to form 1,2-(2,3) diglycerides and then transformed into 2-monoglycerides. Thus, if *sn*-1 and *sn*-3 positions are attached SFAs and *sn*-2 position is attached PUFA, PUFA can not be released while SFAs would be released and absorbed which is not good for human health, unless daily intake of a certain form of TAG with UFAs in the *sn*-1 or *sn*-3 position. While for PL, Phospholipase A1 and A2 act only on *sn*-glycero-3-phospholipids and phospholipase A2 is a dominant enzyme which manifests a specificity for *sn*-2 position. All 1,2-diacyl-*sn*-glycero-3-phospholipids are thus converted into 1-acyl-*sn*-glycero-3-phospholipids, irrespective of the nature of their FAs and their polar headgroup (choline, ethanolamine, etc) (Karleskind, 1993). And as mentioned in 1.1.1 (chapter 1), *sn*-2 position is often attached by PUFA. So PUFA seems more efficient to be absorbed in form of PL. Therefore, although TAGs carry more FAs than PLs (50-100 g/day vs 2-10 g/day), when it comes to FA biodisponibility, PLs are much more efficient carriers than TAGs (Amate *et al*., 2001; Lemaitre-Delaunay *et al*., 1999; Wijendran *et al*., 2002).

2.3 SM molecular species

As we have discussed in 1.1.2 (chapter 1), complex SPLs are composed of only a single FA esterified to a sphingoid backbone. Classes of SPLs are characteristic of the organisms in which they are synthesized. The predominant SPL in mammals is SM, indeed, we didn't find SM in soy PLs. SM contains sphingosine (d18:1), which is an aminodiol with 18 carbon atoms in its chain and an *E*-double bond in position 4. It is the most abundant among the sphingoid constituents of SPLs in human and animal cells (Dyatlovitskaya, 2000). Other sphingolipid bases,

such as sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) in mammals, are reported in smaller amounts (Karlsson, 1970). Therefore, in this part, we chose three animal sources (egg yolk, calf brain and ox liver) for analysis. Because SM is neither in krill oil nor in soy (see Figure 22).

The purification procedure and the detection methods are the same with that of PE (Figure 21). After obtaining pure SM, the molecular mass peaks for SM were detected in these food matrices using positive ion full-scan ESI-MS analysis (Figure 29). Overall, the molecular species of SM, having different polarities, were very well separated. Figure 29A shows a chromatogram of egg yolk SM, revealing a simple composition with few molecular species eluting between 11 and 20 min. Obviously, peak 5 was the main species of egg SM. In contrast, ox liver SM (Figure 29B) and calf brain SM (Figure 29C) showed complex compositions, with at least 13 molecular species peaks. More than four main peaks were clearly seen in the chromatograms. While all molecular species of calf brain SM were evenly separated during 0-62 min, some of the molecular species in ox liver SM had very close retention times appearing intensively, mainly between 10-20 min.

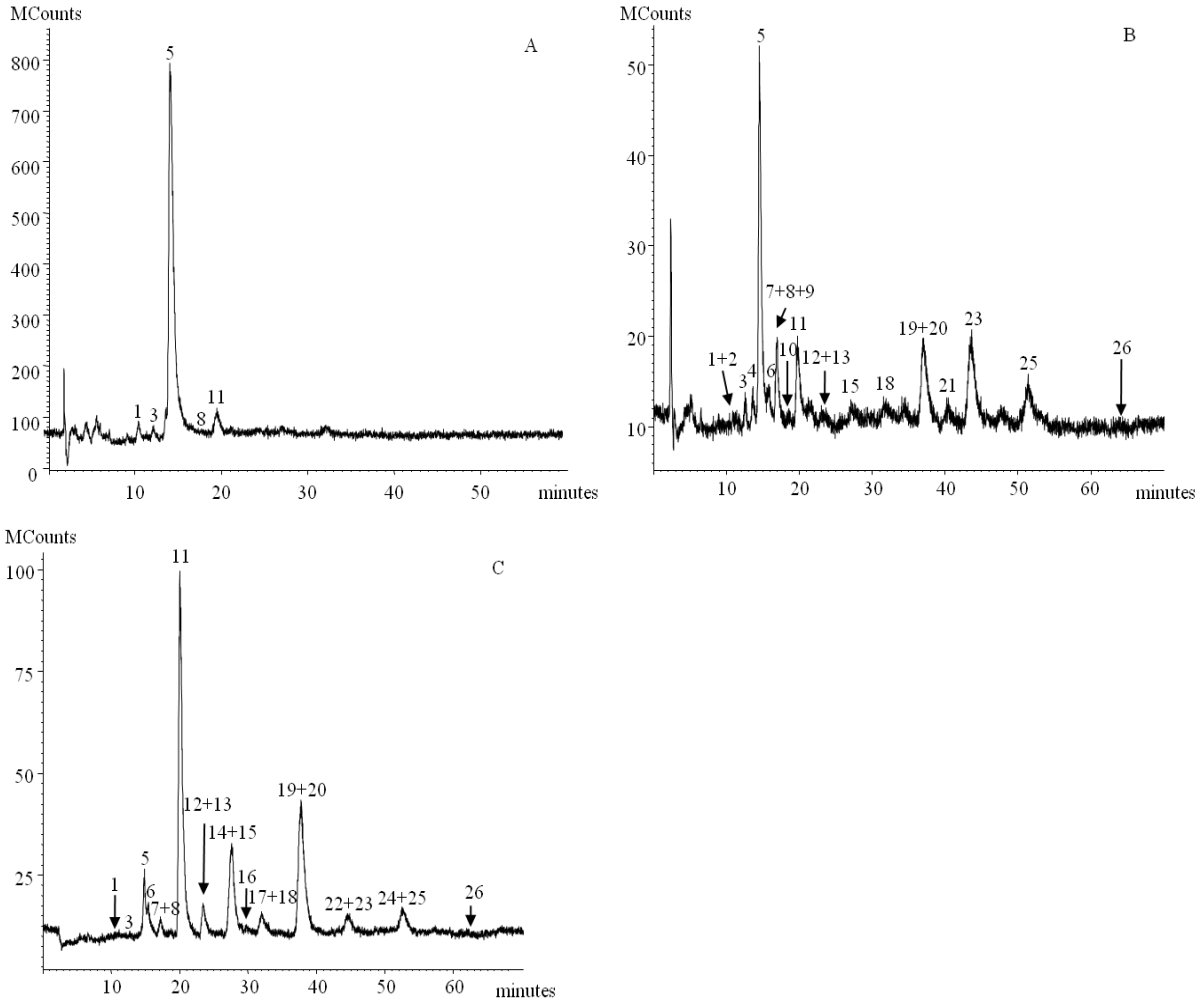


Figure 29: Chromatograms of purified SM: (A) egg yolk, (B) ox liver, (C) calf brain using LC-ESI-MS. Separation was performed using isocratic conditions: CH_3OH containing 5 mM ammonium formate at a rate of 1 mL/min onto a Nucleodur C18HTec (250 mm × 4.6 mm, 3 μm). 1, d18:1-14:0; 2, d18:1-16:1; 3, d18:1-15:0; 4, d18:1-17:1; 5, d18:1-16:0; 6, d18:1-18:1; 7, d18:0-16:0; 8, d18:1-17:0; 9, d18:1-19:1; 10, d18:1-18:0; 11, d18:1-18:0; 12, d18:0-18:0; 13, d18:1-19:0; 14, d18:1-22:1; 15, d18:1-20:0; 16, d18:1-24:2; 17, d18:1-23:1; 18, d18:1-21:0; 19, d18:1-24:1; 20, d18:1-22:0; 21, d18:1-23:0; 22, d18:1-25:1; 23, d18:1-23:0; 24, d18:1-26:1; 25, d18:1-24:0; 26, d18:1-25:0.

2.3.1 Identification of SM molecular species

The structure of SM is quite different with GPL. Hence, the facture mode was very different. Under our experimental conditions, the ESI tandem mass spectra of SM $[M+H]^+$ ions yielded six fragments:

- a fragment ion at *m/z* 264 that is characteristic of the sphingosine long chain base (d18:1) (Hsu and Turk, 2000). This fragment represented a loss of the polar headgroup $[HPO_4(CH_2)_2N(CH_3)_3]^+$, H_2O and the acyl group $[RCO]^+$ corresponding to the FA;

- an $[M+H-183]^+$ ion corresponding to the loss of the polar headgroup;
- a fragment ion at *m/z* 282 corresponding to the loss of the polar headgroup and acyl group;
- an $[M+H-18]^+$ ion resulting from the loss of H_2O;
- an $[M+H-77]^+$ ion corresponding to the loss of trimethylamine and H_2O;
- an $[M+H-282]^+$ ion corresponding to the loss of the acyl group and a hydroxyl group.

Representative MS^2 fragmentation spectra of SM's molecular species are presented in Figure 30. The product ion spectra of reference (d18:1-18:1)SM at *m/z* 729.7 (RT = 15.4 min) clearly showed the fragment ion at *m/z* 264.3, reflecting 18:1 long chain base. Two fragment ions at *m/z* 711.1 and *m/z* 546.5 presumably reflected the loss of H_2O and the polar headgroup, respectively. The tandem mass spectrum also contained fragment ions at *m/z* 447.9, *m/z* 282.1 and *m/z* 652.4, which were related to the loss of the fatty acid, of both the polar headgroup and acyl group, and of both the trimethylamine and H_2O molecule, respectively. However, the analysis of the reference (d18:1-18:1)SM used showed that this standard is not pure (72.7% pure), with a chromatographic profile clearly revealing the presence of other SM species, such as (d18:1-20:1)SM (9.0%), (d18:1-16:1)SM (6.6%), (d18:0-18:1)SM (4.7%), (d18:1-18:2)SM (4.1%) and (d18:1-17:1)SM (2.9%) (Figure 31).

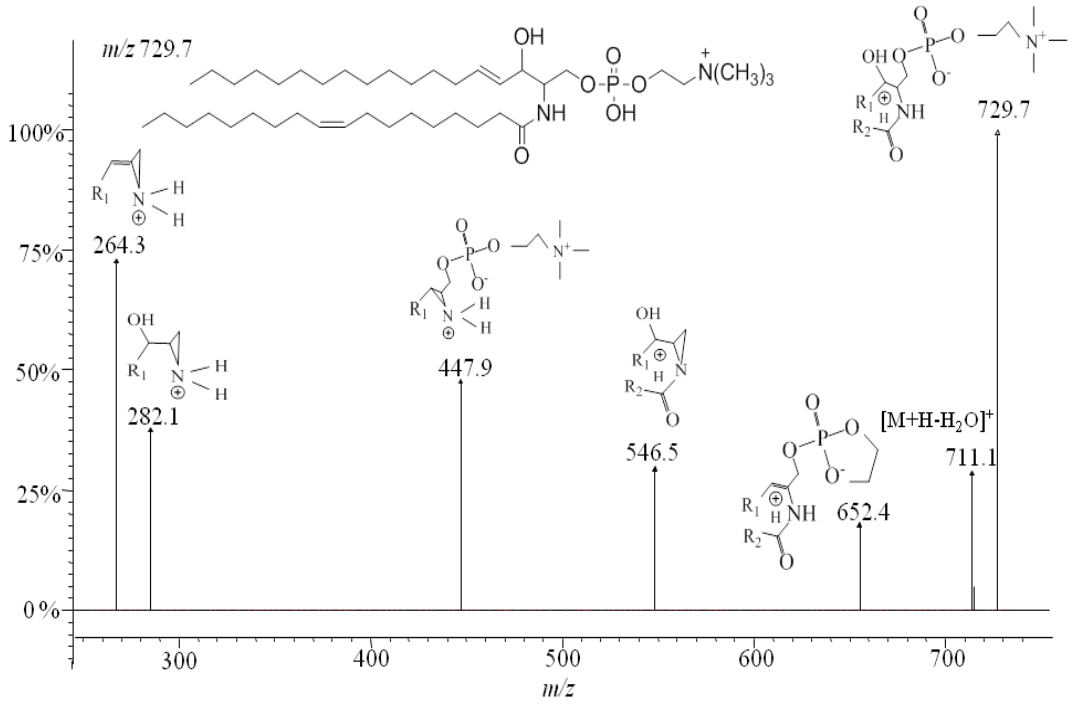


Figure 30: Product ions obtained in positive mode in ESI-MS^2 between *m/z* 200 and 750 from fragmentation of reference SM (d18:1-18:1) at *m/z* 729.7. $R_1 = C_{15}H_{29}$; $R_2 = C_{17}H_{33}$.

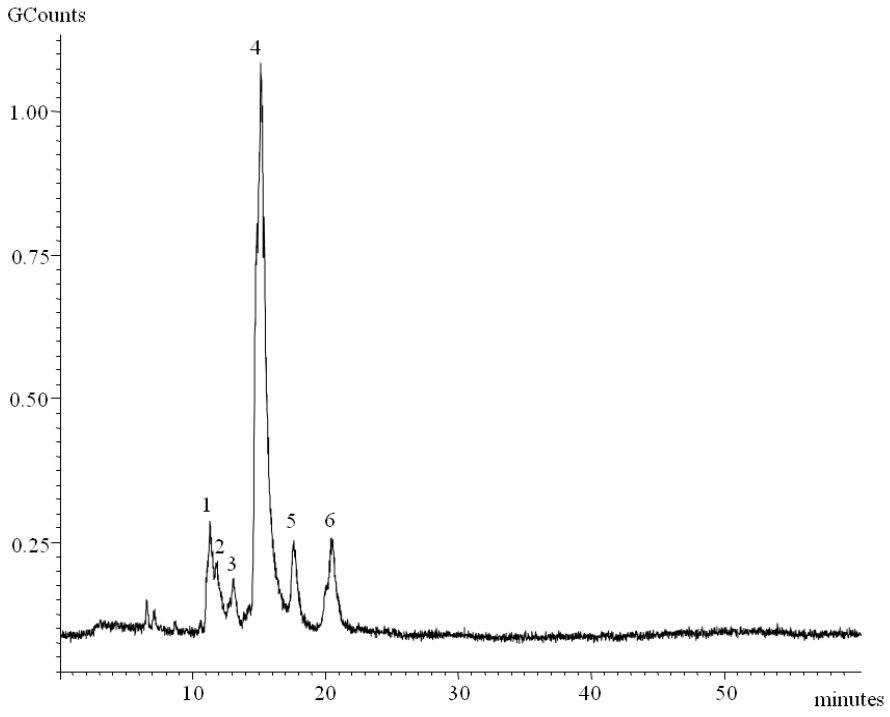


Figure 31: Chromatograms of reference SM (d18:1-18:1) using LC-ESI-MS. Separation was performed using isocratic conditions: CH_3OH containing 5 mM ammonium formate at a rate of 1 mL/min onto a Nucleodur, C18HTec (250 mm × 4.6 mm, 3 μm). 1, d18:1-16:1; 2, d18:1-18:2; 3, d18:1-17:1; 4, d18:1-18:1; 5, d18:0-18:1; 6, d18:1-20:1.

Another representative MS^2 fragmentation spectrum of (d18:1-16:0)SM from egg yolk (*m/z* 703.6, RT = 14.8 min) was given in Figure 32. It also clearly showed six fragments apart from $[M+H]^+$, the fracture mode was the same with Figure 30.

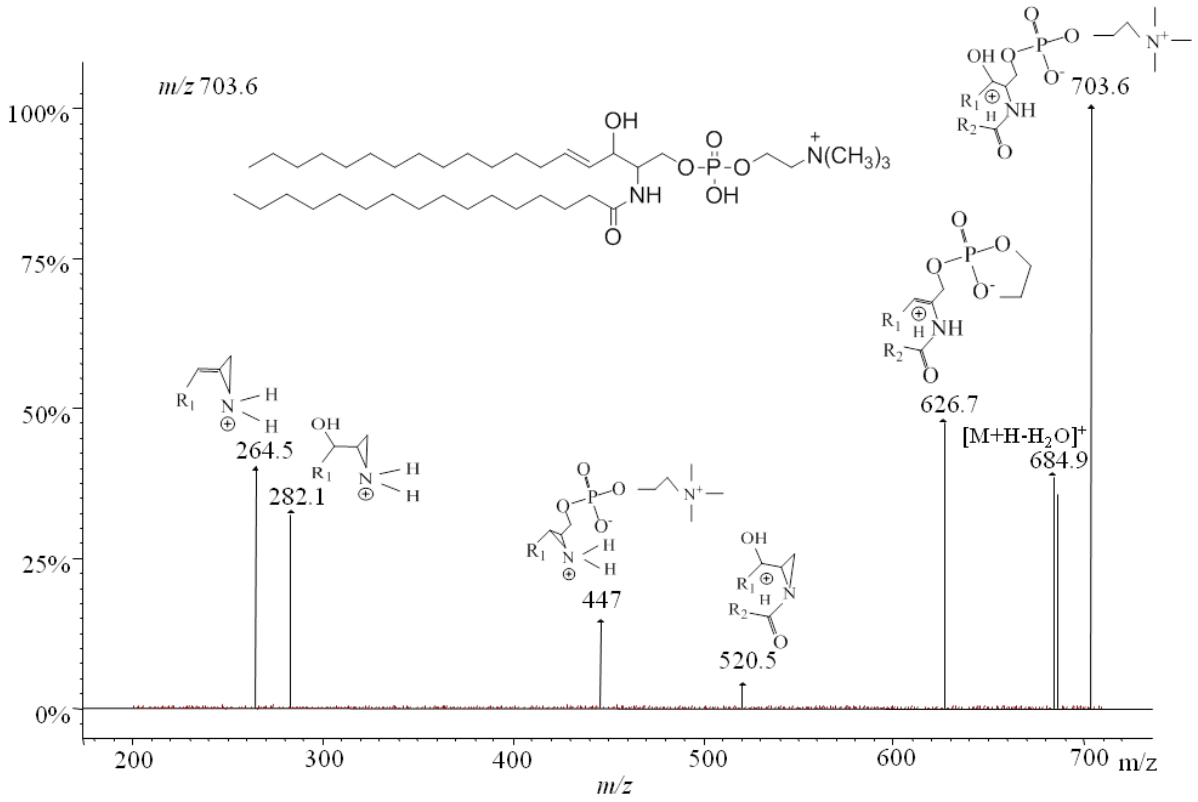


Figure 32: Product ions obtained in positive mode in ESI-MS^2 between *m/z* 200 and 750 from fragmentation of egg yolk SM (d18:1-16:0) at *m/z* 703.6. $R_1 = C_{15}H_{29}$; $R_2 = C_{15}H_{31}$.

2.3.2 Profiles of SM molecular species

The approach used for the reference SM was applied to each *m/z* detected from the purified SM samples, which allowed the identification of a number of constitutive SM molecular species for each food investigated (Table 12). As far as egg yolk SM was concerned, the major species was (d18:1-16:0)SM (94.1%). Other SM species were also identified: (d18:1-18:0)SM (3.0%); (d18:1-14:0)SM (1.4%); (d18:1-15:0)SM (1.0%) and (d18:1-17:0)SM (0.5%). The results obtained here for egg yolk are, to a certain extent, in agreement with our study carried out under the same conditions with the standard egg yolk SM purchased from Sigma-Aldrich (see above). In the latter, the main SM molecular species were also (d18:1-16:0)SM (86.8%), the minor molecular species were (d18:1-18:0)SM (6.9%) and (d18:0-16:0)SM (6.3%).

Table 12: Molecular species profiles of each purified SM

m/z	retention time	molecular	SM (%)			reference
$[M+H]^+$	(min)	species [a]	egg yolk [b]	ox liver [b]	calf brain [b]	(d18:1-18:1)SM
675.7	11.0	d18:1-14:0	1.4	0.5	0.1	nd
689.7	12.8	d18:1-15:0	1.0	1.6	0.1	nd
701.7	11.5	d18:1-16:1	nd [c]	0.8	nd	6.6
703.6	14.8	d18:1-16:0	94.1	25.5	4.4	nd
705.7	17.2	d18:0-16:0	nd	2.7	0.6	nd
715.7	13.4	d18:1-17:1	nd	0.1	nd	2.9
717.7	17.3	d18:1-17:0	0.5	2.3	0.5	nd
727.7	12.0	d18:1-18:2	nd	nd	nd	4.1
729.7	15.4	d18:1-18:1	nd	0.5	2.0	72.7
731.7	17.9	d18:0-18:1	nd	nd	nd	4.7
	18.7	d18:1-18:0	nd	0.5	0.2	nd
	20.0	d18:1-18:0	3.0	5.6	40.7	nd
733.7	23.5	d18:0-18:0	nd	0.5	2.3	nd
743.7	17.3	d18:1-19:1	nd	0.1	nd	nd
745.7	23.5	d18:1-19:0	nd	0.4	0.6	nd
757.7	20.8	d18:1-20:1	nd	nd	nd	9.0
759.7	27.5	d18:1-20:0	nd	1.8	10.8	nd
773.7	32.1	d18:0-21:0	nd	2.3	0.3	nd
785.7	27.1	d18:1-22:1	nd	nd	3.0	nd
787.7	37.9	d18:1-22:0	nd	12.5	6.8	nd
799.7	32.0	d18:1-23:1	nd	nd	2.2	nd
801.7	41.3	d18:1-23:0	nd	3.1	nd	nd
	44.4	d18:1-23:0	nd	19.7	2.5	nd
811.7	29.0	d18:1-24:2	nd	nd	0.6	nd
813.7	37.5	d18:1-24:1	nd	4.9	17.1	nd
815.7	52.5	d18:1-24:0	nd	13.2	4.2	nd
827.7	44.3	d18:1-25:1	nd	nd	0.6	nd
829.7	62.3	d18:1-25:0	nd	1.9	0.2	nd
841.7	52.1	d18:1-26:1	nd	nd	0.3	nd

[a]The position of FAs in the glycerol moiety has not been determined. [b]percentages of all identified molecular species. [c]nd = not detected.

Within ox liver, 21 SM species were clearly identified. SFAs were the most abundant and accounted for 94.1% of total FAs, the rest being made of MUFA (6.4%) (Table 12). Consequently, most SM species contained SFA, with (d18:1-16:0)SM representing the largest proportion (25.5%) of the total SM (Table 12). This result was consistent with the fact that C16:0 (28.2%) was of the predominant FA in ox liver. Other SFA-containing SM species included (d18:1-22:0)SM (12.5%), (d18:1-24:0)SM (13.2%) and (d18:1-23:0)SM (19.7%). The most abundant MUFA-containing species was (d18:1-24:1)SM, with a proportion of 4.9%. To our knowledge, this is the first report on SM molecular species in ox liver.

It is known that different SM subclasses exist depending on the sphingoid bases component (Warneck and Heinz, 2003). One of these subclasses is based on sphinganine (d18:0)SM, with an *m/z* 266 characteristic fragment ion. In ox liver, this fragment ion was present among others in the chromatographic peak at 17.2 min ($[M+H]^+$=705.7), which allowed the identification of (d18:0-16:0)SM (2.7%) (Table 12). Other (d18:0)SM species were also identified: (d18:0-21:0)SM (2.3%) and (d18:0-18:0)SM (0.5%). When applied to the used (d18:1-18:1)SM reference, this analysis showed that it contained as high as 4.7% of (d18:0-18:1)SM (*m/z* 731.7).

As mentioned above, one of the most important SM species in ox liver was (d18:1-23:0)SM, which corresponded to a clear chromatographic peak at 44.4 min (*m/z* 801.7). Another peak with the same mass was also observed at 41.3 min. The presence of the fragment ion *m/z* 264.3 showed clearly that this peak corresponded to (d18:1)SM. In addition, a *m/z* 433.4 fragment ion, corresponding to $[M+H-RCO^-OH]^+$, confirmed the presence of C23:0 as FA. These results suggested that the two molecules observed at 41.3 min and 44.4 min were isomers of (d18:1-23:0)SM, the difference between them being probably due to the relative position of the double bond in sphingosine (d18:1). Unfortunately, the technique used in this study did not allow a more precise determination of the chemical structure of the sphingosine base. A similar observation was also made for (d18:1-18:0)SM, with two possible isomers at 18.7 min (0.5%) and 20.0 min (5.6%) (Table 14). It is noteworthy that the chromatographic separation allowed three different SM species [2 (d18:1-18:0)SM isomers and (d18:0-18:1)SM] of the same mass (*m/z* 731.7) to be separated, although their chemical structures could not be completely resolved.

As opposed to ox liver, calf brain showed high proportion of MUFA-containing SM species, with (d18:1-24:1)SM (17.1%) being second to (d18:1-18:0)SM (40.7%) (Table 12). Reports showed that 18:0, 24:1 and 24:0 were the most abundant FAs in bovine brain SM

(Karlsson *et al*., 1998; Jungalwala, 1979). Yet, while this was consistent with our results regarding 18:0 and 24:1, the content of (d18:1-24:0)SM in our case was only 4.2%, which is less than (d18:1-20:0)SM (10.8%), (d18:1-22:0)SM (6.8%) and (d18:1-16:0)SM (4.4%). As with ox liver, (d18:0)SM species were also identified: (d18:0-18:0)SM (2.3%); (d18:0-16:0)SM (0.6%) and (d18:0-21:0)SM (0.3%). Furthermore, the suggested (d18:1-18:0)SM isomers at 18.7 min and 20.0 min discussed previously for ox liver, were also identified in calf brain.

The molecular species of SM are composed of only a single FA. So the pattern of FA is worth investigating. It is interesting to note that the FAs of SM in all the food matrices which we studied here were almost SFAs. In egg yolk SM, all the FAs were saturated, and in calf brain and ox liver, the proportion of SFAs accounted for a large proportion, 74.3% and 94.1%, respectively. Moreover, the most important SFAs in the three food matrices were C16:0 and C18:0. Besides, the remain FAs of them were nearly MUFAs, accounted for 25.2% and 6.4%, respectively, although calf brain SM contained traces of PUFA, C24:2 (0.6%). Our results were in agreement with previous studies. For example, Byrdwell and Perry (2006) investigated molecular species of SM in bovine brain and egg yolk. The results showed that by far the most abundant FAs of SM in bovine brain and egg yolk were saturated. Futhermore, this phenomenon also existed in other tissues. Isaac *et al*. (2003) determined SM molecular species of human brain and also found C18:0 and C16:0 were the major FAs in SM. Hirvisalo and Renkonen (1970), Jungalwala *et al*. (1979) and Sweeley et *al*. (1963) determined the FAs composition of human serum SM and showed that C16:0 FA was predominant and the total SFAs accounted for a large proportion in all. Based on the above cases, we consider that FAs composition of SM in animals is mainly saturated, this may be with the relevant of its biofunction and its location in organism. Because FA composition has a major impact on membrance characteristics, and a previous study showed that PLs predominantly carrying SFAs have been involved in apoptosis and cellular proliferation (Mollinedo *et al*. 2006).

Three sources of SM were determined in this part, egg yolk SM and calf brain SM have already been studied previously, but to our knowledge, the information of ox liver SM is limited. Moreover, the FAs composition of SM in the three sources are different. It is noted that different molecular species of SM are characteristic of different sources. As shown previously, (d18:1-16:0)SM was the predominant species in egg yolk, ox liver and human serum, while (d18:1-18:0)SM was the most abundant in calf brain and human brain.

Sphingosine (d18:1) is the most abundant among the sphingoid constituents of SM in

mammals. And here we paid attention to the determination of d18:1 base of SM. In fact, SM also contains other sphingoid bases, such as sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) and even other long-chain backbones (16:1, 17:1, 19:1…), which are reported in very smaller amounts. In our study, it is interesting to find several sphinganines, they are d18:0-16:0, d18:0-18:1, d18:0-d18:0 and d18:0-20:0. As for whether it exists other SM family in the three matrices, we suppose that it should try to explore other analytical method. So it will be a challenge and interesting work to continue to find new SM family in further study.

Conclusion

This chapter is the starting point for the screening of PUFA-PL nature sources. The investigation of these sources focuses on different aspects, such as PL composition, content of each PL classes, composition of FA and percentage of molecular species.

Five sources (soy, egg yolk, calf brain, ox liver and krill oil) of PL were studied, because these sources were rich in PL. GPLs like PE and PC commonly existed in the five sources and they accounted for a relatively high amounts in PL classes. Except krill oil, other four sources also contain PI. While PS with little amount was only presented in ox liver and calf brain. Hence, considering the proportion of each PL classes, we tended to analyse PE, because PC has already been studied by Le Grandois in our laboratory. As for SPLs, SM which was a major class was analyzed.

In order to explore potential sources of polyunsaturated PL, the analytical method for FA composition of molecular species has been established. This chapter describes a series of analytical methods allowing the purification of PE and SM from different food matrices and then determining PE and SM molecular species by RP-HPLC coupled with ESI-MS^2.

The first step of the purification was to extract TL from various food matrices. Usually, we used SLE-Folch method to extract TL, which is a most popular extraction procedure. PLE is a new technique that has a lot of advantages, such as reduces solvent consumption and sample preparation time. Therefore, part A of this chapter was aimed to apply PLE method to extract TL from different food matrices and compare PLE method with SLE-Folch method. Our studies show that PLE is more efficient for TL and PL extraction than SLE-Folch method, and PLE method can be a better alternative to the SLE-Folch method.

The second step of the purification was to isolate PL from TL. The isolation step was performed with adsorption column chromatography using non polar solvent to remove NL and polar solvent to elute PL. This method is very simple to obtain a large number of purified PL, and the purity of PL was above 95%.

The third step of the purification was to purify PE and SM from PL. Semi-preparative chromatography can separate different PL classes very well. Because the proportion of PE and SM is high than other PL classes, it is easy to get a relatively large yield in a short time.

The PE and SM molecular species profiles were determined by use of LC-ESI-MS^2. Positive ion mode and negative ion mode were both tested for the optimization of the MS signal. Full-scan mass spectra and MS/MS spectra were acquired in order to obtain the maximum number of available transitions for each sample. The best results in terms of sensitivity were obtained always with the positive ionization mode, using the $[M+H]^+$ as the precursor ion. In our study, soy, egg yolk, calf brain, ox liver and krill oil were chosen as research objects. SM just exists in egg yolk, calf brain and ox liver, so we collected and purified their molecular content. While PE exists in all of the five food matrices, we purified it from each food matrix. 6, 7, 11 and 8 PE molecular species were identified in soy, egg yolk, ox liver and krill oil, respectively. While in calf brain, we found 14 PE molecular species, but we didn't identify all of them.

The method developed for the analysis of molecular species can not only identify FA on glycerol backbone, but also FA on sphingoid backbone. The molecular species of soy PE almost contain C18:2 and C18:3 FAs. And the molecular species of egg yolk PE, ox liver PE and krill oil PE were not only rich in EFAs, but also in LC-PUFAs, especially in krill oil PE, which contains predominant EPA and DHA. As far as SM is concerned, although the predominant FAs of SM in all the sources studied are SFAs, each species contains sphingosine (d18:1).

The method developed for the determination of different natural sources of PE and SM molecular species is fast. The step of purification of PL with absorption column chromatography is very simple and allows the recovery of a relatively high yields of pure PLs. The PL classes are separated very well by semi-prepative chromatography. It is convenient to collect PE and SM, and their purity is above 95%. In fact, we have also collected PI and PS, but due to their very small amount in PL classes, the collected amount was too low for LC-MS analysis. Finally, we use LC-MS^2 to identify the molecular species of PE and SM. Both molecular species of the two classes are well separated. A single solvent (methanol), containing 5 mM ammonium formate as mobile phase was sufficient to get a nice chromatographic resolution and avoid the use of complex mixtures, containing highly toxic solvent. Therefore, our method is a first step forward the principles of green chemistry.

Chapter 3: Study of thermal oxidation of phospholipids and triacylglycerols

Introduction

Lipid oxidation is a major cause of quality deterioration in many natural and processed foods (Asghar *et al*., 1988). It leads to the development of undesirable off-flavours and potentially toxic reactions (Halliwell *et al*., 1995). Therefore, in order to improve food quality, it is very important to analyze lipid oxidation products. In food, lipid oxidation starts with the formation of hydroperoxides, followed by further oxidative changes of the alkyl chains of FAs or formation of dimeric compounds or volatile compounds. Overall, the products of lipid oxidation can be divided into non-volatile oxidized compounds (non-VOCs) and VOCs. Non-VOCs have been already studied by Le Grandois *et al*., (2010). Therefore, in this chapter, we pay attention to study the formation of VOCs.

VOCs are always formed in substantial amounts during processes for food preparation involving high temperature (Márquez-Ruiz *et al*., 1995) and are suspected of impairing the nutritional value and organoleptic quality of fats and oils (Velasco *et al*., 2002). Thus, numerous studies have been carried out on measuring the VOCs of different kinds of oils (Iglesias *et al*., 2007; Bydwell *et al*., 2001), such as soybean oil (Lee *et al*., 1995), olive oil (Morales *et al*., 1997) and almond oil (Beltrán *et al*., 2011). The main component of oil is TAG. The difference between PL and TAG is that the *sn*-3 chain of PL is a phosphate and polar headgroup, it gives PL great nutritional interest that TAG do not have, such as the prevention of cardiovascular risk, the function and growth of the nervous cells, the maintenance of the cognitive functions of the memory and so on. Nowadays, food supplementation with PL rises however PLs with PUFAs are very stable during thermal processing in food industry. Thus, it is significant to study the oxidative stability of PL during heating process.

In this part, headspace-solid phase microextraction (HS-SPME) combined with GC-MS, was developed and applied to detect VOCs of PLs during heat treatment as a step towards understanding the behaviour of PL with regard to thermal oxidation. The comparation of VOCs of TAGs with those of PLs could provide more information on the influences of different polar headgroup on the glycerol backbone.

In the first step, in order to obtain the maximum extraction yield of VOCs, the effect of extraction temperature and duration on the production of VOCs from oxidized SOPC and SLPC were determined, VOCs were identified by MS and by concomitant analysis of authentic standards, and their origin were identified by homogeneous TAGs.

In the second step, a comparative investigation of the oxidative PLs stability with their corresponding TAGs was carried out. Firstly, the formation kinetics of these oxidized products and degradation of the starting materials (TAG and PL pure species) were determined. Secondly, we compared the oxidative stability of analog molecular species of TAG and PL to assess the molecular environment of the FAs effect on their resistance to oxidation. The kinetics of the precursors losses (TAG and PL pure species) were investigated using LC-ESI-MS.

1.Material and method

1.1 Material

Volatile standards (> 95%) (including pentanal, hexanal, heptanal, (E)-2-hexenal, (E)-2-heptenal, (E)-2-octenal, (E)-2-decenal, (E, E)-2,4-decadienal, 1-pentanol, 1-hexanol, 1-octen-3-ol, 1-heptanol, 1-octanol, 2-decanone, 2-undecanone, 1-octen-3-one, octanal, nonanal, 2-pentyl-furan, (E)-2-undecenal, (E)-3-octen-2-one, 5-ethyldihydro-2(3H)-furanone and dihydro-5-propyl-2(3H)-furanone were purchased from Sigma-Aldrich (St. Louis, MO, USA).

PL molecules (99.9%), including 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC), 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine (SOPE), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (SLPE), 1-stearoyl-2-oleyl-*sn*-glycero-3-phosphate (SOPA) and 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphate (SLPA), were purchased from Avanti Polar Lipids (Alabaster, USA). Tristearin C18:0 (SSS) and Triolein C18:1 (OOO) were supplied by Sigma-Aldrich (St. Louis, MO, USA). 1,3-stearoyl-2-oleyl-glycerol (SOS), 1,3-stearoyl-2-linoleoyl-glycerol (SLS) and Trilinolein C18:2 (LLL) (99.9%) was obtained from Larodan (Malmö, Sweden). HPLC grade methanol and ammonium formate were purchased from VWR (Strasbourg, France). Chloroform (Sigma-Aldrich, Steinheim, Germany) was of analytical grade.

The three SPME fibers tested in this study were: 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB), 75 µm carboxen/polydimethylsiloxane (CAR/PDMS) and 100 µm polydimethylsiloxane (PDMS). They were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.2 Sample preparation

Solutions of 10 mg mL^{-1} were prepared in amber vial with all the standards of PL or TAG molecular species. 100 µL of these solutions (1.0 mg of sample) were placed into a 20 mL dark glass injection vial previously desorbed uncapped at 200°C during 2 h in an oven. After evaporation of chloroform at room temperature in the fume chamber, each injection vial was sealed with an aluminium crimp cap provided with a polytetrafluoroethylene/silicone septum (Agilent Technologies, Massy, France) and kept at -20°C until oxidative treatment.

1.3 Oxidative treatments

20 mL dark injection stoppered vial containing the samples of PL or TAG molecular species (1.0 mg) prepared previously were oxidized in an oven heated at a specified temperatures comprised between 100°C and 200°C for durations ranging 0 to 180 min. Other mono-unsaturated PLs and TAGs, such as SOPE, SOPA and SOS were oxidized at 175 °C from 0 min to 180 min, di-unsaturated PLs and TAGs, such as SLPE, SLPA and SLS were oxidized at 125 °C from 0 min to 180 min. After oxidation, samples were cooled down quickly in ice for 5 min, and left at room temperature for 5 min, and finally injected in HS-SPME-GC-MS (Figure 33) for VOCs analysis. To evaluate the remaining PLs and TAGs, 1.0 mL of methanol was added to the 20 mL dark injection vial. 20 µL of this solution was added to 980 µL of methanol, then analyzed by LC-MS. All samples were analyzed in triplicate.

1.4 HS-SPME technology

HS-SPME is an equilibrium technique that requires a preceding optimization of the extraction parameters which might affect extraction efficiencies, in order to obtain high recoveries of volatiles. SPME parameters including fiber type, extraction temperature and extraction time were determined.

1.4.1 Selection of fiber coating

Prior to analysis, the SPME fiber was preconditioned in the bakeout oven of the combi-pal injector at the temperature and during the time suggested by the manufacturers: 250°C, 30 min for PDMS/DVB and PDMS, 300°C, 60 min for CAR/PDMS.

1.0 mg of SOPC (150°C, 30 min) was selected as the sample for comparison of the performance of all fibers. The fiber that presented the most complete profile of SOPC volatile compounds was chosen to the study. The extraction was carried out for 25 min at 50°C and each measurement was repeated three times. The evaluation of the SPME fiber performance was made in terms of the number of detectable VOCs in the extract and their total peak areas The PDMS/DVB fiber revealed to be the most suitable and was subsequently used in all further experiments.

1.4.2 Selection of extraction temperature and duration

In order to obtain the maximum extraction yield for total VOCs, extraction times and temperatures were investigated. The extraction temperature profile was constructed with 25 min as extraction duration, at 40°C, 50°C and 60°C. The extraction duration was carried out during 10 min, 25 min and 40 min at 50 °C. Desorption time was set at 10 min.

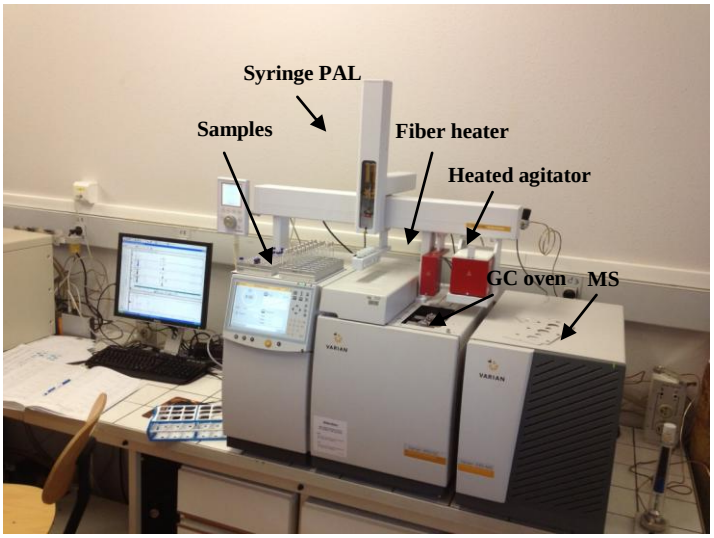


Figure 33: HS-SPME-GC-MS

1.5 GC-MS analysis

GC-MS analysis was performed in a 450 Varian gas chromatograph. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The injector temperature was set at 250°C and operated in the splitless mode. The separation of VOCs was performed on a DB-wax column (60 m × 0.25 mm, 0.15 µm, Agilent, USA). The oven temperature program started at 40°C and raised at a rate of 4°C min^{-1} to 220°C (held for 5 min), and then raised at a rate of 4°C min^{-1} to 240°C (held for 17 min). The detection was performed by an Agilent 240 mass selective detector (Ion trap) in positive electron impact mode (+EI) with 70 eV of electron beam energy. The electron multiplier was set by the auto tune procedure. MS data were collected, in a full scan mode, over the *m/z* range from 50 to 315. Transfer line temperature, trap temperature, ion source temperature were 110, 150 and 150°C, respectively.

VOCs were identified by matching the experimental mass spectra with the reference mass spectra in the National Institute of Standards and Technology (NIST) mass spectral library. More

than half VOCs identified in this way were then confirmed by comparing their mass spectra and retention times with those obtained from authentic reference compounds (STD) under the same analytical conditions.

1.6 LC-MS analysis

After oxidative treatment of the samples, the remaining PLs and TAGs were evaluated by using a Varian Prostar HPLC system made of two 210 solvent delivery modules, a 410 autosampler, and a 1200 L triple quadrupole mass spectrometer with electrospray ionization (ESI) operated in positive ion mode (Agilent, Les Ulis, France). High-purity nitrogen (99.99%, Domnik Hunter) was used as nebulizing gas, set at 317 kPa, and as drying gas, set at 300 °C. Separation was performed on a Zorbax column C8 (250 mm × 4.6 mm, 5 μm, Agilent) using an isocratic elution of 100% methanol containing 5 mM ammonium formate at a flow rate of 1 mL min^{-1}. A split system allowed the HPLC effluent to enter the mass spectrometer at a flow rate of 0.2 mL min^{-1}. 20 µL of each oxidized sample were injected. Spectral data was acquired both in full scan between m/z 500 and 1000, and in SIM mode by extracting ion *m/z* 788.6 for SOPC, *m/z* 786.6 for SLPC, *m/z* 746.6 for SOPE, *m/z* 744.6 for SLPE, *m/z* 725.6 for SOPA, *m/z* 723.6 for SLPA *m/z* 889.8 for SOS and *m/z* 887.8 for SLS. The percentage of the remaining precursors was determined for those PL molecules at each oxidation time as follows:

$$\% = \frac{(\text{area})_t}{(\text{area})_{t^\circ}} \times 100$$

where $(\text{area})_t$ is the area of the peak corresponding to the ion *m/z* of the starting material of PLs and TAGs after oxidation and $(\text{area})_{t^\circ}$ is the area of the same ion m/z without oxidative treatment.

1.7 Statistical analysis

Samples were oxidized in triplicate. Data reported were obtained from triplicate samples and expressed as mean and relative standard deviation or standard error of the mean. Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA) was used for data analyses.

2. Results and discussion

Lipid oxidation is influenced by antioxidants, metals, photosensitisers and oxygen content, heat intensity and light energy and the number of double bonds in FAs (López *et al.*, 2006; Merrill *et al.*, 2008). Lipid oxidation may be caused by heat treatment, one of the most common food processing. Recently. Beltrán *et al.* (2011) proposed to heat samples in closed vials for oxidative treatment. In our work, lipids were heated in the presence of oxygen in 20 mL closed HS-SPME injection vials. The process is then to be regarded as thermal oxidation of lipids.

2.1 HS-GC-MS methodology

At the beginning of this study, we used HS-GC-MS to analyze VOCs, and found a very weak signal and very few VOCs could be detected. Figure 34 shows a full scan chromatogram obtained after analysis of an oxidized SOPC (150°C, 30 min) by HS-GC-MS. We can see from this figure that the signal of VOCs was very weak. Only 7 VOCs were found, such as 2-nonanone, nonanal, 1-heptanol, 2-decanone, 1-octanol, (E)-2-decenal and (E)-2-undecenal. Therefore, we investigated the use of HS-SPME-GC-MS to analyze VOCs of PLs and TAGs in the following study.

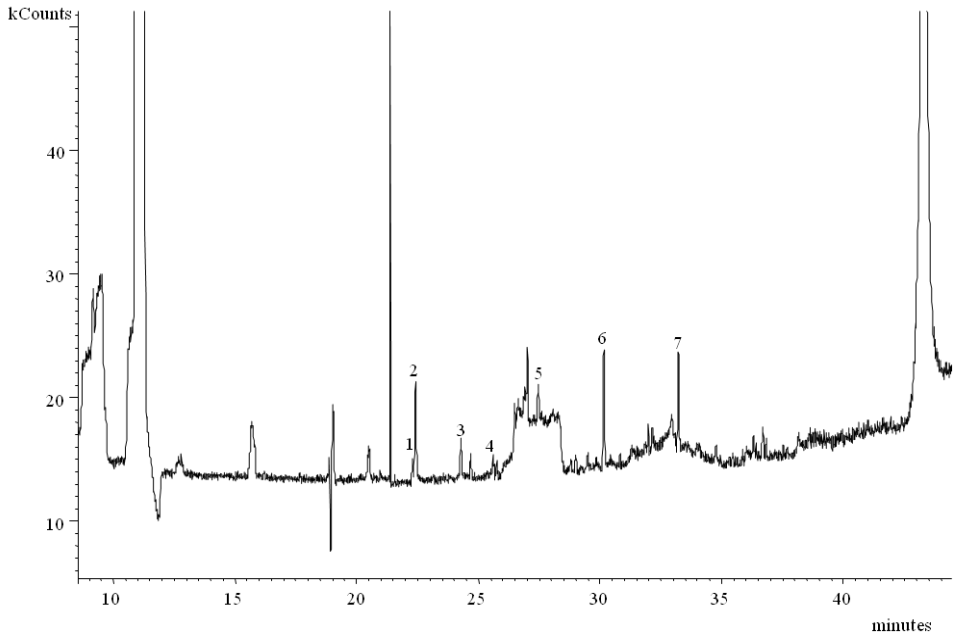


Figure 34: Chromatogram of VOCs produced from oxidized SOPC by HS-GC-MS. 1, 2-nonanone; 2, nonanal; 3, 1-heptanol; 4, 2-decanone; 5, 1-octanol; 6, (E)-2-decenal; 7, (E)-2-undecenal. 1 mg of oxidized SOPC (150 °C, 30 min) were incubated at 50°C for 25 min under rotational shaking (speed of 500 rpm). 1 mL of the vial headspace was injected into the GC inlet heated at 200°C.

2.2 HS-SPME methodology

2.2.1 Selection of SPME fiber

Three types of SPME fibers (i.e. 65 µm PDMS/DVB, 75 µm CAR/PDMS, and 100 µm PDMS) were tested and compared in this study to determine which one was the most efficient to extract VOCs. The comparison of the SPME fiber performance was made in terms of the number of identifiable compounds in the extract and the peak areas of volatile compounds. Figure 35 shows the full-scan chromatograms of VOCs of an oxidized SOPC sample with different fibers and Table 13 shows the peak areas of the different compounds extracted by HS-SPME procedure. Among the three SPME fibers tested, a total of 19 compounds were detected with PDMS/DVB and PDMS, while only 11 compounds were detected with CAR/PDMS. Between PDMS/DVB and PDMS, the first fiber coating showed better signal response for most of the detected compounds. Consequently, PDMS/DVB was selected for further studies.

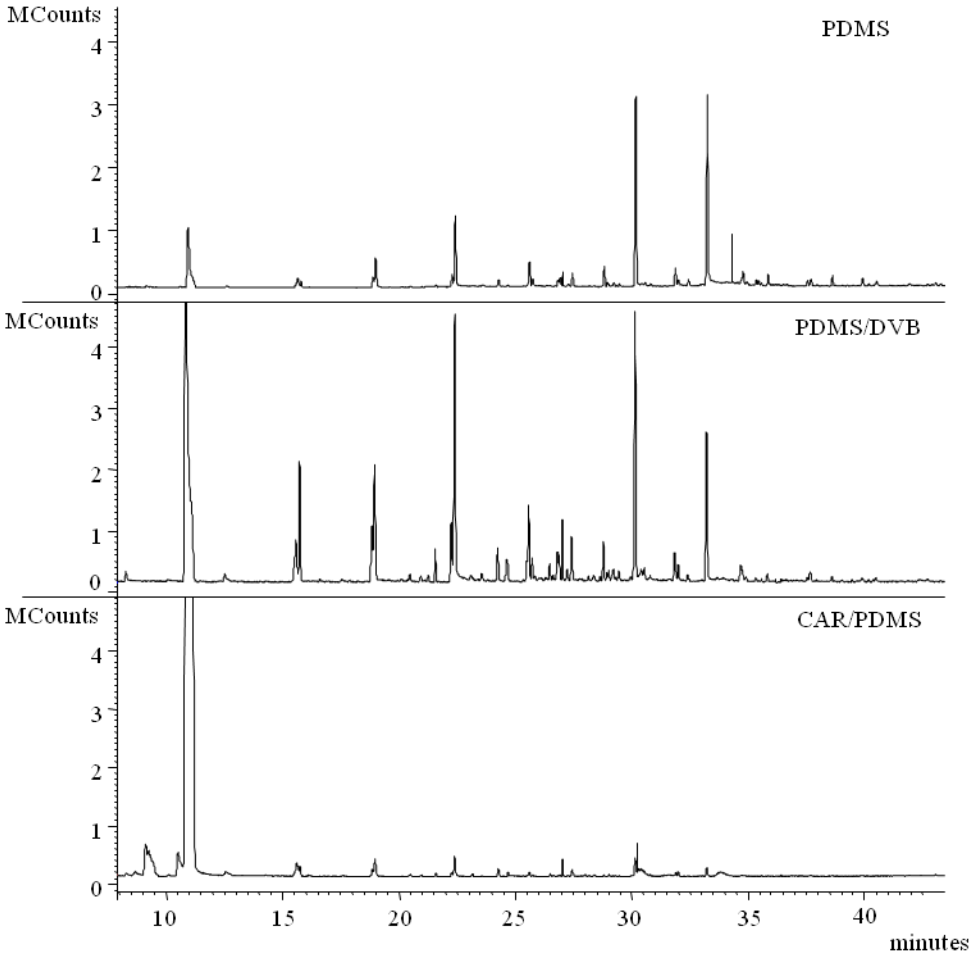


Figure 35: Chromatogram of VOCs produced from oxidized SOPC extracted using PDMS fiber, PDMS/DVB fiber and CAR/PDMS fiber. The SPME extraction time: 25 min; extraction temperature: 50 °C.

Table 13: VOCs of oxidized SOPC identified by HS-SPME using different fiber coating

RT (min)	Compounds	Peak area ($\times 10^4$)		
		PDMS/DVB	CAR/PDMS	PDMS
12.6	hexanal	42.4±2.1	5.1±0.1	3.4±0.1
15.7	heptanal	334±24	16.6±0.3	23.2±0.6
18.9	2-octanone	280±6	4.4±0.1	12.2±0.2
19.0	octanal	687±12	16.2±0.2	58.1±1.7
21.0	1-hexanol	37.7±0.4	2.1±0.1	1.3±0.0
22.3	2-nonanone	167±1	2.7±0.1	20.7±0.8
22.4	nonanal	777±5	18.4±0.2	143±6
24.3	1-heptanol	281±10	9.6±0.3	15.3±0.5
25.6	2-decanone	217±7	2.1±0.2	49±2
25.3	3-decen-1-ol	127±2	8.8±1.0	25.8±2.3
26.9	2-nonenal	26.6±2.3	nd	6.4±1.1
27.5	1-octanol	286±4	7.2±0.2	30.7±0.6
28.8	2-undecanone	88.4±0.2	nd	45.7±0.8
30.2	(E)-2-decenal	972±22	nd	480±2
31.8	2-dodecanone	57.4±2.4	nd	3.7±0.3
33.3	(E)-2-undecenal	462±18	nd	483±3
34.8	dihydro-5-propyl-2(3H)-furanone	66±1	nd	26.6±0.4
37.7	5-butyldihydro-2(3H)-furanone	72.6±0.1	nd	8±1
40.5	dihydro-5-pentyl-2(3H)-furanone	28.7±5.6	nd	29.8±9.3

nd: not detected

2.2.2 Selection of extraction temperature and duration

The SPME process is strongly influenced by the temperature of extraction. Indeed the partition coefficients are temperature-dependent, and the extraction of the analytes by the fibre coating is an exothermic process (Steenson *et al.*, 2002). Three different extraction temperatures (40, 50 and 60°C) were used to investigate the effect of this parameter on VOCs extraction. Figure 36 reports the total VOCs extracted at these temperatures from oxidized SOPC. A maximum peak area of total VOCs (sum of all peak areas) was observed at 50°C. Thus, the temperature of 50°C was chosen for further experiments.

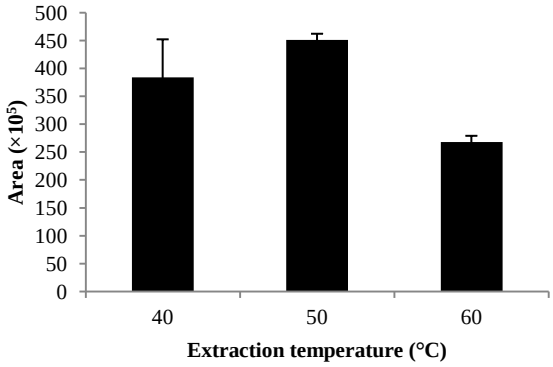


Figure 36: Effect of extraction temperature on total peak areas of VOCs from oxidized SOPC extracted by PDMS/DVB fiber for 25 min at 40-60 °C. All samples were analyzed in triplicate. Results are given as mean ± SD.

Extraction duration is another important factor affecting SPME processes since it affects the mass transfer of the analytes onto the fiber. Optimum time is required for the fiber to reach an equilibrium with the headspace (Achouri *et al.*, 2006). Three extraction durations (10 min, 25 min and 40 min) were investigated. The results showed that the total peak areas of VOCs were bigger with 25 min of extraction than with 10 or 40 min of extraction (Figure 37). Therefore, 25 min was selected for further experiments.

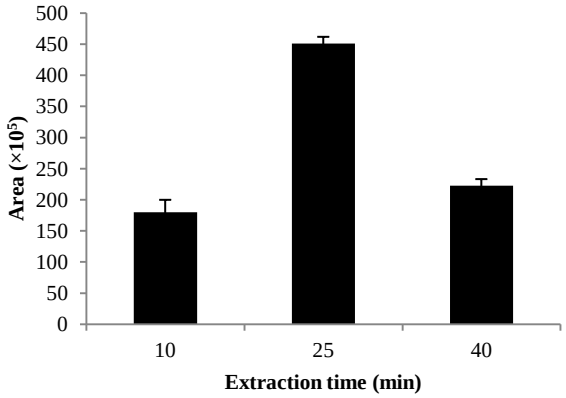


Figure 37: Effect of extraction duration on total peak aeras of VOCs from oxidized SOPC extracted at 50 °C by PDMS/DVB fiber during 10-40 min. All samples were analyzed in triplicate. Results are given as mean ± SD.

In summary, the best analytical conditions for VOCs extraction are the use of PDMS/DVB fiber, with an extraction temperature of 50 °C and an extraction duration of 25 min. These experimental conditions will be used to study the VOCs formation from oxidized PL and TAGs.

2.3 Analysis of VOCs from oxidized phospholipids and triacylglycerides

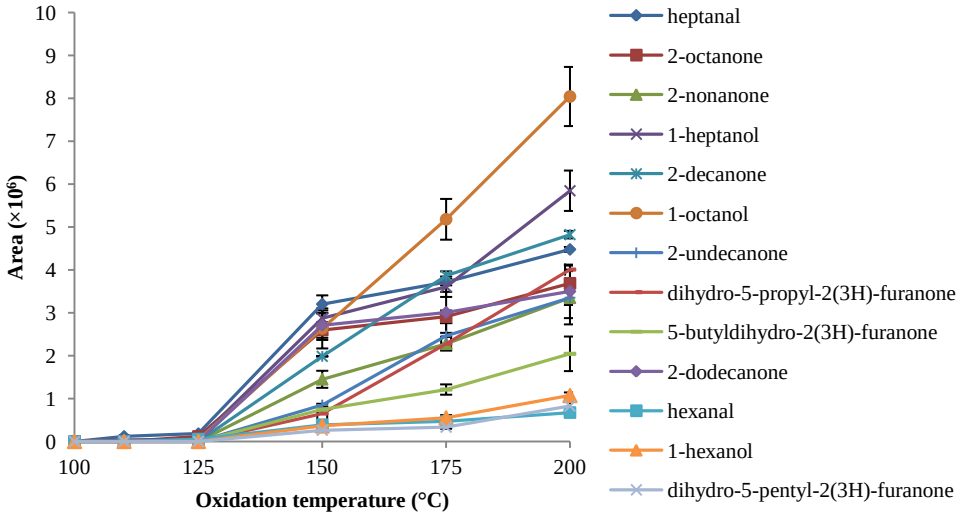
2.3.1 Effect of oxidation temperature

Six different temperatures from 100 to 200 °C were used to examine the effect of oxidation temperature (30 min as oxidation duration) on the formation of VOCs from oxidized SOPC and SLPC.

Two different types of curves were obtained for both SOPC and SLPC, and were shown in Figure 38 and Figure 39, respectively. For SOPC, the peak areas of VOCs had no significant change before 125 °C, but after 125 °C, the amounts of VOCs increased rapidly. One group included the products that increased gradually with the increase of temperature (Figure 38A). The other group included the products that increased rapidly and reached their maximum at 175 °C, then decreased after 175 °C (Figure 38B). Figure 39 gives two types of curves of VOCs peak areas of oxidized SLPC in the function of oxidation temperature. Figure 39A showed that peak areas of VOCs increased sharply until 125 °C and then kept stable or decreased. Figure 39B showed the group of VOCs that increased gradually with the increase of temperature. (E, E)-2,4-decadienal had a relative high amounts in the same group (Figure 39B), in order to let the low content of VOCs show clearly, the curves of (E, E)-2,4-decadienal and other VOCs were separated in two figures. It can be observed (Figure 38A and 38B) that no significant formation of

VOCs from oxidized SOPC was observed at a temperature before 125 °C, but at this temperature (125°C), the formation of certain VOCs from SLPC already reached their maximum (Figure 39A). Although some VOCs of either oxidized SOPC or SLPC increased with the increase of temperature, above 175°C (for SOPC) or 125°C (for SLPC) we can observe the decrease of the relatively high amount of VOCs, such as nonanal, (E)-2-decenal, octanal and (E)-2-undecenal (Figure 38B) and hexanal, (E)-2-octenal, (E)-2-heptenal and (E)-3-octen-2-one (Figure 39A). Thus, for further studies, 175 °C and 125 °C were chosen as oxidation temperature respectively for SOPC and SLPC. Furthermore, except in 2.3.2 (chapter 3), 175 °C and 125 °C were also used for the investigation of oxidation kinetics of other PLs (SOPE and SOPA /SLPE and SLPA) and their corresponding TAGs (SOS and SLS).

(A)



(B)

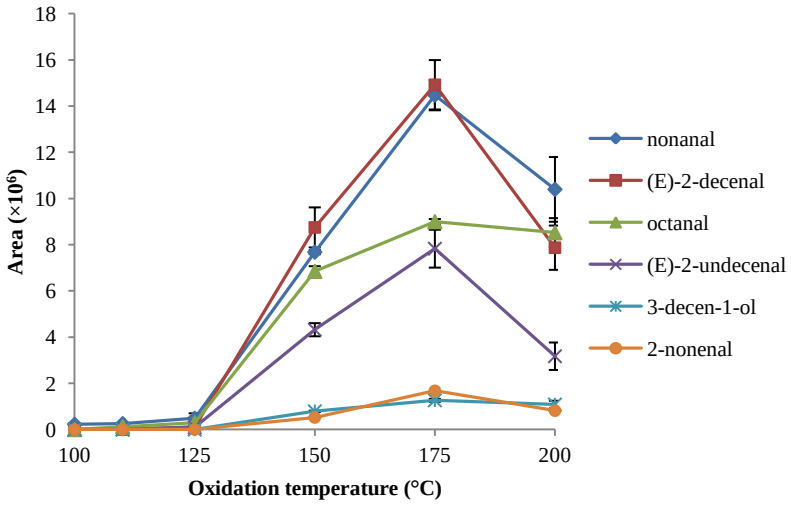
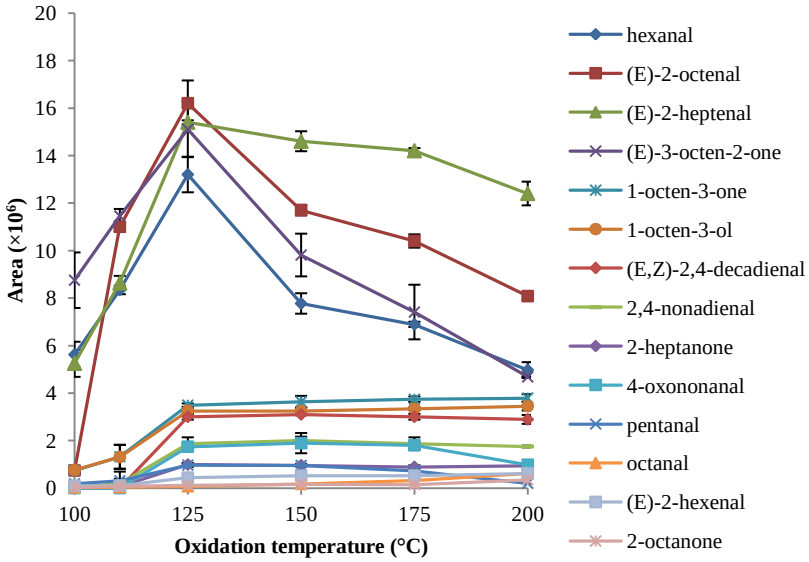
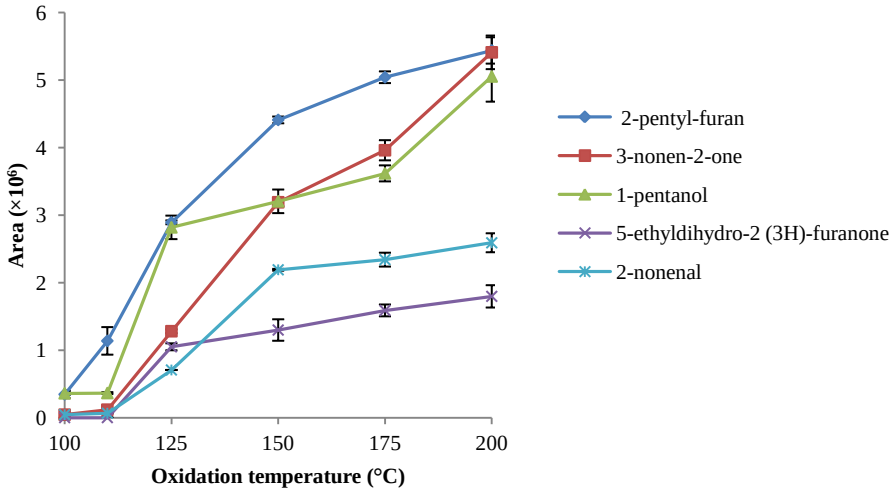


Figure 38: Profiles of VOCs produced from SOPC monitored from 100°C to 200°C (oxidation duration: 30 min). Two types of curves were presented in Figure 38A and 38B.

(A)



(B)



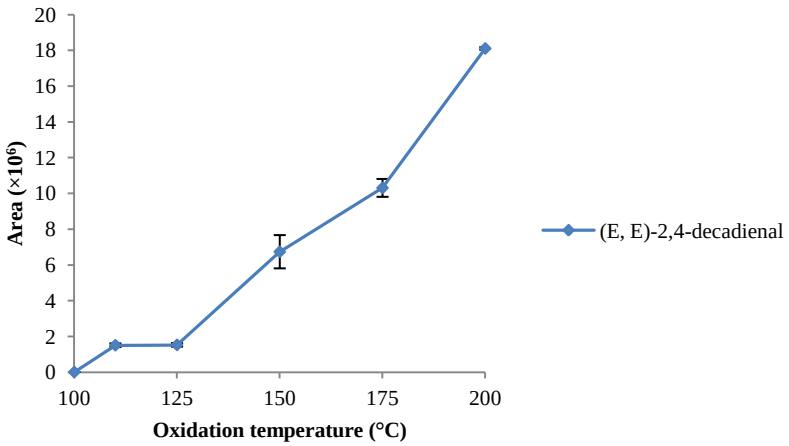


Figure 39: Profiles of VOCs produced from SLPC monitored from 100°C to 200°C (oxidation duration: 30 min). Two types of curves were presented in Figure 39A and 39B.

2.3.2 Identification of VOCs and their origin

The separation and identification of VOCs were performed by HS-SPME-GC-MS. Typical GC chromatograms of VOCs from oxidized SOPC and SLPC are shown in Figure 40. In order to identify the origin of the formation of VOCs, the corresponding TAGs (SSS, OOO and LLL) were tested under the same conditions with SOPC and SLPC (175°C, 30 min). A total of 35 VOCs were tentatively detected in this study. They belonged to the following chemical group: aldehydes (15 compounds), alcohols (6 compounds), ketones (13 compounds) and furans (1 compound).

As shown in Table 14, 19 VOCs were from oxidized SOPC. The most abundant VOCs were aldehydes, such as (E)-2-decenal (18.6%), nonanal (18.1%), octanal (11.3%) and (E)-2-undecenal (9.75%) which accounted for 57.75% of total VOCs. These aldehydes were also observed from oxidized OOO, and they were not detected from oxidized SSS. Therefore, the origin of them was from the central mono-unsaturated oleic acid chain of SOPC, and the result was consistent with Varlet *et al*. (2007) who demonstrated that (E)-2-undecenal, (E)-2-decenal, nonanal and octanal were derived from oleic acid with an intermediary 8-hydroperoxide, 9-hydroperoxide, 10-hydroperoxide and 11-hydroperoxide, respectively. Table 14 showed that aldehydes were also the main VOCs formed form oxidized SLPC. (E)-2-heptenal (17.39%), (E)-2-octenal (12.71%), (E, E)-2,4-decadienal (12.6%) and hexanal (8.41%) accounted for more than half of total VOCs detected in oxidized SLPC. It was clear by comparing the results with the VOCs of LLL that were formed from the central di-unsaturated linoleic acid chain of SLPC, and this result was in agreement with already published data (Meynier *et al*., 1998; Beltran *et al*., 2005).

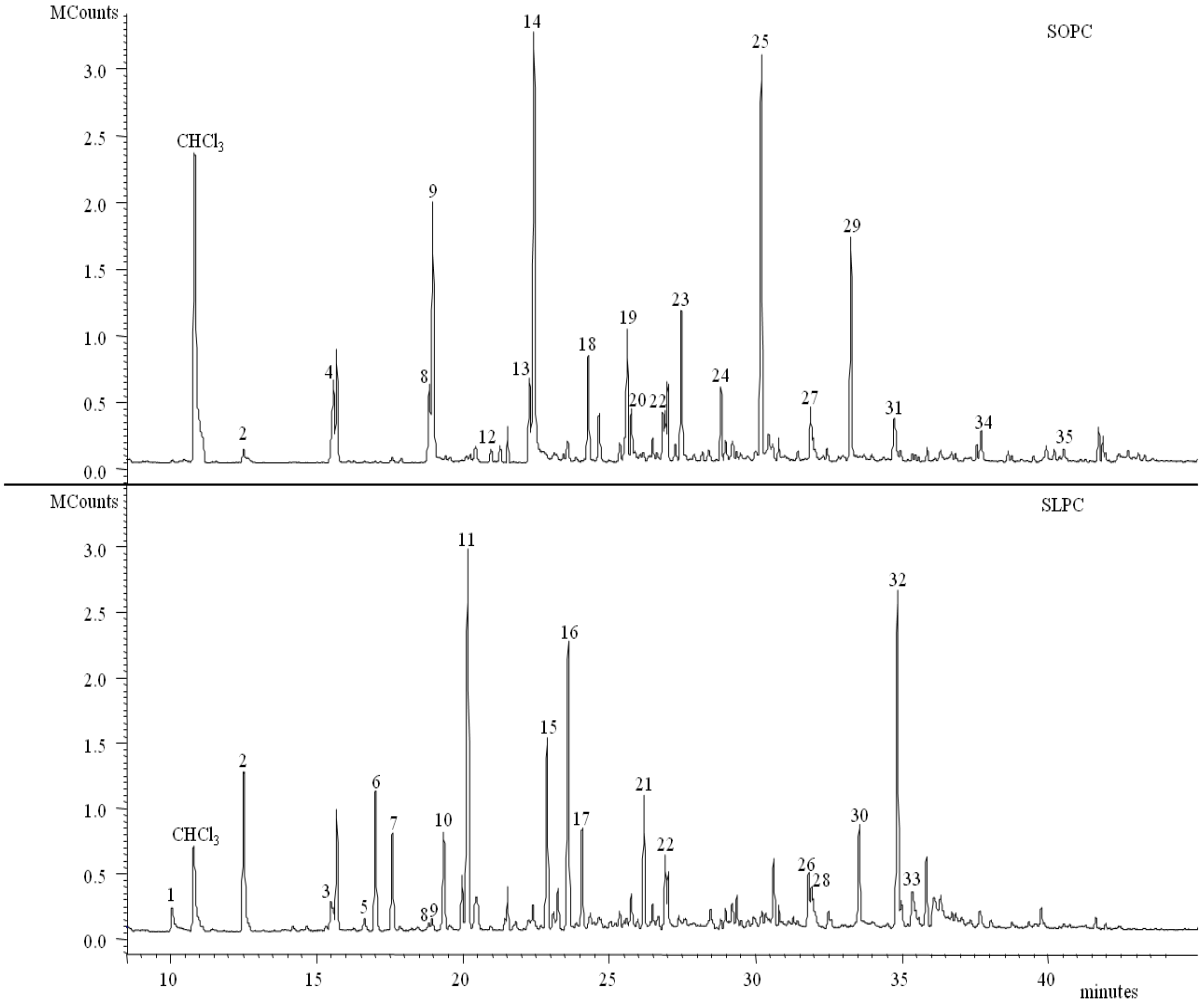


Figure 40: Total ion chromatograms of VOCs extracted from oxidized SOPC and SLPC (175°C, 30 min). 1, pentanal; 2, hexanal; 3, 2-heptanone; 4, heptanal; 5, (E)-2-hexenal; 6, 2-pentyl-furan; 7, 1-pentanol; 8, 2-octanone; 9, octanal; 10, 1-octen-3-one; 11, (E)-2-heptenal; 12, 1-hexanol; 13, 2-nonanone; 14, nonanal; 15, (E)-3-octen-2-one; 16, (E)-2-octenal; 17, 1-octen-3-ol; 18, 1-heptanol; 19, 2-decanone; 20, 3-decen-1-ol; 21, 3-nonen-2-one; 22, 2-nonenal; 23, 1-octanol; 24, 2-undecanone; 25, (E)-2-decenal; 26, 2,4-nonadienal; 27, 2-dodecanone; 28, 5-ethyldihydro-2(3H)-furanone; 29, (E)-2-undecenal; 30, (E, Z)-2,4-decadienal; 31, dihydro-5-propyl-2(3H)-furanone; 32, (E, E)-2,4-decadienal; 33, 4-oxononanal; 34, 5-butyldihydro-2(3H)-furanone; 35, dihydro-5-pentyl-2(3H)-furanone.

Ketones were also present in the VOCs of oxidized SOPC and SLPC. As shown in Table 14, (E)-3-octen-2-one (9.0%), was a major VOCs of oxidized SLPC. It was generated from linoleic acid chain of oxidized SLPC. Moreover, it is noteworthy that almost all of 2-saturated ketones were found in the VOCs of oxidized SOPC, and not in oxidized SLPC, except 2-heptanone and 2-octanone. The result can be explained by the competitive oxidation equilibrium between *sn*-1 and *sn*-2 FA chain of PC. It is recognized that in the same oxidation conditions, linoleic acid was easier to be oxidized than oleic acid. The competition between stearic acid and

oleic acid for VOCs oxidation was relatively weak by comparing between stearic acid and linoleic acid. Therefore, a higher proportion of total 2-saturated ketones were formed from SOPC than that from SLPC.

In addition, four furanones were detected in our study. Among them, dihydro-5-propyl-2(3H)-furanone and 5-ethyldihydro-2(3H)-furanone (2.84% and 1.9%, respectively) were identified. 5-ethyldihydro-2(3H)-furanone was derived from linoleic acid chain of SLPC, other three furanones were formed from oleic chain of SOPC. To our knowledge, these four furanones were not detected in previous studies of fats and oils. However, Garcia-Esteban *et al*. (2004) have detected these furanones in dry cured ham volatile compounds.

Furthermore, 2-pentyl-furan was also identifed. It was derived from linoleic acid chain of SLPC, and commonly presented in volatiles of many fats and oils (Iglesias *et al*., 2007; Lee *et al*., 1995; Hartvigsen *et al*., 2000; Steenson *et al*., 2002). Frankel (2005) proposed the formation of pentyl furan by the degradation of 9-OOH to form a cyclic peroxide and further pentyl furaldehyde, which can be decomposed to pentyl furan and formaldehyde. We identified 2-pentyl-furan in the oxidized SLPC and it was formed from linoleic acid chain, while formaldehyde was not detected in our samples.

Table 14: Identification of VOCs from oxidized SOPC and SLPC and their origin

Peak No.	VOCs	Identification	RT (min)	SOPC (%)[a]	SLPC (%)[a]	SSS (%)[a]	OOO (%)[a]	LLL (%)[a]
Aldehyde								
1	*pentanal*	*MS, STD*[c]	*10.1*	*nd*	*< 1%*	*nd*	*nd*	*3.4±0.1*
2	***hexanal***	***MS, STD***	***12.5***	***< 1%***	***8.41±0.05***	***nd***	***< 1%***	***17.1±0.4***
4	heptanal	MS, STD	15.6	4.65±0.28	nd[b]	nd	3.68±0.03	nd
9	***octanal***	***MS, STD***	***19.0***	***11.3±0.7***	***< 1%***	***nd***	***6.76±0.06***	***< 1%***
14	nonanal	MS, STD	22.4	18.1±0.4	nd	nd	13.5±2.1	nd
5	*(E)-2-hexenal*	*MS, STD*	*16.6*	*nd*	*< 1%*	*nd*	*nd*	*< 1%*
11	*(E)-2-heptenal*	*MS, STD*	*20.2*	*nd*	*17.39±0.38*	*nd*	*nd*	*13.8±0.3*
16	*(E)-2-octenal*	*MS, STD*	*23.6*	*nd*	*12.71±0.29*	*nd*	*nd*	*12.4±0.1*
22	***2-nonenal***	***MS***	***26.9***	***< 1%***	***2.86±0.12***	***nd***	***2.66±0.01***	***1.04±0.05***
25	(E)-2-decenal	MS, STD	30.2	18.6±0.2	nd	nd	37.8±1.5	nd
29	(E)-2-undecenal	MS, STD	33.3	9.75±0.44	nd	nd	26.8±0.8	nd
26	*2,4-nonadienal*	*MS*	*31.8*	*nd*	*2.28±0.29*	*nd*	*nd*	*2.44±0.24*
30	*(E, Z)-2,4-decadienal*	*MS*	*33.5*	*nd*	*3.66±0.17*	*nd*	*nd*	*6.8±0.1*
32	*(E, E)-2,4-decadienal*	*MS, STD*	*34.9*	*nd*	*12.6±0.7*	*nd*	*nd*	*24.35±0.02*
33	*4-oxononanal*	*MS*	*35.3*	*nd*	*2.12±0.12*	*nd*	*nd*	*< 1%*
Alcohol								
7	*1-pentanol*	*MS, STD*	*17.6*	*nd*	*4.41±0.06*	*nd*	*nd*	*2.14±0.01*
12	1-hexanol	MS, STD	21.0	< 1%	nd	nd	< 1%	nd
17	*1-octen-3-ol*	*MS, STD*	*24.1*	*nd*	*4.1±0.1*	*nd*	*nd*	*3.69±0.03*
18	1-heptanol	MS, STD	24.3	4.5±0.1	nd	nd	2.03±0.03	nd
20	3-decen-1-ol	MS	25.8	1.71±0.08	nd	nd	< 1%	nd
23	1-octanol	MS, STD	27.5	6.46±0.23	nd	nd	3.61±1.10	nd
Ketone								
3	**2-heptanone**	**MS**	**15.5**	**nd**	**1.1±0.1**	**14.2±0.7**	**nd**	**nd**
8	**2-octanone**	**MS**	**18.8**	**3.63±0.10**	**< 1%**	**18.4±1.2**	**nd**	**< 1%**
13	**2-nonanone**	**MS**	**22.3**	**2.92±0.03**	**nd**	**22.5±0.3**	**nd**	**nd**
19	**2-decanone**	**MS, STD**	**25.6**	**4.82±0.26**	**nd**	**20.4±1.0**	**nd**	**nd**
24	**2-undecanone**	**MS, STD**	**28.8**	**3.07±0.18**	**nd**	**15.1±1.3**	**nd**	**nd**
27	**2-dodecanone**	**MS**	**31.9**	**3.74±0.38**	**nd**	**9.4±0.5**	**nd**	**nd**
10	*1-octen-3-one*	*MS, STD*	*19.4*	*nd*	*4.56±0.22*	*nd*	*nd*	*1.36±0.12*
15	*(E)-3-octen-2-one*	*MS, STD*	*22.9*	*nd*	*9.0±1.2*	*nd*	*nd*	*< 1%*
21	*3-nonen-2-one*	*MS*	*26.2*	*nd*	*4.84±0.27*	*nd*	*nd*	*2.2±1.1*
28	*5-ethyldihydro-2(3H)-furanone*	*MS, STD*	*31.9*	*nd*	*1.9±0.1*	*nd*	*nd*	*< 1%*
31	dihydro-5-propyl-2(3H)-furanone	MS, STD	34.8	2.84±0.16	nd	nd	< 1%	nd
34	5-butyldihydro-2(3H)-furanone	MS	37.7	1.51±0.07	nd	nd	< 1%	nd
35	dihydro-5-pentyl-2(3H)-furanone	MS	40.5	< 1%	nd	nd	< 1%	nd
Furan								
6	*2-pentyl-furan*	*MS, STD*	*17.0*	*nd*	*6.2±0.2*	*nd*	*nd*	*6.32±0.03*

[a]Compounds were produced by SOPC, SLPC, SSS, OOO and LLL heated at 175°C for 30 min.
[b]nd: not detected.
[c]STD : Retention time and spectra of authentic reference compounds.
Compounds in italic originated from linoleic acid.
Compounds in bold face originated from stearic acid.
Comounds in normal originated form oleic acid.

Different products have their typical flavor, which is one of the most important quality criteria of food products. The information about the volatile flavor compounds is desired in order to monitor flavor quality and ripeness of fruits and vegetables, and to provide quality control for fresh and processed products. A previous study (Carrapiso *et al*., 2002) showed that aldehydes were the main contributors to Iberian ham flavor due to the low olfaction thresholds and distinctive odour characters (e.g. rancid, sweet, floral notes). Lee *et al*. (1995) reported that nonanal contributed the greatest individual effect on the flavor intensity of fresh and oxidized oils, followed by 2,4-heptadienal and 2-heptenal. Nóbrega *et al*. (2007) showed that (E, E)-2,4-decadienal, (E, Z)-2,4-decadienal, (E, Z)-2,6-nonadienal, 1-octanol and (E)-2-nonenal were characteristic aromas of bullfrog meat. Cheng (2010) showed that acetaldehyde, diacetyl, acetoin, acetone, and 2-butanone contributed to the typical aroma and flavor of yogurt. Jordán *et al*. (2003) characterized the aromatic profile in commercial guava essence and fresh fruit puree and demonstrated that (E)-2-hexenal was more significant to the aroma of the commercial essence than of the fresh fruit puree. Hartvigsen *et al*. (2000) demonstrated total 27 volatiles, including 1-octen-3-one, 1-octen-3-ol, 1-penten-3-one,… contributed to the development of unpleasant fishy and reancid off-flavors. Therefore, the developed HS-SPME-GC-MS analytical method allows the detection of characteristic off-flavors of PL or TAG molecular species and the monitoring of their degradation during thermal oxidation.

2.3.3 Kinetics of the formation of VOCs during oxidation

The oxidation kinetics of PLs and TAGs were investigated by analyzing HS-SPME-GC-MS peak areas of all VOCs produced as a function of oxidation time. As previously mentioned in 2.3.1, some VOCs had a relative high amounts occurrence in one chemical group, in order to let the low content of VOCs appear clearly, the curves of relative high content and low content VOCs were separated in two figures, respectively.

2.3.3.1 SOPC, SOPE, SOPA and SOS

The UFA chains in PLs are the primary targets of oxidation. Similar to TAGs oxidation, PL oxidation may occur through enzymatic or non-enzymatic reactions. However, enzymatic oxidation of PL can be eliminated due to treatment. Oxidation products can be classified into three main categories (Henna *et al*., 2011). The first categorie included long chain non-volatile products that preserve the PL skeleton, and which may result from insertion of oxygen followed by rearrangement or cleavage of PL hydroperoxides leading to epoxy, polyhydroxy, hydoxy or keto

derivatives of PL. Le Grandois *et al.* (2010) identified oxidation products such as hydroxyl, keto and epoxy groups of oxidized SOPC and SLPC. The second categorie included short-chain volatile products, such as aldehydes, ketones and so on. These products were formed by cleavage of unsaturated fatty acids. The third categorie included products formed by reaction on the polar headgroup, such as amino group. Therefore, the formation of different PL oxidation products depended on the predominating oxidative process (Domingues *et al.*, 2008).

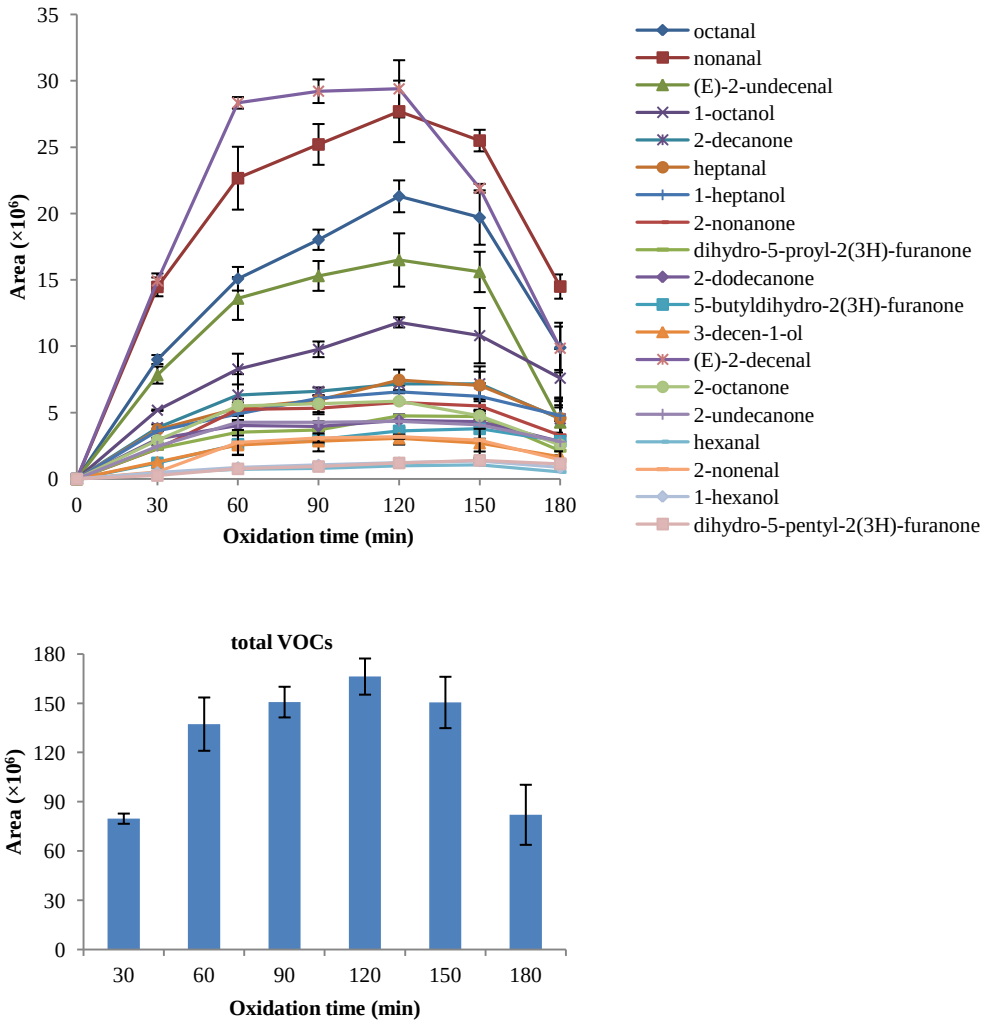


Figure 41: Profiles of VOCs produced from SOPC monitored from 0 min to 180 min (oxidation temperature: 175 °C).

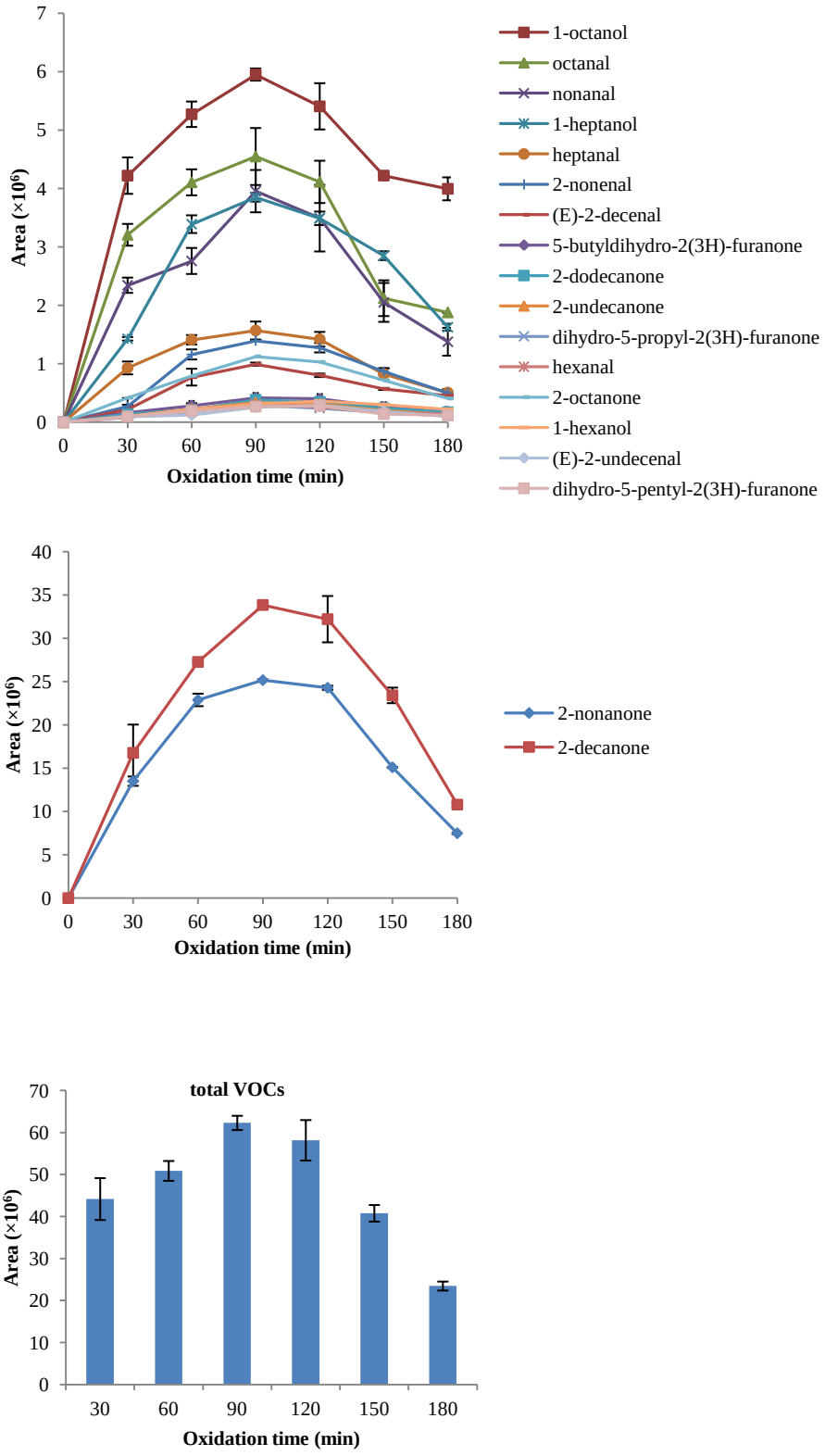


Figure 42: Profiles of VOCs produced from SOPE monitored from 0 min to 180 min (oxidation temperature: 175 °C).

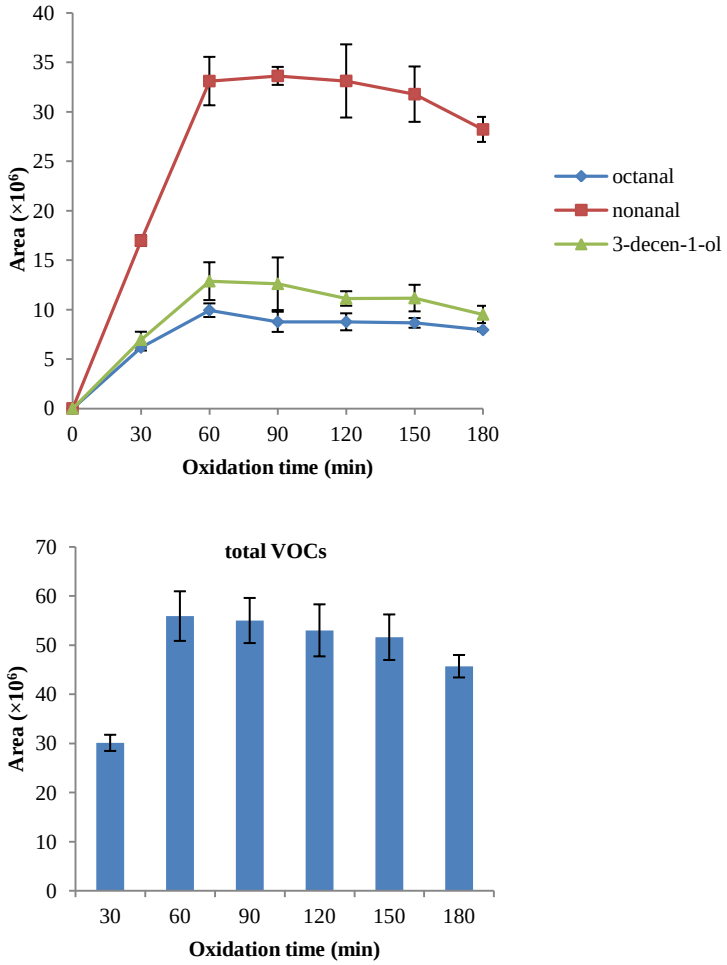


Figure 43: Profiles of VOCs produced from SOPA monitored from 0 min to 180 min (oxidation temperature: 175 °C).

In order to compare the kinetic curves of VOCs from oxidized SOPC, the samples of SOPE, SOPA and SOS were heated at the same temperature (175 °C) during 0-180 min. 19, 18 and 3 VOCs were detected respectively from oxidized SOPC, SOPE, and SOPA. 2-decenal, nonanal, octanal, 2-undecenal and 1-octanol were the major VOCs of oxidized SOPC. All the VOCs increased rapidly during 0-60 min and reached their maximum at 120 min, then kept stable or decreased slowly until 150 min but decreased rapidly after 150 min (Figure 41). For SOPE (Figure 42), 2-nonanone and 2-decanone were very abundant, following 1-octanol, octanal, 1-heptanol and nonanal. It was clearly observed that all the VOCs increased rapidly until 90 min of oxidation and stabilized during 90-120 min, then decreased rapidly after 120 min. For SOPA, only 3 VOCs were detected (Figure 43). Nonanal accounted for a relative high amount among them. The three VOCs increased rapidly before 60 min, then stabilized.

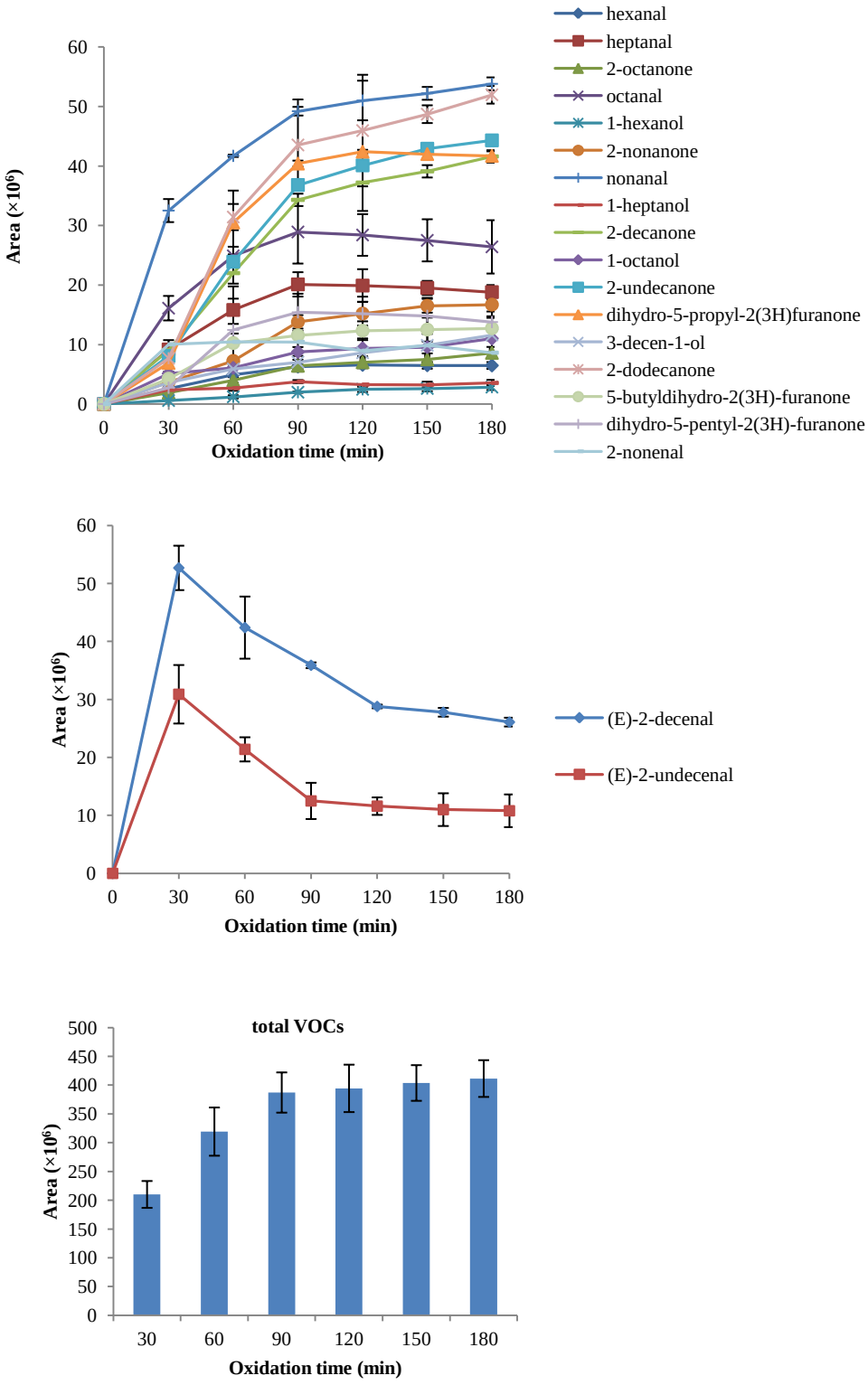


Figure 44: Profiles of VOCs produced from SOS monitored from 0 min to 180 min (oxidation temperature: 175 °C).

As far as VOCs of oxidized SOS were concerned, 19 VOCs were detected (Figure 44). The VOCs can be separated into two groups: one group included (E)-2-decenal and (E)-2-undecenal, which increased in a sharp way before 30 min and decreased rapidly until 90 min, then

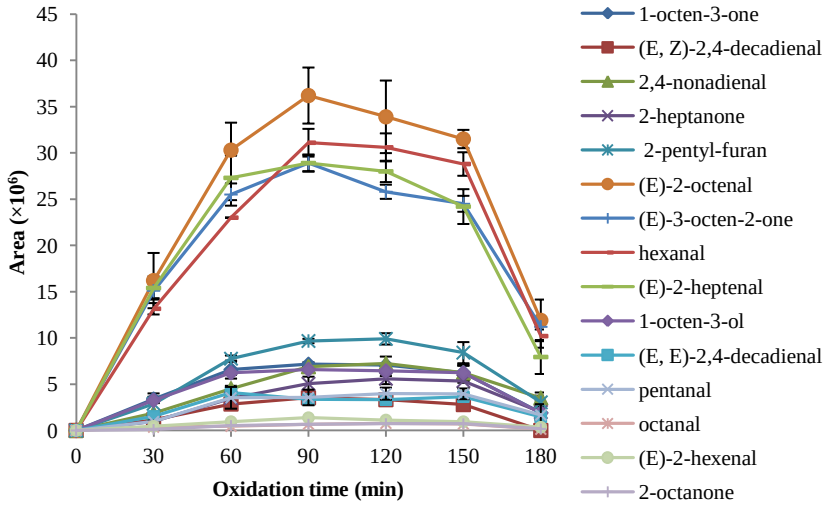
reached equilibrium; the other groups included the products that increased gradually until 90 min and then stabilized. Table 14 showed that oxidized OOO produced large amounts of (E)-2-decenal and (E)-2-undecenal (175 °C, 30 min). Among the VOCs of oxidized SOS, (E)-2-decenal and (E)-2-undecenal accounted also for a large proportion at the same oxidative condition (175 °C, 30 min) (Figure 43). There is a competition among *sn*-1, *sn*-2 and *sn*-3 position of SOS. *sn*-1 and *sn*-3 position of SOS being stearic acid which is not easy to be oxidized, while *sn*-2 postion of SOS is oleic acid which can be easily oxidized. The peak areas of (E)-2-undecenal and (E)-2-decenal from oxidized SOPC in the first 30 min oxidation accounted for a high proportion, but from oxidized SOPE, their peak areas were low. That demonstrated that choline group in *sn*-3 position of glycerol have similar influence than stearic acid for the formation of (E)-2-undecenal and (E)-2-decenal.

The results showed that the total amounts of VOCs from oxidized SOPC, SOPE and SOPA reached their maximum in 120 min, 90 min and 60 min, respectively (Figure 41, 42 and 43), and the total amouts of VOCs from oxidized SOS reached equilibrum in 90 min (Figure 44). It demonstrated that the primary formation of hydroperoxide of SOPC was most resistant to decompose than others in the same temperature.

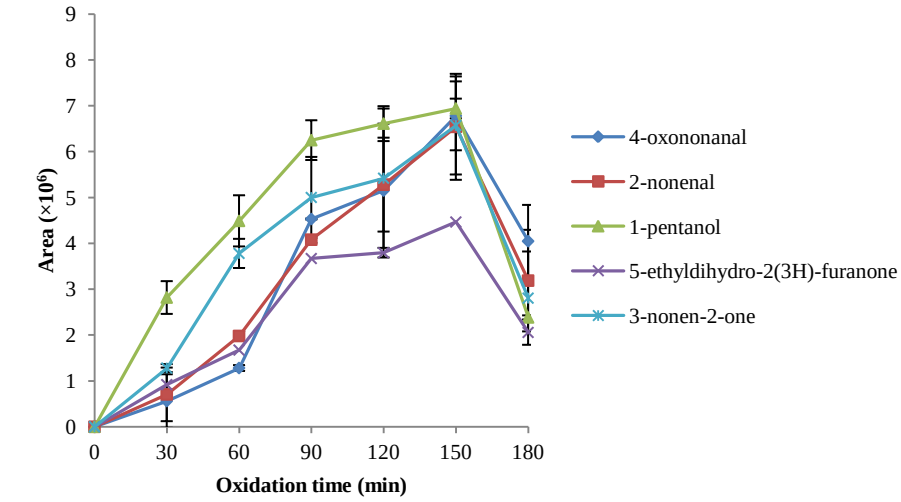
2.3.3.2 SLPC, SLPE, SLPA and SLS

Figure 45 shows kinetics of production of VOCs from oxidized SLPC. 20 VOCs were detected, among them, (E)-2-octanal, hexanal, (E)-2-heptenal and (E)-3-octen-2-one were abundant. Two groups were obtained: one group (Figure 45A) that rapidly reached maximum at 90 min of oxidation and kept stable or decreased slowly until 150 min, then, decreased sharply after 150 min; the other group (Figure 45B) that increased gradually until 150 min and then decreased rapidly.

(A)



(B)



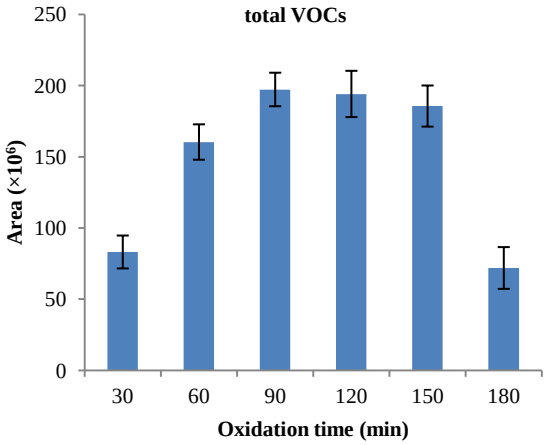


Figure 45: Profiles of VOCs produced from SLPC monitored from 0 min to 180 min (oxidation temperature: 125 °C).

15 VOCs were detected from oxidized SLPE (Figure 46). 2-heptanone, hexanal, (E)-3-octen-2-one were abundant. All the VOCs reached their maximum at 90 min, decreased rapidly during 90-120 min and stabilized or decreased slowly after 120 min.

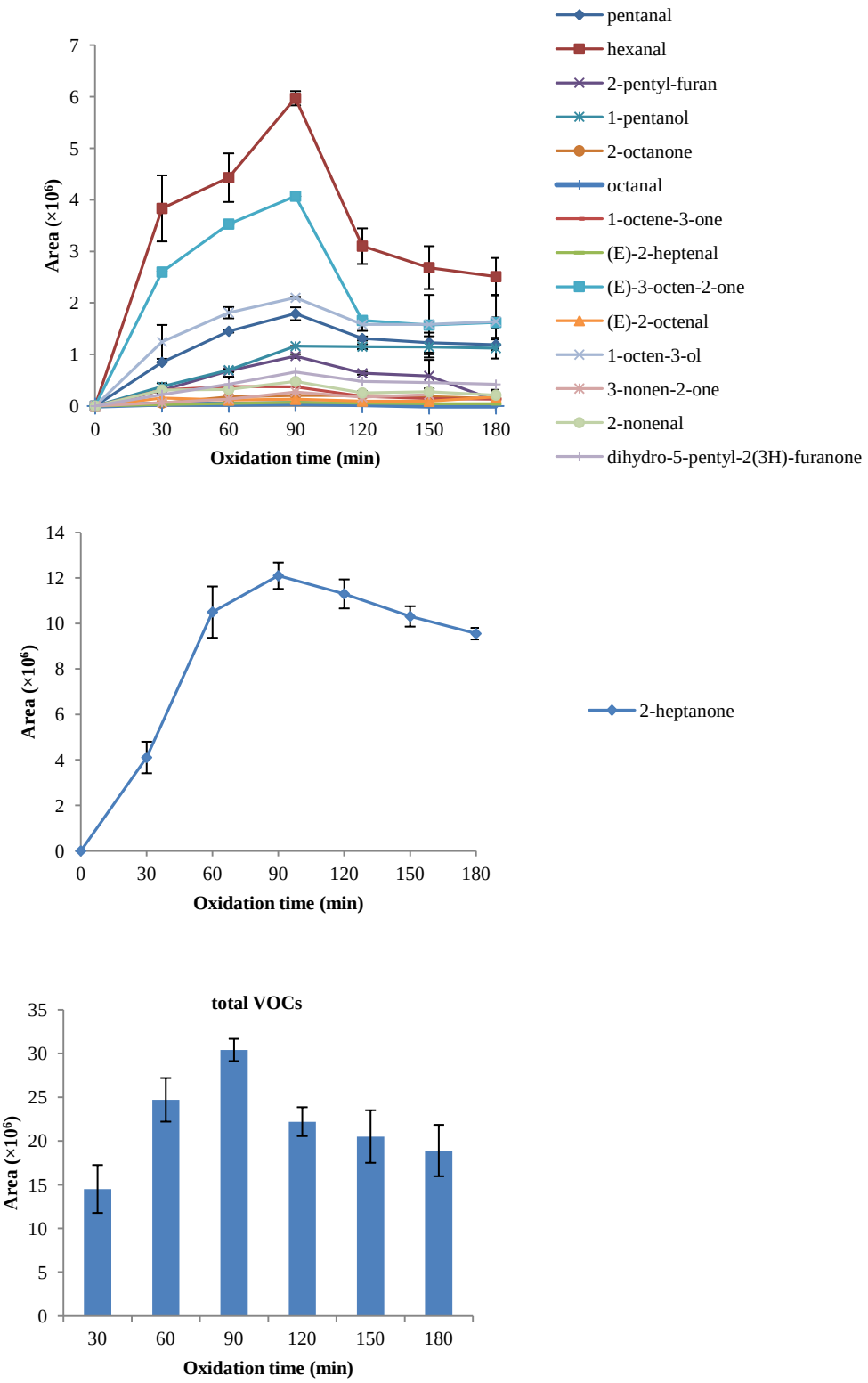


Figure 46: Profiles of VOCs produced from SLPE monitored from 0 min to 180 min (oxidation temperature: 125 °C).

13 VOCs were detected from oxidized SLPA and these compounds were included in two groups. One group included the products that increased raplidly before 60 or 90 min of oxidation, then kept stable or decreased slowly (Figure 47). The other group included 2-nonenal and 2-pentyl-furan which increased with the increase of oxidation time. That was likely that these two VOCs might be derived from the primary decomposition products.

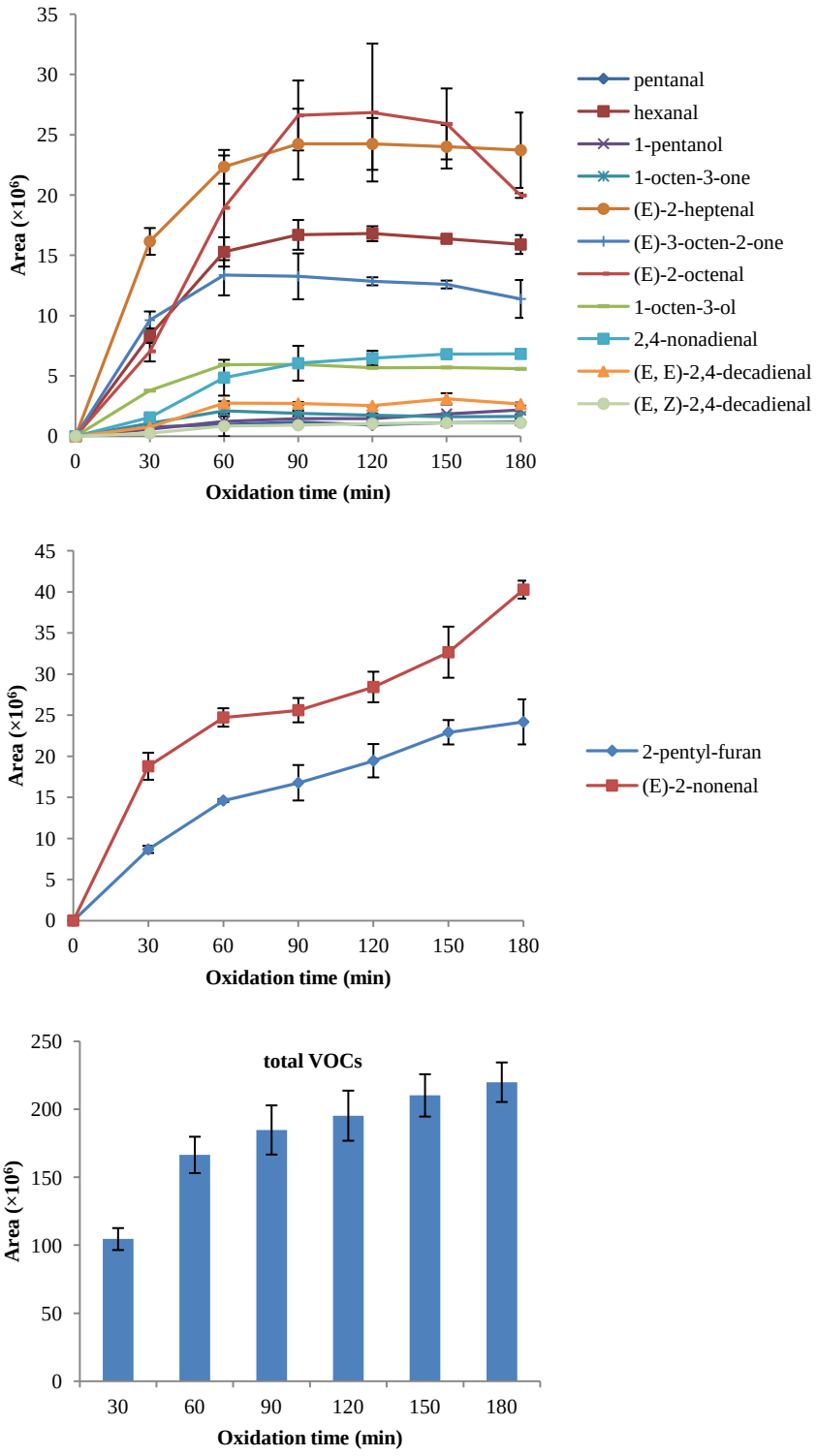
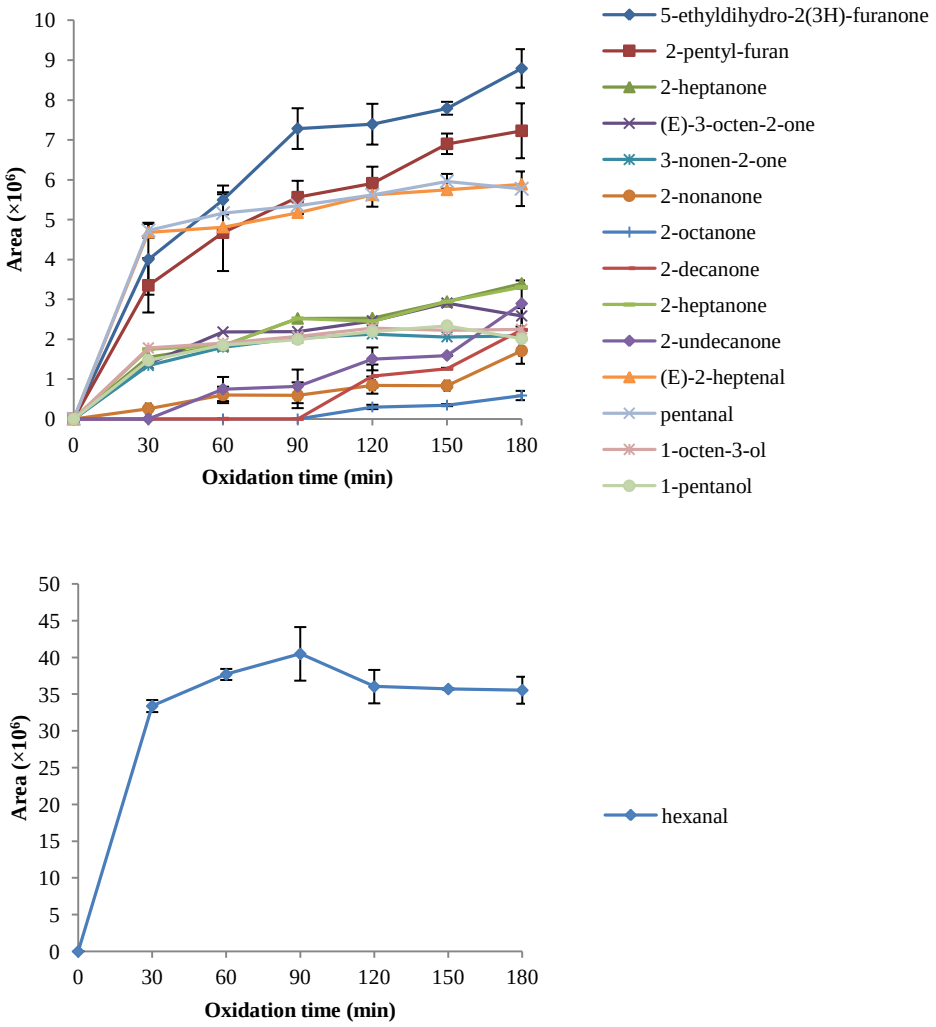


Figure 47: Profiles of VOCs produced from SLPA monitored from 0 min to 180 min (oxidation temperature: 125 °C).

As far as SLS oxidation products are concerned, 18 VOCs were detected (Figure 48). Hexanal was very abundant among the VOCs. It increased in a sharp way in the first 30 min, reached its maximum at 90 min, then decreased and stabilized. The amount of (E)-2-octenal, 2,4-nonadienal, (E, Z)-2,4-decadienal and (E, E)-2,4-decadienal reached a maximum after 30 min of oxidation, then decreased and stabilized. Other VOCs increased gradually with the increase of oxidation time.



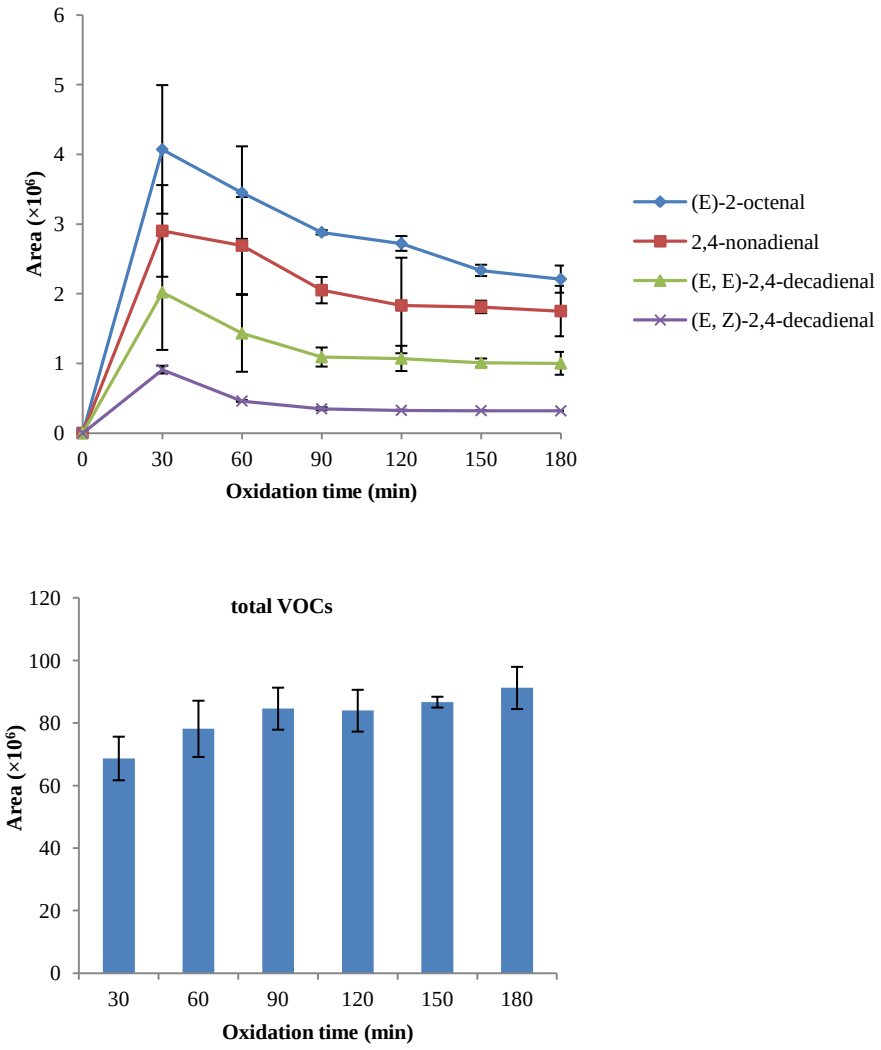


Figure 48: Profiles of VOCs produced from SLS monitored from 0 min to 180 min (oxidation temperature: 125 °C).

2,4-alkadienals, 2,4-nonadienal, (E, E)-2,4-decadienal and (E, Z)-2,4-decadienal, which were formed from linoleic acid, were present in the VOCs of SLPC, SLPA and SLS, but not in SLPE. That demonstrated that ethanolamine headgroup has impact on the formation of 2,4-alkadienals. Thus, in further study, it is interesting to investigate whether other PE molecules or PE mixture can produce 2,4-alkadienals. If 2,4-alkadienals are not formed from other PE molecules or PE mixtures, it would be used as a measuring indicators to distinguish food composition containing PE or not.

The results showed that the total amounts of VOCs from oxidized SLPE were lower than those from oxidized SLPC, SLPA and SLS (Figure 45-48). It implied that most hydroperoxides of SLPE might decompose into non-VOCs.

Overall, the process of thermal oxidation of PL is in a way a competition between formation and decomposition of hydroperoxides. Hydroperoxides formed from the oxidation of lipid are unstable and will rapidly decompose to form VOCs and non-VOCs. In fact, either VOCs or non-VOCs formed at low temperature can further decompose into other VOCs at high temperature or with extension of oxidation time. There are two additional ways to increase the content of VOCs: one way consists in unsaturated aldehydes which undergo autoxidation and provide additional sources of volatile compounds. Further decomposition of 2-aldehydes probably produce saturated aldehyde. For example, according to Figure 49, 2-nonenal, (E)- 2-hexenal, (E)-2-heptenal and (E)- 2-decenal may produce octanal, pentanal, hexanal and nonanal, respectively (Frankel, 2005).

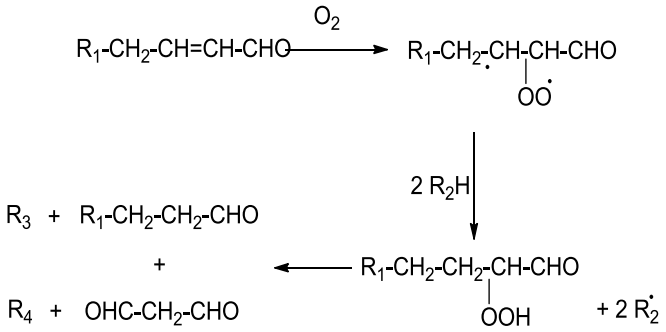


Figure 49: Oxidation of 2-alkenals (Frankel, 2005).

The second way consists in non-VOCs, such as hydroperoxy epoxides, which undergo further decomposition into volatile products (Frankel, 2005). For example, Le Grandois *et al.* (2010) studied the kinetics of SLPC oxidation during heat treatment at 125°C and demonstrated that hydroperoxy epoxides decreased rapidly after 30 min of oxidation. This implied these compounds to be not stable and probably degrade into volatile products. This could explain our results that why VOCs of SLPC increased rapidly after 30 min of oxidation. Therefore, VOCs reached maximum in a short time and then achieved equilibrium, while some VOCs maintained a steady growth with the increase of oxidation time could be explained by the fact that primary hydroperoxides (epoxy, oxo and hydroxyl) further decomposed into VOCs. When primary hydroperoxides decompose into secondary thermally stable VOCs, the concentration of these VOCs increased with the increase of the oxidation time. If secondary VOCs also decompose into tertiary products, the rate of secondary VOCs production became dramatically lower and even may became constant. This phenomenon will last as long as primary hydroperoxides are thermo-formed from precursor PL. But during the PL oxidation, the concentration of PLs precursor

vanished so as the concentration of hydroperoxides That's why some VOCs concentration decreased with the increase of oxidation time.

However, lipid oxidation is a complex process and the exact mechanism of production of VOCs is still not very clear. The proportion of VOCs produced by secondary oxidation products and the further decompostion of each VOCs are something that needs further investigation. Indeed, these factors would affect the kinetics of VOCs of PL during thermal treatment, and both the nutritional and organoleptic qualities of processed foods.

In summary, the kinetics of most of VOCs formed from oxidized PL, either mono-unsaturated PC (PE) or di-unsaturated PC (PE), were in this trendency: firstly, VOCs increased rapidly or gradually until achieved a maximum and kept balance, then decreased more or less. While the kinetics of formation of VOCs from oxidized PA were similar with TAGs. The VOCs increased in first 30 or 60 min and then reached equilibrium. Therefore, the formation kinetics of VOCs of PE and PC were different from those of PA and TAG, and this might be related to the polar headgroup, because PE and PC contain amino group.

2.4 Influence of the constituent in the position *sn*-3

The percentage of the starting materials SOPC, SOPE, SOPA and SOS (SLPC, SLPE, SLPA and SLS) during oxidation process was investigated by LC-MS. The remaining percentage of SOPC, SOPE, SOPA and SOS were calculated and compared for each oxidation time (Figure 50). The peak area of SOPC decreased gradually over time of oxidation, and at 180 min, SOPC amounts decreased by 60.4%. As for SOPE, the peak area decreased rapidly during 0-90 min (decreased by 94.1%) and almost dispeared after 120 min. For SOPA, the peak area decreased sharply by 91.4% in a short time of 5 min and disappeared after 15 min. The comparison of remaining percentages of each oxidation duration obviously demonstrated the oxidative stability in the order SOPC >SOPE > SOS > SOPA.

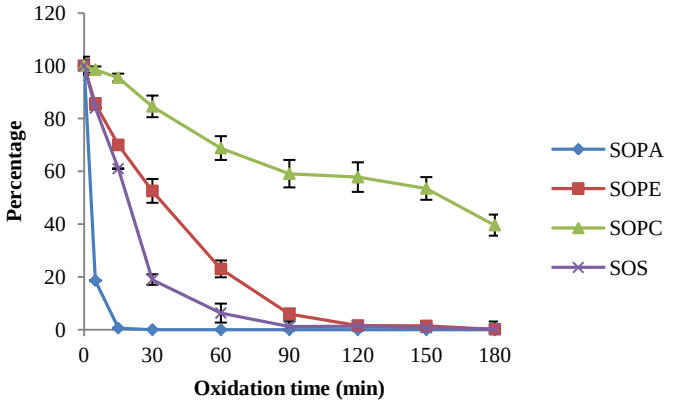


Figure 50: Kinetics of SOPC, SOPE, SOPA and SOS oxidation during heat treatment at 175 °C.

Then, we compared the kinectis of degradation of SLS with SLPE, SLPA and SLPC to observe whether this tendency present in the molecules with two double bonds.

The kinetics of degradation of SLPC, SLPE, SLPA and SLS were compared (Figure 51). It appeared that SLS and SLPA decreased rapidly and disappeared within 90 min of oxidation, while SLPC and SLPE decreased more gently down to 21.3% and 19.9%, respectively after 180 min oxidation. Similar to the tendency of SOPC, SOPE, SOPA and SOS, the oxidative stability of di-unsaturated molecules were in the order: SLPC >SLPE > SLS > SLPA (Figure 51).

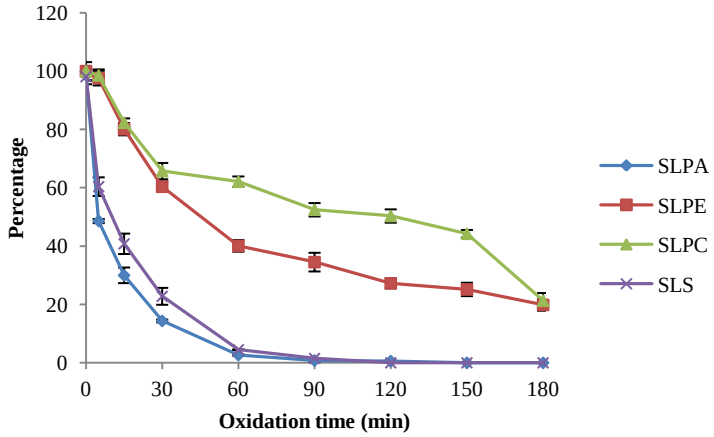


Figure 51: Kinetics of SLPC, SLPE, SLPA and SLS oxidation during heat treatment at 125 °C.

By comparison with the oxidative stabilities of mono-unsaturated molecules and di-unsaturated molecules (Figure 50 and 51), it can be concluded that the FAs in form of PC and PE are more resistant to oxidation than in form of TAG. Indeed, the percentage of remaining starting material of PC and PE were higher than TAG and their degradation speeds were lower. Our

results were consistent with the results of Lyberg *et al.* (2005) who evaluated the oxidation stability of DHA incorporated into PC, PE and TAG. The results demonstrated that DHA was protected against hydroperoxide formation when it was incorporated at *sn*-1 or *sn*-2 position of either PC or PE. While incorporation of DHA at any positions of a TAG was not an appropriate solution for protecting DHA.

Moreover, either mono-unsaturated molecules or di-unsaturated molecules, PE and PC were more stable than their counterparts in form of PA during thermal oxidation. This reason could be that PA doesn't contain any amino group. Our finding was consistent with a previous study (Nwosu *et al*., 1997; Saito and Ishihara, 1997). Furthermore, PC showed more resistance to oxidation than PE. The result was in agreement with the previous study of Yin *et al.* (1993) who investigated the influence of the polar headgroup on the PL oxidation stability, and reported that PL containing choline (PC) showed a slower oxidation rate than that containing ethanolamine (PE).

Another point noted was that TAG was more stable than PA. It can be explained by the *sn*-3 position of glycerol. In our study, TAG contained a SFA which is quite resistant to oxidation, while PA as the acid form of phosphate is easier to be oxidized.

Furthermore, the starting material PA and TAG decreased rapidly before 90 min and then nearly disappeared (Figure 50 and 51), while the total amounts of VOCs of PA and TAG have no significant change after 90 min of oxidation (Figure 43, 44, 47 and 48). Especially, only 3 VOCs were detected from oxidized SOPA. Most of PA and TAG might then be degraded into non-volatile compounds, which may be interesting to be analyzed in further studies.

Conclusion

The object of this chapter was to evaluate the differences in the thermal oxidative stability between two types of glycerolipids, PL and TAG. HS-SPME followed by GC-MS, was used to investigate VOCs of PL and TAG. At first, the influence of several parameters on the efficiency of extraction of VOCs, such as type of fiber, extraction duration and temperature were studied. The best results were obtained with the fiber of 65 µm PDMS/DVB for 25 min of extraction at 50 °C. Then, the VOCs of PL were identified with authentic reference compounds (STD) and the National Institute of Standards and Technology (NIST) mass spectral library. Meanwhile, the VOCs of TAG were also be identified because these oxydative products were similar with that presented in PL. Then, the effect of oxidation temperature on the yield of VOCs of PL were investigated, and their kinetics of formation were determined in the chosen oxidation conditions (175 °C for monounsaturated molecular species and 125 °C for di-unsaturated species). This first step allowed the evaluation of the stability of oxidative products formed over time and thereby allowed us to understand the behaviour of PL with regard to thermal oxidation. All the results obtained allowed a comparison of the stability between the PL and TAG. It appeared that FAs in form of PL were more stable than that of TAG. The stabilities in descending order was: PC > PE > TAG > PA in the study of the stability of SOPC, SOPE, SOPA and SOS (SLPC, SLPE, SLPA and SLS). In fact, reasons leading to the difference of oxidative stability between PL and TAG are the difference in *sn*-3 position (PL contains a polar headgroup while TAG contains a SFA). TAG was more stable than PA. Certainly, because TAG contains a SFA able to reduce oxidation in *sn*-3 position, while PA is the acid form of phosphate which is easier to be oxidized. Therefore, TAG was more resistant to oxidation than PA.

Evaluting the influence of the polar headgroup nature on the oxidative stability of GPLs, it appeared clearly that PA was very unstable, the reason being that both PC and PE contained an amino group, while PA didn't contained it. Other PL classes, such as PS and PI, just because their polar headgroups are very different from those of PC or PE. They may have very different kinetics of formation of oxidative products. It will be interesting to investigate the oxidation of PS and PI in a further study because the results would bring new information and explainations for oxidation mechanism influenced by different polar headgroup. It will be also interesting to evaluate the oxidation of more unsaturated PLs which include more than two double bonds at *sn*-1 position, *sn*-2 position or both positions of the PLs, and compare with their corresponding TAGs. The results in this chapter can gave us a enlightenment that PE and PC would be highly

interesting as food additives to stabilize PUFA, which has a considerable impact on health and need to be protected to avoid oxidation.

GENERAL CONCLUSION

PLs are rich in PUFA and are an important source of PUFA. Supplementation of food products with PUFA-rich PLs has recently emerged as an interesting way of increasing the health benefits of human body and thereby received more and more attention. Another important source of PUFA is TAGs, which are the major carriers of FAs. Although TAGs carry more FAs than that by PLs, the biodisponibility of FAs of PLs are much more efficient than that of TAGs. Moreover, cellular permeability to PUFA and their intracellular level were indeed much higher when linked to PL than to TAG. It is therefore necessary to explore and characterize the sources of PUFA-PLs so as to assess whether the interest attributed to them is justified or not.

In the first part of this thesis, we developed methods to analyze PLs from different food sources. Five potential sources of PUFA-PLs were studied: soy, calf brain, ox liver, egg yolk and krill oil. Preparation was different for each sample depending on its physical state. The first step was to develop an extraction method to improve the recoveries of TLs and PLs from these matrices. We used PLE method to extract TLs and PLs from various food matrices and determined the extraction efficiency by comparison with a standard liquid extraction method (SLE-Folch method). Our finding showed that PLE method was more efficient than SLE-Folch method. Then, the optimal condition of PLE method for extraction was determined. The classes of PL extracts from the matrices studied were identified and quantified by HPLC-ELSD. The number of classes identified varied depending on the food considered. In ox liver and calf brain, five classes were identified, with PC and PE being the major ones and PI, PS and SM being less abundent. Four classes (PE, PI, PC and SM) were found in egg yolk, three (PE, PI and PC) in soy, and two (PE and PC) in krill oil. PE and PC are the main PL classes and account for a relatively high proportion, while SM is only present in mammal sources.

After extraction of TLs from food matrices, we identifed the molecular species of PE and SM. The first step was to isolate PLs from TLs by use of both adsorption column and semi-preparative chromatrographies to collect and purify PE and SM from PLs. The purity of them was checked by analytical chromatography. The next step was to identify the molecular species of PE and SM by reversed phase-HPLC-ESI-MS^2. Methanol containing 5 mmol L^{-1} ammonium formate was used as the mobile phase. In krill oil PE, we found a special molecule (*m/z* 750.7). In order to identify its structure, we collected it in the corresponding HPLC fraction between 16.9 and 17.7 min and analyzed it by HRMS. Finally, we inferred that this molecule was probably C16:0(alkyl)-22:6(acyl)PakE. Moreover, soy PE contained a high amounts of EFAs, egg yolk PE, ox liver PE and krill oil PE were not only rich in EFAs, but also rich in LC-PUFAs. Especially in krill oil PE, where EPA and DHA were predominant. The method developed for the analysis of molecular species can not only identify FA on glycerol backbone, but also FA on sphingoid backbone. The

SM molecular species of ox liver, calf brain and egg yolk were identified as well. All the predominant SM species of them contained SFAs.

Food supplementation with PL-PUFA rises the question of their stability during processing, storage and cooking. In order to have a comprehensive study of PLs, the thermal oxidation stability of PLs were studied.

Lipid oxidation starts with the formation of hydroperoxides, these unstable compounds break rapidly into nonvolatile and volatile products. As non-volatile products was already studied by LC-ESI-MS, in the laboratory, my thesis focused on the volatile products of PLs. The VOCs were identified by HS-SPME-GC-MS. The first step was to establish the suitable conditions for extraction VOCs from oxidized PCs. Then, the effect of oxidation temperature on the yields of VOCs of PC were investigated, and their kinetics of formation were determined. The origins of VOCs were studied by comparing the results with those obtained from the corresponding TAGs under the same experimental conditions.

The FAs in form of PE and PC were more stable than in the form of TAG during thermal oxidation. Certainly, because PC and PE contain amino groups, so they were more resistant to oxidation than TAGs containing stearic acid in *sn*-3 position. On the opposite, while PA being the acid form of phosphate appeared to be easier to be oxidized than TAGs.

This thesis is a step forward to find potential sources of PUFA-PLs for food supplementation. The results reflected that PUFA-PL as food suppplementation in food will be a good choice.

Furthermore, there remain many aspects which need to be studied in further works. In GPLs, PE and PC account for relatively high proportions. The characterization of PC was investigated in a previous study in the laboratory. In my thesis, I focus on the second abundant class (PE) of GPL. However, other two GPLs, PS and PI, which account a relatively low proportion, were not studied. In fact, the polar headgroups (PS and PI) are very different from those of PC and PE. It will be very interesting to investigate not only the composition of FAs in PS or PI molecular species, but also their oxydation by the study of thermal oxidation of SOPS and SOPI. Such study can provide more information of the influence of the polar headgroup on the thermal oxidation stability of PLs. In addition, differences in chainlength could affect the thermodynamic properties of PLs. Evaluation of the effect of FA composition of PLs on their

oxidative stability, especially EPA/DHA-PL, will be interesting and significant. Individual PL as antioxidant often are added to lipid-containing products to delay the rate of oxidation. However, the investigation of antioxidant capacity of PLs in other food model systems such as high protein or high-sugar food are not common. The chelating properties of primary or free amino headgroup of PL with food components would have a profound impact on the antioxidant mechanism. Therefore, we considered another focus of the further study which could be on the evaluation of oxidative stability of various food models supplemented with PLs, the analysis of the volatile compounds from maillard reaction and the elaboration of the antioxidant mechanism of each individual PL.

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Résume

Parmi les nombreux effets bénéfiques des phospholipides on retient essentiellement leur role dans la protection du système cardio-vasculaire, l'amélioration de la mémoire et de l'apprentissage. Ces effets peuvent s'expliquer en partie par le fait que les phospholipides sont riches en acide gras polyinsaturés (acides eicosapentaénoïque et docosahexaénoïque) ou les acides gras essentiels. C'est pourquoi il nous est apparu comme étant important d'explorer des sources naturelles potentielles contenant des phospholipides polyinsaturés destinées au développement de produits alimentaires nouveaux supplémentés en phospholipides.

Ce travail a permis de mettre au point des méthodes d'extraction, de purification et de séparation des phospholipides afin de caractèrises les sources naturelles de phospholipides polyinsaturés. Une supplémentation des aliments avec des phospholipides polyinsaturés ne peut être envisagé sans avoir une bonne connaissance de leur stabilité au cours de la transformation et de la conservation des aliments. Nous avons donc développé des méthodes d'identification des produits de dégradation des phospholipides et de détermination de leur cinétique d'oxydation pendant les traitements thermiques. Ainsi, nous avons montré que les phospholipides sont plus stable que les triacylglycérides, ceci étant du probablement à la présence d'un groupe aminé dans la tête polaire.

Mots-clés : Phospholipides, extraction, analyse chromatographique, espèces moléculaire, oxydation, stabilité, volatile composé oxydé

Abstract

Among the numerous beneficial effects of phospholipides can highlight their role in protecting the cardiovascular system and improving memory and learning. These effects can be partially explained by the fact that phospholipids are rich in polyunsaturated (eicosapentaenoic and docosahexaenoic acids) or essential fatty acids. Thus, it is very significant to explore potential food sources containing polyunsaturated rich phospholipids for the development new food products supplemented with phospholipids.

This work allowed a serie method for the extraction, purification, separation and quantification of phospholipids in order to characterize the polyunsaturated phospholipid sources. Food supplementation with polyunsaturated phospholipids rises however the question of their stability during food processing at high temperature. Then we have developed methods to identify phospholipids degradation products and determine their kinetics of the oxidation during thermal treatment which is a widespread food processing.

Phospholipids (PE and PC) are more stable than triacylglyceride, this is due to the presence of an amino group in the molecule.

Keywords: Phospholipids, extraction, chromatographic analysis, molecular species, oxidation, stability, volatile oxidized compound